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## FOREWORD

The Second R & D Programme, indirect action in the field of environment, was adopted in 1976 \* for a five year period, 1976-1980, and revised in 1979 \*\* with a total allocation of 20.8 million units of account.

The programme was divided into two phases, the first ended on 31st December 1978 \*\*\*, the second one on 31st December 1980. The cost-sharing research contracts concluded with institutions and laboratories of member countries dealt with the various topics (see Table of Contents) included in the four research areas of the programme.

1. Research aimed at the establishment of criteria (exposure-effect relationships) for pollutants and environmental chemicals
2. Research concerning environmental information management
3. Research concerning the reduction and prevention of pollution and nuisances including clean technologies
4. Research concerning the protection and improvement of the natural environment

This volume contains the summary reports concerning the research carried out during the second phase of the programme. The texts are in the form submitted by the contractors. The results, as those obtained for previous programmes, provide a scientific and technical support to the European Community policy on the environment.

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\* Official Journal of the European Communities no. L74 of 20.3.76  
\*\* Official Journal of the European Communities no. L258 of 13.10.79  
\*\*\* See Report EUR-6388, 1980, concerning the first phase

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**RESEARCH AREA '1' : ESTABLISHMENT OF CRITERIA**

Contractor: Inst. f. Ingenieurbiologie, Universität Karlsruhe

Contract n<sup>o</sup>: 172-77-1 ENV D

Project leader: Prof. Dr. L. Hartmann

Title of project: Synergistic effects of heavy metal ions on the activity of bacteria and other aquatic micro-organisms

---

Pollution of natural waters by heavy metals hardly ever occurs by only one pollutant. Under these conditions synergistic and antagonistic interferences between the different inhibitors are the rule, simple summation of effects being rather an exception as shown in a previous report. A valid prognosis on the inhibitory effects of heavy metal pollution, therefore, must include the mutual interactions between the different heavy metals.

To interpret and mathematically describe such systems, the effects of six different heavy metal combinations on acetate consumption by different biocommunities were investigated. In every case a reliable mathematical description of the reaction could be developed, using the standard laws of noncompetitive enzyme inhibition as a mathematical model. However, the experiments proved that the type of reaction as well as the kinetic parameters depend not only on the heavy metal combination tested but also on the biocommunity affected. A mathematical description developed for an individual case, therefore, may not be generalized but can only be applied to a similar system.

Considering this, a survey on the whole range of possible interferences between two noncompetitive inhibitors was attempted. Such a complete survey may be helpful in the evaluation, interpretation and mathematical description of inhibition by heavy metal combinations in general. It can be given, because up to now the mathematical model "noncompetitive enzyme inhibition" proved to be suitable, and, on the basis of this model, only a limited amount of different reactions is possible.

According to the fundamentals of enzyme inhibition inhibitors as well as the substrate are reversibly bound to the enzyme, forming an enzyme-inhibitor complex. In noncompetitive inhibition the reaction of inhibitor and catalyzing element does not affect the binding but the conversion of substrate; in other words: substrate can be bound to the enzyme-inhibitor complex as well as to the free



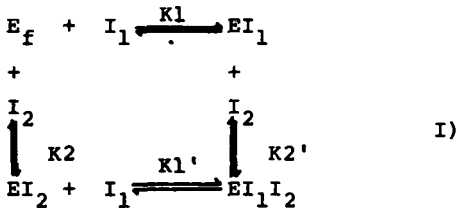
enzyme, but only if it is bound to free enzyme, it will be converted into product. The maximal reaction velocity of a noncompetitive inhibited reaction, therefore, depends on the amount of free enzyme, which may be calculated from the equilibrium between enzyme, inhibitor and the enzyme-inhibitor complex. Thus the velocity equation is derived.

If two noncompetitive inhibitors take part in the reaction, the derivation of the velocity equation may be done analogously, calculating the amount of free enzyme from the equilibrium conditions between enzyme, inhibitors and the different enzyme-inhibitor complexes, occurring in the reaction scheme.

On the basis of the mathematical model the question of interferences between two different heavy metals thus may be reduced to the question: which reactions may occur between the enzyme and two noncompetitive inhibitors?

A specification of the different reaction types possible and the corresponding velocity equations is given below. The heavy metal combinations experimentally found to cause such reactions are given as examples.

1. Under the assumption that interference between the inhibitors is mutual, the following reaction scheme may be postulated:



If, as implicated in the assumption of mutual interference,  $\frac{K1}{K1'} = \frac{K2}{K2'}$ , the corresponding velocity equation can be derived

$$\text{as: } v_{\text{max}_{I_1, I_2}} = \frac{v_{\text{max}}}{\frac{I_1}{K1} + \frac{I_2}{K2} + \frac{I_1 I_2}{K1 K2'} + 1}$$

A reaction of this type may be synergistic, antagonistic or additive, depending on the relation between K1 and K1' resp. K2 and K2'.

1.a. If the different inhibitors do not interfere,  $I_1$  will be bound to the  $EI_2$ -complex as readily as to the free enzyme ( $E_f$ ). In this case

$$K_1 = K_1'; K_2 = K_2' \text{ and } \frac{K_1}{K_1'} = \frac{K_2}{K_2'} = 1 .$$

This type of reaction may be defined as summation. The combination Zn / Hg was the only heavy metal combination found to react according to this type.

1.b. If the previous binding of one inhibitor hinders the binding of the second one, the reaction is antagonistic.

$$K_1' > K_1; K_2' > K_2 \text{ and } \frac{K_1}{K_1'} = \frac{K_2}{K_2'} < 1$$

This type of reaction was found with the combination Hg / Cu.

1.c. If the binding of the second inhibitor is facilitated, the reaction is synergistic.

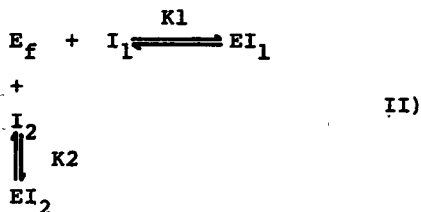
$$K_1' < K_1; K_2' < K_2 \text{ and } \frac{K_1}{K_1'} = \frac{K_2}{K_2'} > 1$$

Reactions of this type were caused by two heavy metal combinations: Zn / Cu and Pb / Cu.

2. The binding of one inhibitor may totally prevent the binding of a second one. Both inhibitors then will compete for the enzyme. This reaction type may be defined as "competitive antagonism" and be regarded as an extreme case of reaction scheme I, as

$$K_1' = K_2' = \infty \text{ and } \frac{K_1}{K_1'} = \frac{K_2}{K_2'} = 0 .$$

The reaction scheme may be written as

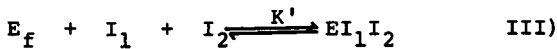


and the corresponding velocity equation reads:

$$V_{\max_{I_1, I_2}} = \frac{V_{\max}}{\frac{I_1}{K_1} + \frac{I_2}{K_2} + 1}$$

This type of reaction was found with the combination Zn / Cd and, with some special biocommunities only, with the combination Zn / Hg.

3. Extreme synergism will occur, if the binding of the second inhibitor is thus enhanced that it takes place immediately after the first inhibitor is bound. The formation of an  $EI_1$ - resp. an  $EI_2$ -complex then will be only an intermediate stage in the formation of the  $EI_1I_2$ -complex and, therefore, may be neglected in the formulation of the reaction scheme:

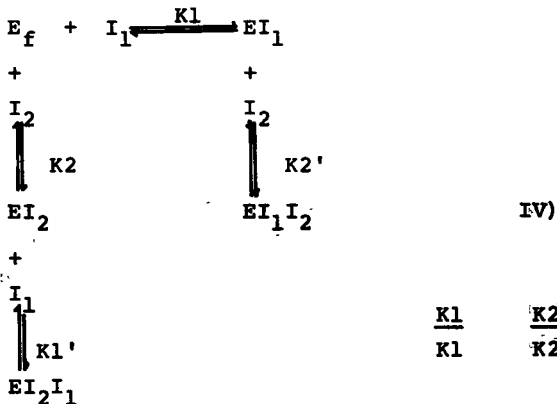


The corresponding velocity equation reads:

$$V_{\max_{I_1, I_2}} = \frac{V_{\max}}{\frac{I_1I_2}{K'} + 1}$$

Reactions of this type were found with the combination Zn / Pb.

4. Interference between the inhibitors need not necessarily be mutual; the form of the  $EI_1I_2$ -complex may depend on which inhibitor is bound first. In this case the reaction scheme reads

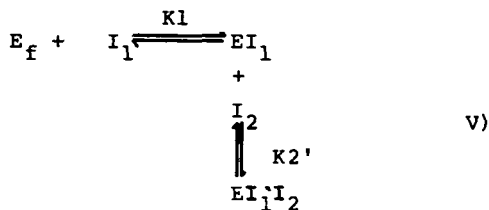


and the velocity equation can be written:

$$V_{\max_{I_1, I_2}} = \frac{V_{\max}}{\frac{I_1}{K_1} + \frac{I_2}{K_2} + \frac{I_1 I_2}{K_1 K_2'} + \frac{I_1 I_2}{K_1' K_2} + 1}$$

A reaction of this type may be synergistic or antagonistic. However, only the synergistic type could be verified by experiment. It was caused, with one special biocommunity only, by the combination Zn / Cu.

5. The binding of the second inhibitor may depend on the previous binding of the first one, in other words: one inhibitor only causes an effect, if the other inhibitor is present. The reaction scheme then may be formulated as



the corresponding velocity equation being:

$$V_{\max_{I_1 I_2}} = \frac{V_{\max}}{\frac{I_1}{K_1} + \frac{I_1 I_2}{K_1 K_2'} + 1}$$

This reaction type could only be verified in some special cases with the combination Zn / Pb. In these cases Pb alone did not cause any measurable inhibitory effects.

From the experimental results the conclusion may be drawn that reactions of type 4. and 5. occur as rarely as does simple summation. In most cases interference was found to be mutual, yielding reactions of type 1.b, 1.c, 2. and 3.

To give a complete survey, two possible deviations need to be mentioned that may occur in every type of reaction discussed hitherto:

- Two or more  $I_1$  and/or  $I_2$  ions may take part in the reaction. In this case the inhibitor concentration will enter in the equation exponentially ( $I^n$ ).

- $I_1$  and/or  $I_2$  may cause an inhibition of the partly noncompetitive type. In this case conversion of substrate bound to the  $EI_1$  and/or  $EI_2$ -complex is not totally prevented but only slowed down. To derive the velocity equation the amount of  $EI$  then has to be calculated as well as the amount of  $E_f$ .

### Definitions of Symbols

$V_{max}$	maximal reaction velocity
$V_{max}_{I_1, I_2}$	maximal reaction velocity in presence of two inhibitors
$I_1 ; I_2$	inhibitors
$EI_1 ; EI_2 ; EI_1I_2$	enzyme-inhibitor complexes
$K_1 ; K_2$	inhibitor constants; dissociation constants of the $EI_1$ and the $EI_2$ complex
$K_1' ; K_2'$	dissociation constants of the $EI_1I_2$ -complex

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- G. Engelmann "Inhibition by Mixtures of Heavy Metals" submitted for publication in the Journal of the Environmental Engineering Division

**Contractor:** Landesanstalt für Umweltschutz Baden-Württemberg  
**Contract no.:** ENV 418-80 D (B)  
**Project leader:** Dr. Werner Obländer  
Prof. Dr. Werner Weisweiler  
**Title of project:** "Investigation about the Behaviour of Thallium and other heavy Metals in thermic Processes"

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### **Objective of the research**

This research project is concerned with thermic processes, particularly cement manufacturing in relation to charge stocks, emissions and admixtures in the presence of heavy metals like thallium, lead and cadmium.

A special task in this research project is to find analytical methods to determine quantitatively the trace elements especially thallium, lead and cadmium in limestone, sand, cement, dust and other materials of interest for the cement industry. This includes the digestion of the inorganic materials, the separation of the trace elements from the matrix and finally the quantitative determination of these elements.

### **Materials and methods**

Location for measuring and sampling as well as material balances have been fixed for the pursuit of heavy metal pathes in cement production. An overall balance of all units - kiln, preheating, and filter - has been accomplished. The location for measuring and sampling sites in the plant are plotted in the material balances ( Fig. 1 ).

At the sites 5 and 7 the sampling material has to be separated from the gas stream. Because the gas has a high water content, it must be taken care, that condensation does not occur . To avoid mistakes during sampling, it is also necessary to suck off the gas with the velocity it has in the pipe. This demands a high apparative expense.

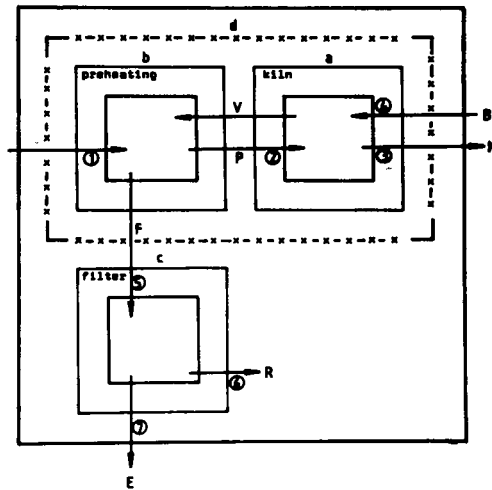


Fig. 1: Material balance flow with kiln, preheating and filter, including the points of measurement and sampling

- |               |              |               |
|---------------|--------------|---------------|
| 1 Raw meal    | 2 Kiln inlet | 3 Clincer     |
| 4 Fuel        | 5 Raw gas    | 6 Filter dust |
| 7 Treated gas |              |               |

Capital letters stand for the mass-rates of heavy metals ( g/h )

- B carried by fuel or by substitute fuel
- E carried by treated gas
- F carried by raw gas
- K carried by clincer
- P carried by raw meal after preheating
- R carried by filter dust
- T carried by raw meal
- V carried by hot gas

A digestion procedure with fluoric acid is essential for the analysis of the materials namely cement, sand and dust, which are predominantly silicates by nature.

The decomposition is carried out in an autoclave lined with polytetrafluorethylene. A mixture of fluoric acid, nitric acid and hydrochloric acid is used for digestion.

To separate the trace elements thallium, lead and cadmium from the matrix, an extraction procedure is adapted. This procedure consists of complex formation of thallium, lead and cadmium with ammonium-pyrrolidin dithiocarbamate (APDC) and diethylammonium diethyldithiocarbamate (DDTC) and the extraction of the complexes in liquid phase using methyl-isobutyl-ketone (MIBK) as solvent. For the simultaneous extraction of the three elements the optimal pH-value is found to be about 5. In the organic MIBK-extract the three elements thallium, lead and cadmium are quantitatively determined by flame atomic absorption spectroscopy. In the organic extract, the detection limit for thallium was found to be 0,07  $\mu\text{g/ml}$ , for lead 0,1  $\mu\text{g/ml}$ , and for cadmium 0,0015  $\mu\text{g/ml}$ .

## Results

Using the procedures described before the concentrations of thallium, lead and cadmium determined in raw meal after preheating, in clinker, in filter dust and in raw gas are as follows:

Range of heavy metal concentrations in raw meal after preheating, this means at kiln inlet:

Tl:	from 50 to 300 $\mu\text{g per g}$
Pb:	from < 5 to 25 $\mu\text{g per g}$
Cd:	all samples < 0,5 $\mu\text{g per g}$

Range of heavy metal concentrations in clinker:

Tl:	all samples < 10 $\mu\text{g per g}$
Pb:	from 20 to 40 $\mu\text{g per g}$
Cd:	all samples < 0,5 $\mu\text{g per g}$

Range of heavy metal concentrations in filter dust:

Tl:	from 300 to 3000 $\mu\text{g per g}$
Pb:	from < 10 to 20 $\mu\text{g per g}$
Cd:	all samples < 0,5 $\mu\text{g per g}$



**Range of heavy metal concentrations in raw gas:**

Tl:	from 200 to 4000 $\mu\text{g}$ per g
Pb:	from < 10 to 20 $\mu\text{g}$ per g
Cd:	from 1 to 40 $\mu\text{g}$ per g

**Additional Comments**

In cases where the described method is not sensitive enough to determine the exact heavy metal concentrations - as for example for cadmium in most of all sampling materials or for thallium in clinker - another method using the flameless atomic absorption spectroscopy namely the heated graphite atomizer has to be tested in future.

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Wachter, Ch., Weisweiler, W. :  
"Verfahren zur Extraktion von Thallium sowie zur Simultan-Extraktion von Blei, Cadmium und Thallium für die quantitative Analyse dieser Elemente mit der Flammen-Atomabsorptions-Spektroskopie"  
("Extraction of thallium and simultaneous extraction of lead, cadmium and thallium for quantiative analysis of these elements by flame atomic absorption spectroscopy")

Contractor : *Université de Bordeaux I - Faculté des Sciences - 351. cours de la Libération - 33405 - TALENCE - France*

Contrat n° 211 - 77 - 1 ENV F

Project leader : *Prof. R. MARTY - Directeur du Laboratoire d'Ecologie et Ecophysiologie animales*

Title of project : *Experimental trophic chain in a freshwater environment : fundamental and practical research*

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Objectives :

*In situ ecotoxicological studies of the contamination of natural aquatic systems by mercury derivatives reveal a number of phenomena typical of this type of pollution, i.e. :*

*- the concentrations of the derivatives in the free state in the environment - basically in inorganic form - are low ; the most frequent values monitored range from 0.01 to 1  $\mu\text{g}\cdot\text{l}^{-1}$  (ppb).*

*- suspended matter and sediment have a significant storage capacity ; almost all the heavy metals discharged into the aquatic environment accumulate in this way.*

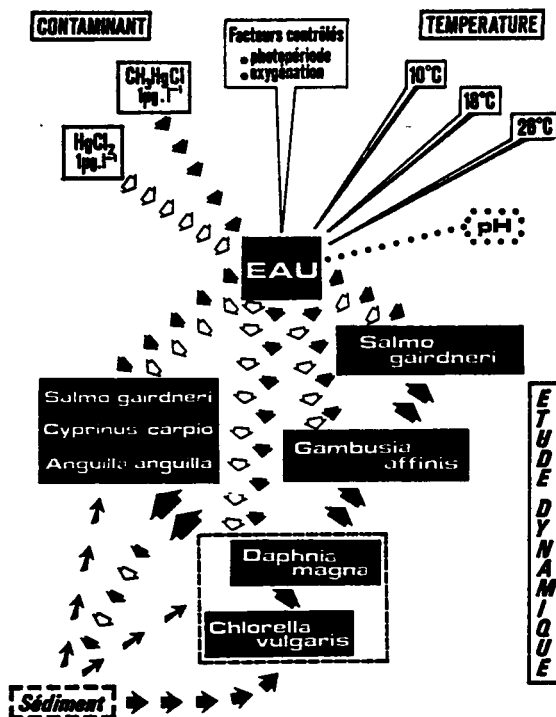
*- the inorganic and organic forms in which mercury is present vary widely according to the various compartments of the ecosystem. This complex "chemical speciation" is governed by the main abiotic and biotic factors (i.e., pH, temperature, salinity, concentration of the derivatives, bacterial processes etc.).*

*- the organisms which make up the biocenoses have a mercury bio-accumulation capacity which varies according to the species (biological concentrators), the chemical forms in which the substance is present and the abiotic conditions of the environment.*

*- as one moves up the trophic chains, bio-amplification or biomagnification of the mercury concentrations in the organisms may occur. The scale of this phenomenon is considerable in some cases, producing concentration factors of the order of 500 000, e.g. at Minamata (Japan). Furthermore, the mercury which accumulates in these third or fourth order consumers is nearly always in methyl form.*

*The setting-up of an experimental trophic chain in the laboratory will enable an ecotoxicological study to be made of mechanisms involved in bio-accumulation and the transfer of mercury derivatives in relation to the principal abiotic and biotic parameters.*

*Following the earlier research (Report on Contrat EEC N°211-77-ENV.F-1977/79) a comparative study of  $\text{HgCl}_2$  and  $\text{CH}_3\text{HgCl}$  at a concentration of  $1 \mu\text{g}\cdot\text{l}^{-1}$  in water (1 ppb) is now in progress. Figure 1 shows the structure of the model used and the main parameters measured.*



*Figure 1 : Experimental trophic chain : structure and parameters measured.*

*Since a perfect simulation of the ecosystems is impossible, the basic aim of this experimental approach - under simplified conditions which are monitored and gradually made more complex - is to gain a better understanding, from both the qualitative and quantitative points of view, of the phenomena occurring in aquatic systems, which are heterogeneous and highly complex.*

**Material and methods :**

*The experiments are designed to quantify the amounts of mercury directly accumulated (1 ppb in water -  $HgCl_2$  and  $CH_3HgCl$ ) by each link in the chain and also the transfers which occur in the trophic chain after general contamination (direct and trophic) and the concentration factors in the different consumer levels.*

*The experimental conditions are varied for each species studied depending on ecophysiological requirements, rates of development and population characteristics, i.e. :*

- Chlorella vulgaris : synthetic medium (1 litre Erlenmeyer flask), 25 mg dry weight/l (d.o.  $35.10^{-3}$  at 665 nm), photoperiod 16hrs (7hrs-23hrs)/24hrs, temperature : 10, 18 or 26°C, accumulation dynamics over 24 hours with additional analysis up to 144hrs.

- Daphnia magna : Synthetic medium (1 litre Erlenmeyer flask), 300 water-fleas (Daphnia sp. : 6 days old) per unit, photoperiod 16hrs/24hrs, temperature : 10°C, 18°C or 26°C ; accumulation dynamics over four days.

- The fish links in the trophic chain : various species were used, i.e. : Salmo gairdneri (fry and adult fish), Cyprinus carpio, Gambusia affinis. Since the comparative study of the two mercury derivatives required similar concentrations of the metal in water, it was necessary to set up automated direct contamination systems (Figure 2) in view of the differing time histories of the two derivatives in an aqueous solution ( $1 \mu\text{g.l}^{-1}$ ).

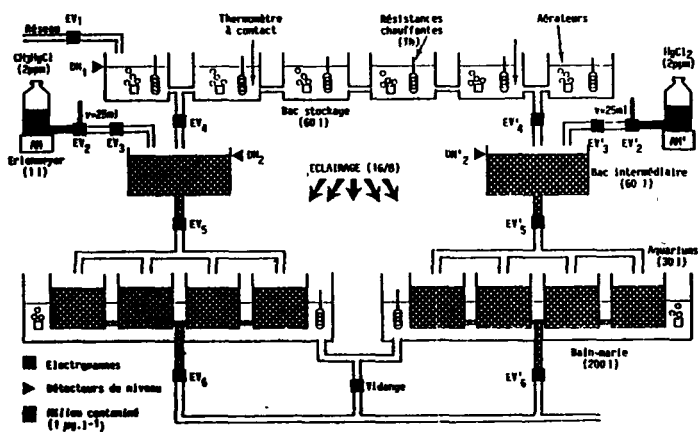


Figure 2 : Diagram of automated direct contamination modules.

The experiments are based on a dynamic study of the bioaccumulation of the two derivatives in relation to the two contamination routes over a period of 30 days at temperatures of 10°C, 18°C and 26°C respectively.

In the case of Salmo gairdneri, the mercury distribution in the main organs was investigated : liver, brain, gills, muscle, hind-gut, blood, spleen, kidney and also global - with a view to determining their typical ecotoxicological behaviour in relation to time, ambient temperature, species, etc.

In the case of algae and daphnia, the results obtained from a study of each parameter, i.e. the specific mercury derivative, temperature, contamination route, exposure time, are based on an analysis of the six experimental tanks. In the case of fish, the number is determined according to a maximum load (biomass/volume of the contaminated media/replacement cycle), ranging from 6 individuals per tank (adult trout : average weight : approximately 30 g) to 24 individuals per tank (trout fry and gambusia ; average weight : approximately 2 g).

The mercury concentration readings are expressed in  $\mu\text{g}\cdot\text{g}^{-1}$  (fresh weight) and in  $\mu\text{g}\cdot\text{l}^{-1}$ ; this enables the concentration factors in the organisms or organs investigated to be calculated, i.e.  $\mu\text{g}\cdot\text{g}^{-1} / \mu\text{g}\cdot\text{l}^{-1}$ . The comparisons have been made on the basis of average samples, taking account of the distribution (confidence interval  $P = 0.05$  plus homogeneity tests). A factorial method of analysing the relationships has been used to make a simultaneous comparison in terms of the various parameters of distribution of the two derivatives in the body tissue.

Since 1978, flameless atomic absorption (VARIAN AA 175) has been used to determine the amount of mercury present in the water and organism samples. The detection limit is normally 5 ng for a 50 ml sample.

### Main results :

In order to illustrate the importance of the main parameters investigated, we give below some examples of our results extracted from the published reports :

- Primary producer : - Chlorella vulgaris : Analysis of the bio-accumulation of mercury shows the importance of the exposure time factor. The nature of the derivative, and the ambient temperature, also affect this process, i.e. :  $\text{CH}_3\text{HgCl}$  accumulates more readily than  $\text{HgCl}_2$ , and the phenomenon is augmented by any rise in temperature (Figures 3 and 4).

- Primary consumer : - Daphnia magna : Contamination of this "link" in the trophic chain leads to a distinct predominance of the organic derivative over the mineral derivative. Furthermore, the temperature factor strongly influences this transfer balance by a factor of between 3 and 10 (Figure 5).

- Second and third order consumers : By using the experimental trophic chain, the respective significance of the direct and trophic contamination routes may be quantified in terms of exposure time and ambient temperature. Accordingly, in the case of Gambusia affinis, after direct exposure and trophic contamination (contaminated daphnia), over a 30-day period at 26°C, the recorded concentration exceeded 25 000. A comparison between  $\text{HgCl}_2$  and  $\text{CH}_3\text{HgCl}$  was also carried out on the Salmo gairdneri link ; as an example, Figure 6 shows the tissue concentrations of mercury after direct contamination ( $1 \mu\text{g}\cdot\text{l}^{-1}$ ) at 10°C and after 30 days' exposure.

### Conclusion :

Our results show that each parameter studied, i.e. contamination period, chemical nature of contaminant, ambient temperature, contamination routes, species and trophic levels - causes significant change in the mercury bio-accumulation processes.

Our research is now being directed towards a more complex version of the present ecotoxicological model (incorporation of the "sediment" factor, systemic approach) ; a long-term study of the tissue decontamination processes is also under way.

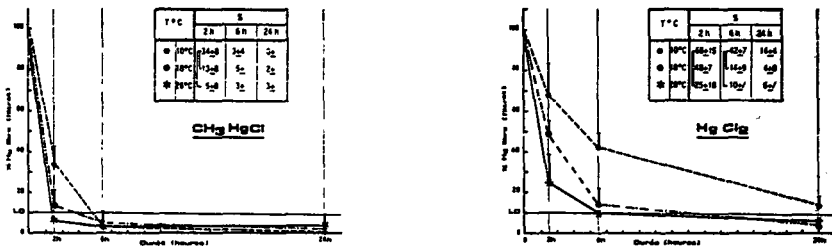


Fig. 3 et 4 : Dynamic of mercury bioaccumulation by *Chlorella vulgaris*.

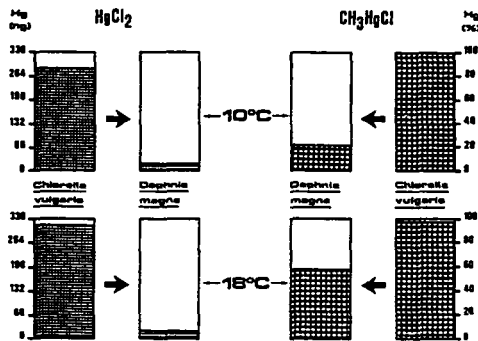


Fig. 5 :  $HgCl_2$  and  $CH_3HgCl$  transfert between *Chlorella vulgaris* and *Daphnia magna*.

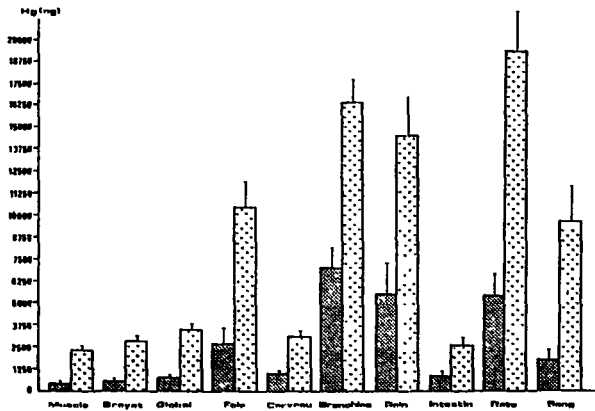


Fig. 6 : Mercury tissular repartition (*Salmo gairdneri*) after 30 days of direct contamination with  $HgCl_2$  and  $CH_3HgCl$  (10°C).

List of publications and oral communications :

1. Chaînes trophiques expérimentales (études écotoxicologiques fondamentales et appliquées) - Symbioses IX, n°1, 1977, 57-70.
2. Modèles expérimentaux en écotoxicologie : chaînes trophiques en milieu limnique. Bulletin d'Ecologie 8(4), 1977, 401-414.
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4. Experimental trophic chains in freshwater environment : their ecotoxicological interests both fundamental and practical. Research seminar (ecological tests relevant to the implementation of proposed regulations concerning environmental chemicals : evaluation and research needs) Berlin-Ouest, décembre 1977.
5. Ecotoxicological studies on an experimental trophic chain in freshwater environment - Workshop European Communities - Water Research Center - Windermere (Angleterre) - 1977.
6. Ecotoxicological action of mercury by-products on different animal tissues - International workshop on monitoring environmental materials and specimen banking - Berlin-Ouest, octobre 1978.
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13. Chaîne trophique expérimentale en milieu limnique : contamination directe d'un consommateur de troisième ordre (Salmo gairdneri) par le méthylmercure - Water, Air and Soil Pollution 14, 1980, 339-347.
14. Chaîne trophique expérimentale en milieu limnique : contaminations directe et trophique d'un consommateur de troisième ordre (Salmo gairdneri) par le méthylmercure. Water, Air and Soil Pollution 14, 1980, 349-357.
15. Bioaccumulation et bioamplification des dérivés du mercure par un consommateur de troisième ordre : Salmo gairdneri. Incidences du facteur température. Water Research 14, 1980, 61-65.
16. Symposium International sur les "principes à appliquer pour l'interprétation des résultats d'essais en écotoxicologie", Sophia-Antipolis (France), Octobre 1980.
17. Freshwater Contact Group, National Institute for Physical Research Dublin (Irlande), Octobre 1980.
18. Contamination d'une chaîne trophique expérimentale par le méthylmercure : importance du système "Producteur-Consommateur primaire" - Environmental Pollution (Series A) 24, 1981, 193-206.



Contractor: ASSORENI (former SNAMPROGETTI)

Contract: n° 293-76-1-ENVI

Project Leader: P. Garibaldi

Title of project: Environmental tracing of petrol lead

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The Isotopic Lead Experiment (ILE) is a large-scale, stable isotope tracer experiment using as antiknock agent added to gasolines, an alkyl lead produced with lead from the Australian Broken Hill mine.

This lead has an isotopic composition ( $Pb-206/Pb-207 = 1.04$ ) which is significantly different from that of the lead found in the environment of the test area ( $Pb-206/Pb-207 = 1.18$ ) at the beginning of the experiment. The test area is the Region of Piedmont in Northwest Italy. The primary goal of the ILE project is to know how much of the lead in the human blood comes from motor traffic. Other objectives are the determination of the pathways of lead from fuel through air, soil, vegetation and food to the human blood.

The sampling is focused on the central part of the test area, namely the city of Turin and its surroundings within a radius of 40 km, and extended to a few remote areas. The sampling plan includes the source, the environment and the population. The samples collected until December 1980 are listed in Table I.

TABLE 1 SAMPLES TAKEN FROM JULY 1974 TO DECEMBER 1980

---

<u>Source</u>		<u>Environment</u>	
metallic lead	326	Airborne particulate	6826
alkyl lead	74	soil	148
gasoline at refineries	192	river sediments	28
gasoline from retailers	2332	vegetation	391
		rain	412
<u>Population</u>		snow	11
		honey	20
blood	3317	milk	53

---

Overall 14,130 samples have been collected.

The samples are analysed for their lead isotopic composition by mass spectrometry and for their total lead content by X-ray fluorescence and/or by atomic absorption.

Since August 1975 all refineries in North-West Italy were partially supplied (average 50%) with lead alkyl derived from the Australian Lead.

Starting from May 1977 until December 79 about 99% of the alkyl lead supplied to these refineries was manufactured with the Australian lead.

Based on the supply of the Australian lead to the refineries the study can be divided in three phases, as reported in fig. 1.

- the background phase A: 0% Australian lead
- the transition phase B: about 50% Australian lead
- the final phase C: ~ 99% Australian lead

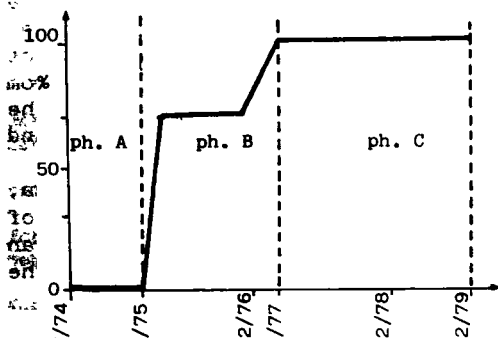


Fig. 1 - AUSTRALIAN LEAD DISTRIBUTED IN NORTH-WEST ITALY

It has been impossible to control the subsequent phase because as of January 1980 the supply of lead alkyl to the refineries in the area was resumed by different companies as before this study started.

The lead in the engine exhaust has been monitored by periodically sampling the gas distribution network in the area and determining the lead isotopic ratio.

A weighted average has been computed by applying importance factors to the market share, to the Company and to the distance from Turin of the sampling area.

The results obtained are illustrated in fig. 4.

## ENVIRONMENT

The atmospheric particulate is the most important part of the environmental samples, because it is the direct way by which lead from gasoline can be absorbed by the human beings.

Samples were taken weekly in the city of Turin in fixed stations placed at eight points with different topographical conditions and traffic intensity, and daily at Ispra; in the other areas of the country portable low volume samplers were used every 3 months, which allowed taking 12 consecutive samples of 24 hours intervals.

In the city of Turin 24 hour samples were taken to evaluate the mean exposure level of the population and 4 hour samples were taken to find the maximum exposure level of the population in hours of heaviest traffic.

The lead concentration values are summarized in Figure 2.

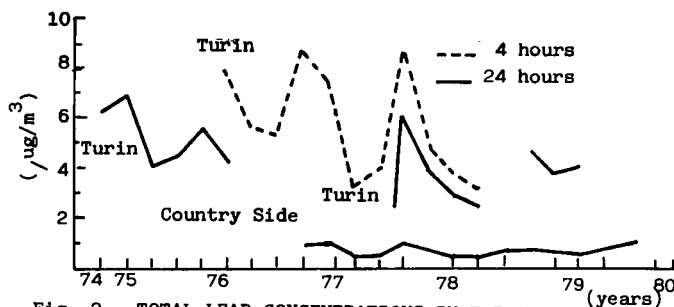


Fig. 2 - TOTAL LEAD CONCENTRATIONS IN THE AIR

The values of the Pb-206/Pb-207 ratio during the experiment are shown in fig. 4. There are two levels of values, one for rural locations of about 1.09 and one for Turin of about

1.06. This last value shows that in the city there is, for the atmospheric particulate, a variation in the isotopic ratio similar to that of gasoline.

The purpose of the examination of other types of environmental samples which will be analysed later, is:

- river sediments: to determine the probable variation of lead concentration and isotopic composition in the samples taken in urban areas, in comparison to those of areas next to the sources of the river, which is an indication of the impact of pollution due to urban industrial areas and motor traffic;
- soil: to evaluate the lead deposited by fall-out between the beginning of the experiment and the sampling date, as well as its possible migration;
- vegetation: to obtain information on the transfer of metals in vegetation by means of the probable variation in isotopic ratio in different parts of the plant;
- precipitations: to provide information on the type of lead present and on the quantity washed out from the atmosphere by means of rain and snow;
- honey: to find an useful indicator of atmospheric pollution, as bees are subject to pollution in a restricted zone;
- milk: to determine the atmospheric pollution of the human beings through the chain air  $\rightarrow$  grass  $\rightarrow$  cow  $\rightarrow$  milk  $\rightarrow$  people.

### POPULATION

Over 3300 samples of blood have been obtained from different groups living in town and in the country.

The population groups examined are:

Group I	Adults sampled in 1974/79 (No Follow-up)	1066
Group II	Adults sampled in 1975/79 (Follow-up)	458

Of each individual of the group II the residence place, sex, age, habits (namely smoking and drinking) and profession are known.

The table 3 gives a general split of the group II.

TABLE 3 - DISTRIBUTION OF ADULT SUBJECTS (FOLLOW-UP) BY RESIDENCE PLACE, SEX, HABITS AND PROFESSIONS

RESIDENCE PLACE	Turin	184	-
	Rural areas	274	-
SEX	Male	374	(Turin 174)
	Female	84	(Turin 10)
HABITS	Smokers	224	(Turin 104)
	Drinkers	359	(Turin 148)
	Smokers and drinkers	188	(Turin 90)
	No smokers, no drinkers	63	(Turin 22)
PROFESSIONS	Farmers, woodcutters, ecc.	12	(Turin -)
	Students, Office workers		
	House-wives, Pensioners	145	(Turin 19)
	Industrial workers	200	(Turin 88)
	Exposed workers	101	(Turin 77)

The distribution of the subjects in terms of overall sampling during the overall project is reported in Table 4.

TABLE 4 - DISTRIBUTION OF ADULT SUBJECTS (FOLLOW-UP) BY NUMBER OF SAMPLINGS

PLACE	1	2	3	4	5	6	7	8	9	10	11	12	TOTAL OF SUBJECTS	TOTAL OF SAMPLINGS
Turin	-	9	88	81	-	1	2	3	-	-	-	-	184	650
Country	-	46	48	63	37	30	18	10	12	6	2	2	274	1273
Total	-	55	136	144	37	31	20	13	12	6	2	2	458	1923

The distribution of lead concentration in blood per locality relative to subjects is given in Table 5. The data are expressed by ranges of concentration of lead in blood and obtained by repeated determinations on the same subject from 1975 to 1980.

TABLE 5 - LEAD IN BLOOD CONCENTRATION ( $\mu\text{g}/100 \text{ ml}$ ). PERCENT DISTRIBUTION FROM 1975 TO 1980 PER LOCALITY RELATIVE TO 458 SUBJECTS

Locality	Subjects	Samplings	0-10	11-20	21-30	31-40	41-50	50
Turin	184	401	-	72	88	19	4	-
Lanzo	40	142	-	7	21	7	4	1
Viù	63	267	1	8	25	21	8	-
Nole	46	168	-	11	21	12	2	-
Santena	27	85	-	11	7	8	1	-
Fiano	21	82	-	8	7	5	-	1
Castagneto	31	138	-	10	15	5	1	-
Druento	27	87	1	13	8	4	1	-
Balangero	19	55	-	7	9	2	1	-

More than 400 samples of blood have been analysed for the isotopic composition.

The analytical results are highly reliable (e.g.  $1.1500 \pm .0008$ ). Quality control tests between CCR-Ispira and Bruxelles University have been made.

A preliminary evaluation of data shows a reduction of 206/207 isotopic ratio in blood that is slightly different in towns and in villages far from Turin less or more than 20 Kms.

The average results are reported in table 6 and fig. 3.

TABLE 6 - RATIO Pb 206/207 IN BLOOD. MEAN VALUES

	1975	1976	1977	1978	1979
Turin	1.1624	-	1.1426	1.1358	1.1284
<20 km	1.1591	1.1492	-	1.1447	1.1384
>20 km	1.1604	1.1509	1.1505	1.1498	1.1458

A summary picture of isotopic composition data is reported in fig. 4, showing the changes in the 206/207 isotopic ratio versus time in the samples divided according to source, environment and population.

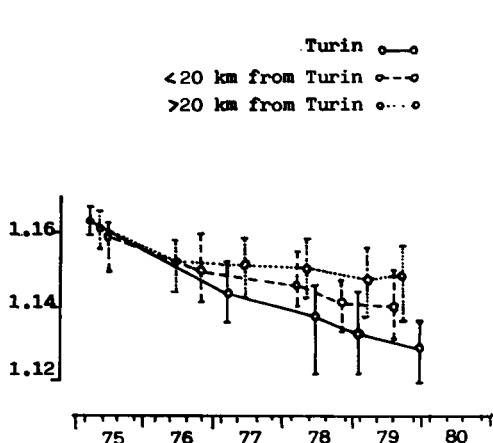


Fig. 3 - BLOOD LEAD RATIO 206/207

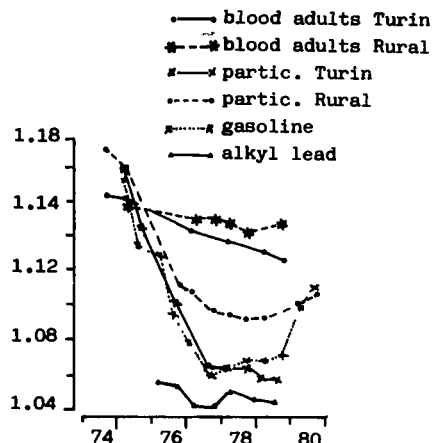


Fig. 4 - Pb 206/207 RATIO

CONCLUSIONS

The isotopic composition technique has offered an innovative and valuable tool for assessing the impact of automotive traffic on the lead pollution of the environment in general and the human beings in particular.

In town most of the airborne lead derives from the engine exhaust.

Lead from other sources contributes to a large extent (about 50%) to the airborne lead in the country side.

As for the human beings, the isotopic ratios of lead in the blood so far determined suggest the following conclusions:

In the population of Turin the reduction of the 206/207 ratio in the blood after 2,5 year of exclusive use of Australian lead was about 30% of the maximum attainable value. This reduction was about 20% and about 15% respectively for the population living in places far from Turin less then 20 kms and more than 20 kms.

The analytical work in progress at CCR-Ispra and the statistical examination of the results will produce in a few months a more accurate assessment of the contribution of traffic to the lead pollution in the environment and in the human beings.

Contractor : The Foundation Institute for Soil Fertility, ~~as follows~~  
Haren (Gr), The Netherlands.  
Contract : N<sup>o</sup> 199-77-1 ENV N  
Project leader : dr. W. Salomons  
Title of the project : Dynamics of heavy metals in fluvial and limnic  
sediment-water systems

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### 1. Objective of the research.

A number of large lakes in Western Europe are fed by more or less contaminated rivers. Examples are Lake Constance, Lake Geneva and Lake IJssel (IJsselmeer). Especially the IJsselmeer in the Netherlands has become seriously contaminated with heavy metals. It is fed by a distributary of the river Rhine. In this lake a number of processes affect the distribution of the heavy metals over the dissolved and solid state (attached to the suspended matter or the deposited sediments). The IJsselmeer will be used as a model to describe the changes in occurrence of heavy metals when a contaminated river flows into a lake. Over a period of two year samples have been taken from the surface waters, the suspended matter, the deposited sediments and from the interstitial waters. In these samples the contents of mercury, lead, cadmium, copper, nickel, zinc and chromium have been determined. Additionally samples from the algae in the lake have been analysed for heavy metals, to determine their influence on the bio-geochemical cycle of heavy metals. The pH-variations in the lake, which determine to a great extent adsorption-desorption processes have been studied with the methods of stable isotope geochemistry. Laboratory experiments have been conducted on the adsorption behaviour of heavy metals onto the suspended matter. Both the results from field and laboratory experiments will be used to construct a model, which simulates the behaviour of the dissolved metals in the lake.

### 2. Materials and methods

A large variety of techniques is used in this combined field and laboratory study. For the sampling of surface waters specially constructed all-teflon made filtration apparatus is used. Suspended matter is sampled in teflon-lined centrifuges. Heavy metals in the sediments, water, suspended matter and algal material are analysed with atomic absorption and with neutron activation analysis. Mass spectrometry is used in the stable isotope studies. For the adsorption studies radiochemical techniques are applied.

### 3 Results

#### 3.1 introduction

Large amounts of trace metals are transported by the river Rhine and its affluent the river IJssel to the North Sea. Before reaching the North Sea the river water from the IJssel spends about 6 months in the artificial lake IJsselmeer, formed in 1932 when the Zuiderzee was separated from the North Sea (figure 1). The surface area of the shallow lake has gradually decreased as a consequence of reclamation projects. The present surface area is 1230 km<sup>2</sup>, including the Ketelmeer (the mouthing area of the river IJssel), which originated in 1955). The mean depth of the lake is 4.5 m and the volume of the water body is 5.5 km<sup>3</sup>. The river IJssel is the main water source and is largely responsible for the heavy metal input (1700 tons/year) into the lake.

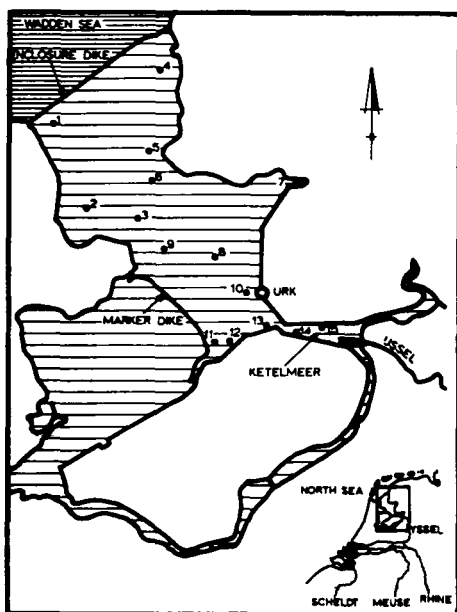


Figure 1. Map of the IJsselmeer showing the sampled localities for the deposited sediments.

#### 3.2 Changes in total dissolved and particulate metal concentrations in the lake.

Heavy metals are transported both in solution and attached to the suspended matter by the river Rhine and the river IJssel. The concentration of metals in the suspended matter depends on the discharge (SALOMONS AND EYSINK 1981). High concentrations are observed during periods of low discharge, and low concentrations during periods of high discharge. During transport in the river system and during transport in the lake changes occur in the ratio of dissolved to particulate metals. In figure 2 the particulate and dissolved cadmium concentrations in the river Rhine at the Dutch-German border are compared with these concentrations in the river IJssel and in some locations of the lake.



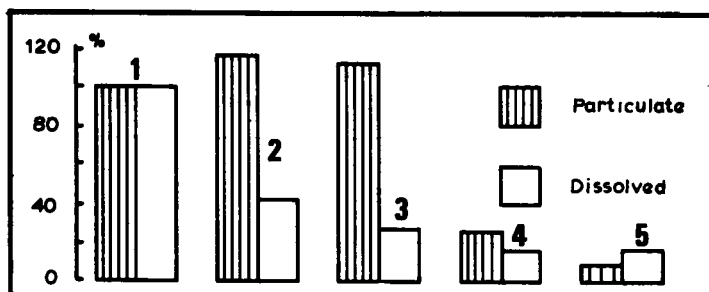


Figure 2. Cadmium concentrations in the river Rhine (1), the river IJssel (2), the Ketelmeer (3), the IJsselmeer (4) and in the water leaving the lake through the sluices in the Enclosure dike (5).

During transport in the river system, the particulate cadmium concentrations increase and those in solution decrease. This change is probably due to an increase in pH of the riverwater. In the Ketelmeer and in the IJsselmeer this decrease in dissolved cadmium concentrations continues. However, also the particulate metal concentrations start to decrease. The decrease in dissolved metals concentrations is reflected in the metal concentrations observed in the bivalve *Dreissena Polymorpha*. This correlation shows the importance of dissolved metal concentrations for the accumulation of metals by organisms.

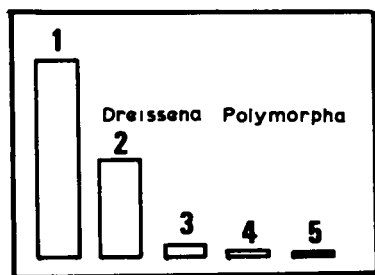


Figure 3. Accumulation of cadmium by *Dreissena Polymorpha* at the localities given in figure 2. (MARQUENIE 1981)

Metal concentrations in the sediments of the IJsselmeer are high in the Ketelmeer area but much lower in the IJsselmeer (table 1).

	Zn	Cu	Cr	Pb	Cd	Ni	Hg	n
Ketelmeer 1977	1960	250	570	320	34	67	5	60
IJsselmeer 1977	430	40	94	73	2.8	30	0.8	85
IJsselmeer 1933	133	19	88	39	0.4	39	--	12

Table 1. Concentrations (ug/g) of trace metals in sediments (data corrected for grain-size differences by the 16  $\mu\text{m}$ -method: all concentrations are given at 50 %  $< 16 \mu\text{m}$  (FÖRSTNER AND SALOMONS 1980)

### 3.3 Processes affecting metal concentrations in the lake

The differences in concentrations of dissolved trace metals between the incoming water from the river IJssel and the water leaving the lake (figure 2, table 2) show that the lake acts as a sink for dissolved metals.

	Zn	Cu	Cr	Pb	Cd
IJssel	40	5.5	2.3	1.5	0.8
Enclosure dike	4.3	2.7	0.3	1.6	<0.1

Table 2. Mean concentrations of dissolved heavy metals ( $\mu\text{g/l}$ )

The decrease in metal concentrations in the lake correlates for Zn, Cd en Cr with the pH (figure 4). The small increase in the pH in the Ketelmeer of 0.3 units, results in a decrease in dissolved cadmium from about 0.8  $\mu\text{g/g}$  to less than 0.1  $\mu\text{g/g}$ .

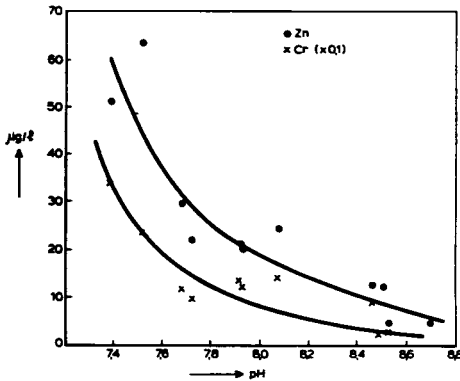


Figure 4. Correlation between dissolved zinc and chromium and the pH.

In the laboratory pH-dependent adsorption processes have also been studied by adding radioactive cadmium and zinc to suspended matter from the lake and measuring the adsorption as a function of the pH. Some results are presented in figure 5.

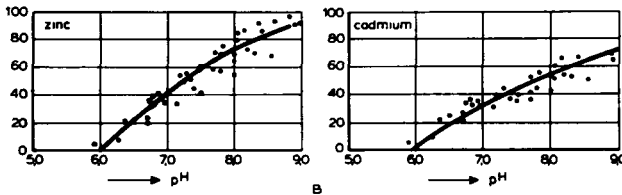


Figure 5. Adsorption (in % of added metal) of cadmium and zinc on suspended matter from the lake.

The processes which determine the pH of the lake (e.g. the carbon cycle) have been studied with the methods of stable isotope geochemistry. The results showed, that algae are the most important factor. During the autumn-winter period the release of pore waters (high alkalinity) by erosion of the deposited sediments have to be taken into account.

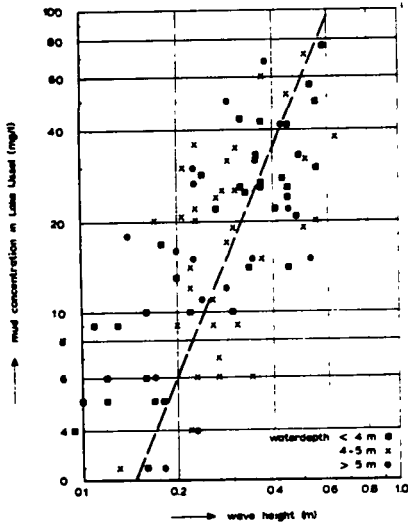


Figure 6. Relationship between waves and the suspended matter content of the water in the lake.

The IJsselmeer is very shallow (mean depth 4.5 m) and sediments are easily stirred up by waves. Figure 6 shows the correlation between wave height and suspended matter concentration.

The erosion of the bottom deposits results into a release of pore waters with a high alkalinity which influences the pH of the surface waters.

The bottom deposits contain low concentrations of heavy metals. The resuspended bottom sediments (low metal concentrations) mix with the recent suspended matter from the river IJssel (high metal concentrations). As a result hardly any metal gradients in the bottom deposits are observed in lake IJssel. This mixing process also explains the decrease in particulate metal concentrations (figure 2) In the IJsselmeer a carbonate precipitation takes place in summer due to the increase in pH upto values of 9 in summer. In fact seasonal variations in the calcium concentrations of the water are observed. The total amount of calcium carbonate which precipitates in the lake is higher than the sediment supply by the river IJssel (SALOMONS AND MOOK 1980). The admixture of carbonates with low metal concentrations to the deposited sediments causes an additional decrease in metal concentrations.

Algae indirectly influence metal concentrations in the lake by changing the pH of the surface waters. However, algae also take up heavy metals from the water. In this way trace metals accumulate with the algal matter in the bottom sediments. By measuring metal concentrations in algal material and correction for the inorganic matter present in the samples an estimate could be made of the removal of dissolved trace metals by algae. Results are presented in figure 7.

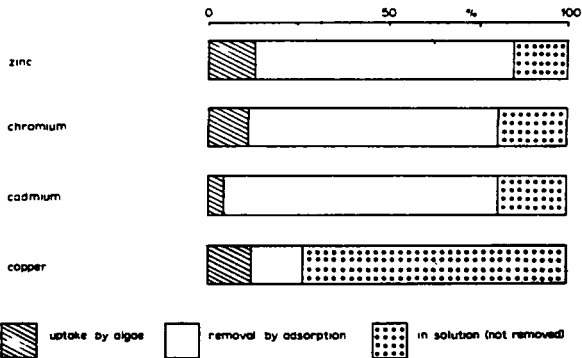


Figure 7. Fate of dissolved heavy metals in the IJsselmeer

Algae appear to account for about 4-14 % of the removal of dissolved metals. For copper, algae are seen to provide an important mechanism for removal, whereas for dissolved cadmium, zinc and chromium, the pH-dependent adsorption is the major process. The adsorption of copper does not depend on the pH between values of 7 and 9 (the range observed in the IJsselmeer), therefore the lake does not act as a major sink for dissolved copper.

#### 4 Conclusions

1. The IJsselmeer acts as a sink for dissolved chromium, zinc and cadmium due to pH-dependent adsorption processes in the lake. The increase in the pH is due to algal blooms. The algae account for a minor part of the removal of trace metals.
2. Physical mixing processes are important in the lake due to its shallowness. The erosion of bottom deposits and their admixture with recent contaminated suspended matter from the river are mainly responsible for the decrease in particulate metal concentrations. A further decrease is caused by the precipitation of carbonates (low metal concentrations) in the lake causing a "dilution" of the high metal concentrations in the sediments.

#### Additional remarks:

- A mathematical model of the processes affecting the dissolved metals will be made in 1981.

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- W. Salomons and W.D. Eysink (1981) *Proc. Holocene sedimentation in the North Sea Basin*. Blackwell. (In press).

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- W. Salomons and W.G. Mook (1980) Biogeochemical processes affecting metal concentrations in lake sediments (IJsselmeer, the Netherlands). *Sci. Total Env.* 16, 217-229.
- W. Salomons and W.D. Eysink (1981) Pathways of mud and particulate metals from rivers to the Southern North Sea; In: *Proceedings Holocene Sedimentation in the North Sea Basin*. Blackwell (in press)
- W. Salomons, W.G. Mook and W.D. Eysink (1981) Biogeochemical and hydrodynamic processes affecting heavy metal in rivers, lakes and estuaries. Delft Hydraulics Laboratory Publication No 253.

#### ORAL COMMUNICATIONS

- W. Salomons and W.G. Mook (1978) Processes affecting trace metals in Lake IJssel 10th International Congress on Sedimentology, Jerusalem 1978
- W. Salomons (1978, 1979, 1980) Several meetings of the "Freshwater Contact Group" in Metz, Ispra and Dublin.
- W. Salomons and W.D. Eysink (1979) Pathways of mud and particulate metals from rivers to the Southern North Sea. Congress on Holocene Sedimentation in the North Sea Basin. Texel, the Netherlands.

Contractor: Association EURATOM-ITAL

Contract No. 255-77-1 ENV N

Project leader: P. Poelstra, subsequently M.J. Frissel

Title of project: The contamination of vegetation, surface water and deep ground water (drinking water) by heavy metals unintentionally released into soils

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### Objective of the research

The aim of the study was to obtain insight in the behaviour of Cd in soil. Within the study the following elements can be considered: a. Cd adsorption and desorption measurements on soils. Data obtained were used to predict accumulation in topsoils and leaching to subsoils. b. Determination of Cd leaching in soil columns. These data were used to verify the predictions stated under a. c. Literature review on Cd uptake studies by crops. The review provided data to predict the uptake of Cd by the crop from data measured under a. d. Further predictions based on assumed Cd input data and calculations of Cd leaching to subsoil and Cd removal by the crop.

### Material and Methods

#### Soils

Five soils were used. Two of these soils have a higher than normal Cd concentration, two of the other soils were specially selected to study the fate of Cd from phosphate fertilizer dressings.

Sandy soil Branschweig: Slightly acid sandy soil used since 1895 for dumping sewage water with an average of 900 mm annually. Exchange capacity top layer 31,6 me per 100 g, at a depth of 100 cm approx. 2 me per 100 g. Total Cd content: top layer 3,5 mg Cd kg<sup>-1</sup>, decreasing to 0,75 mg Cd kg<sup>-1</sup> at a depth of 40 cm, pH 6,5 at the top and 5,4 at a depth of 1 meter.

Peat soil Schoonebeek: N.E. part of the Netherlands. Peaty soil used as permanent pasture, organic matter content for layer 0-100 cm 95%, pH (H<sub>2</sub>O) 5,1. Total Cd concentration 1,4 mg·kg<sup>-1</sup> in the upper 10 cm, decreasing to 0,2 mg·kg<sup>-1</sup> at 20 cm depth.

Clay soil Valburg: Soil from the foreland of the Rhine river, used as permanent pasture. Layer 0-30 cm: Fraction < 2 μm 23%, organic matter 6%, traces CaCO<sub>3</sub>; Layer 30-100 cm: Fraction < 2 μm 20%, organic matter 1%, traces CaCO<sub>3</sub>. pH (H<sub>2</sub>O) of both layers 7,4. Total Cd concentration at soil surface 6,5 mg·kg<sup>-1</sup> decreasing to 1 mg·kg<sup>-1</sup> at a depth of 35 cm, and decreasing further to 0,5 mg·kg<sup>-1</sup> at a depth of 100 cm.

Sandy soil Doetinchem: Old arable land. pH (in artificial rain water) 6,0. Organic matter 0-5 cm 6,9%, 5-10 cm 4,4%, at lower depths 4%. Exchangeable cations in 0-5 cm layer: K 0,4, Na 0,1, Ca 5,4 and Mg 1,1 me per 100 g. Extractable Cd 0-20 cm 0,2 mg per kg soil.

Löss soil Haasdal: Arable land. pH (in artificial rain water) 5,1. Organic matter 0-5 cm 3,8%, 5-10 cm 1,9%. Exchangeable cations in 0-5 cm layer: K 0,2, Na 0,1, Ca 5,1 and Mg 0,2 me per 100 g. Extractable Cd varies between 0,45 and 0,87 mg/kg for the upper 20 cm.

Table 2 - Accumulation of Cd in top cm of soil as percentage of the total amount supplied. Continuous supply. Calculated results based on  $K_D$  values of fig. 3, experimental results see text. Cd level less than 1 mg per kg of soil.

Loess soil, Haasdal				Sandy soil, Doetinchem			
rain supplied				rain supplied			
102,5 ml		200 ml		102,5 ml		200 ml	
calc.	exp.	calc.	exp.	calc.	exp.	calc.	exp.
64,2	42,2	45,4	71,3	89,7	84,9	81,4	67,6

Table 3 - Accumulation of Cd as percentage of the total amount supplied. Continuous supply. Calculated values for an effective amount of precipitation of 1000 cm (25 to 50 years) and a soil density of 1,2 g dry matter per  $\text{cm}^3$  of bulk soil.

$K_D$ $\text{g}\cdot\text{cm}^{-3}$	Accumulated fraction %
25	1,9
50	4,0
100	8,2
200	16,5
400	31,5
800	51,7

### Conclusions

From the adsorption/desorption experiments it appears that adsorption is not a function of the redox potential, but is strongly dependent on the pH. Adsorption curves were linear to  $0,03 \text{ mmole l}^{-1}$  for pH values up to 6,5 and to  $0,01 \text{ mmole l}^{-1}$  for a pH up to 7. The influence of the pH on the sandy soil from Doetinchem and löss soil from Haasdal is shown in fig. 1. The influence of the pH seems even more important than the soil type. The  $K_D$  (distribution ratio of Cd between soil and solution in  $\text{g}\cdot\text{cm}^{-3}$ ) varies from  $25 \text{ g}\cdot\text{cm}^{-3}$  at pH 4,4 to values between 600 and  $700 \text{ g}\cdot\text{cm}^{-3}$  at pH values above 6,2. These differences mean that the leaching properties must also vary by a factor of 20 to 30, depending on pH. The differences in Cd uptake by the crop are probably somewhat less pronounced, however, enormous differences in Cd uptake (for equal Cd levels in the soil) must be expected. This means that general recommendations for permissible Cd levels in soil cannot be based on average values. The use of average values will induce high risks in particular areas, and at the same time induce excessive restrictions in other areas.

Adsorption/Desorption measurements

Adsorption was measured in 0,01 mole·l<sup>-1</sup> CaCl<sub>2</sub> or in a 1,7×10<sup>-4</sup> N mixture of chlorides of Ca, Na and K (ratio 3 : 1 : 1). The pH was varied by HCl or NH<sub>4</sub>OH; pH and Cd in solution were measured after 24 h equilibration. Redox potential was varied by adding small amounts of glucose and keeping the sample vial airtight. Bacterial activity induced low oxygen concentration and low redox potential; equilibration time varied from 15 min to 1 week. For high Cd concentrations stable Cd was determined by atomic absorption, for low Cd concentrations <sup>115m</sup>Cd was used as a tracer.

Column experiments

Transport was observed in undisturbed soil columns, length 120 cm, diameter 12 cm. The soil was non-watersaturated. Rain was applied by a rain simulator. <sup>115m</sup>Cd transport was measured by scanning (non destructively) or by slicing the column and analysing each slide. There were aerobic and anaerobic sections in the column.

Results

The results of the adsorption desorption experiments are shown in table 1 and fig. 1. The results of the leaching experiments with the column are shown in the figs. 2 and 3 and the tables 2 and 3.

Table 1 - Some K<sub>D</sub> values for Cd

Soil	K <sub>D</sub> g·cm <sup>-3</sup>	pH	E <sub>H</sub> mV	Highest conc. in ads./des. series mmole l <sup>-1</sup>	Remarks
Valburg, clay soil	670	7,4		0,01	ads./des.
Schoonebeek, peat soil	230	5,1		0,01	ads./des.
Braunschweig 0-20 cm	33	6,5		0,03	ads./des.
30-40 cm	18	6,5		0,03	ads./des.
0- 5 cm	5,6	5,0	143	0,2	ads./des.
0- 5 cm	4,9	4,8	206	0,2	ads./des.
0- 5 cm	51	6,7	206	0,02	ads./des.
0- 5 cm	1100	8,5	206	0,001	ads./des.
Valburg, clay soil	710				column
Schoonebeek, peat soil	320				column
Braunschweig	33				column
Braunschweig, anaerobic	43				column

The  $K_D$  values which were used for the calculated Cd distribution are shown in the figs. 2 and 3 are compared with the  $K_D$  values obtained from the adsorption/desorption measurements in table 1. The agreement is good, except for anaerobic conditions. More data are presented by Poelstra et al. (1979). From the data it can be concluded that there exists a good agreement between actual leaching and calculated leaching (based on adsorption/desorption measurements). Only the behaviour under anaerobic conditions is unpredictable. With very low redox potentials transport may cease completely.

A literature study showed that Cd is taken up by crops rather well. When a dry matter production of 1 kg per 500 kg transpired water is assumed it appears that between 20 and 100% of the Cd present in the soil solution which is taken up by the crop, is transferred to the crop. This fraction is called the selectivity coefficient. Of course not all Cd is removed together with the crop. Yet it can be concluded that the Cd removal via the crop and by leaching is considerable. There are many situations in which a moderate supply of Cd will be compensated for by a removal of Cd. An example of such a calculation is shown in Fig. 4. The Cd supply is set at  $0,2 \text{ kg}\cdot\text{ha}^{-1}\cdot\text{y}^{-1}$ , the amount of drainage water is set at  $20 \text{ ml}\cdot\text{cm}^{-2}\cdot\text{y}^{-1}$  and the transpiration by the crop at  $20 \text{ ml}\cdot\text{cm}^{-2}\cdot\text{y}^{-1}$ . For more details see Fig. 4 and Poelstra et al. (1979). Fig. 4 shows that for  $K_D$  values as they were found in the sandy soil from Braunschweig the Cd level reaches an equilibrium at about 10 kg Cd per ha (layer 0-30 cm). For  $K_D$  values as measured for the clay soil from Valburg the equilibrium level is not reached even after a period of a few hundred years. (Although it is easy to extend the calculations to longer periods, this is not done; the model is not sophisticated enough for such calculations).

In a special experiment the transport of Cd at levels which might result from Cd containing phosphate fertilizers was investigated. A phosphate fertilization during 25 years, with an intensity of  $100 \text{ kg ha}^{-1} \text{ y}^{-1}$  of P with a fertilizer containing 60 mg Cd per kg P, produces in the top layer of a soil (thickness 1 cm) 1,25 mg Cd per kg of soil. Rainfall during 25 years is (in the Netherlands) about 1800 cm of which approximately one third evaporates without causing any leaching. Another third passes the rooting layer and causes leaching. The last third penetrates into the soil but evaporates via soilpores or is taken up by the crop and transpired. The effective precipitation, i.e. the fraction which contributes to leaching ranges, in a 25 years period, therefore from 600 to 1200 cm. It was impossible to do a leaching experiment with this amount within the period which was available for experimentation. The leaching experiment was therefore performed with 100 and 200 cm respectively. The artificial rain contained an amount of Cd which would induce the previously mentioned 1 mg/kg Cd level. The Cd was labelled with  $^{115m}\text{Cd}$  to facilitate the measurement of the distribution within the soil after leaching. Soils: loess soil from Haasdal and sandy soil from Doetinchem. Calculated and measured results of the Cd accumulation in the upper soil layer are shown in table 2. The agreement between calculations and observed values is rather poor. This is probably caused by sampling errors. The experiments were carried out with undisturbed soil monoliths and it is difficult to separate exactly a one cm slice from the soil surface. The concentration in the 0 to 0,5 cm layer is



about three times as high as in the 0,5 - 1,0 cm layer. Small sampling errors induce therefore large errors in the values of the Cd content. (This difficulty had been previously realized, but time did not permit a longer lasting period with its more accurate measurements). Despite the poor agreement it is assumed that the  $K_D$  values as shown in fig. 1 can be used to predict the fate of Cd which is, together with the fertilizer, added to a soil. Table 3 shows the expected accumulation in the top cm of a soil for 1000 ml of effective precipitation. (This represents thus a period of 25 to 50 years).

It appears that the accumulation is considerably counteracted by leaching. Even with a very high  $K_D$  value of 800 (maximum accumulation) only 50% of the added amount is still present in the upper cm. Table 3 shows once again that the use of mean values for Cd accumulation is not applicable.

#### Publication

POELSTRA, P., M.J. FRISSEL and N. EL-BASSAM. Transport and accumulation of Cd-ions. Z.Pflanz. u. Bodenk. 142 (1979): 848-864.

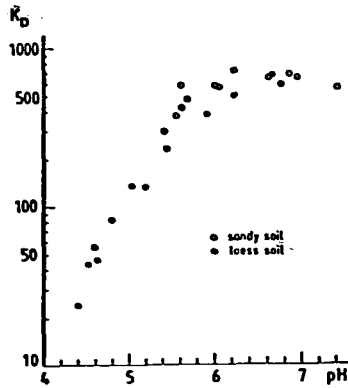


Fig. 1 - The  $K_D$  value of Cd, in  $g \cdot cm^{-3}$ , for a loess soil (Haasdal) and a sandy soil (Doetinchem). Cd level 1 mg Cd per kg soil. Natural pH in simulated rainwater ( $1,7 \times 10^{-4}$  N mixture of NaCl, KCl and  $CaCl_2$ ) of the loess soil is 5,1, of the sandy soil 6,0 respectively.

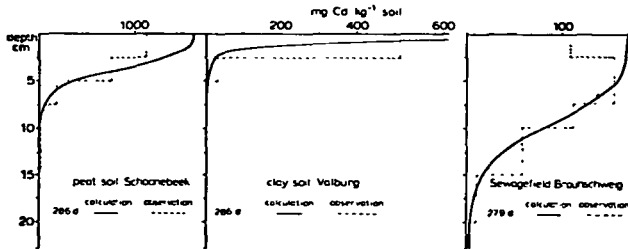


Fig. 2 - Calculated and observed distribution of Cd in undisturbed soil columns. The time elapsed since the beginning of the application of Cd is indicated near the explanation of the curves. Cd concentration of the influent:  $5 \text{ mg} \cdot \text{l}^{-1}$ . Further details: Peat soil: influent rate approx.  $1 \text{ cm} \cdot \text{d}^{-1}$ ,  $K_D = 320$ ; Clay soil: influent rate approx.  $0,7 \text{ cm} \cdot \text{d}^{-1}$ ,  $K_D = 710$ ; Sewage field: influent rate approx.  $2 \text{ cm} \cdot \text{d}^{-1}$  during the first 146 d, thereafter approx.  $0,4 \text{ cm} \cdot \text{d}^{-1}$ ,  $K_D = 33$ .

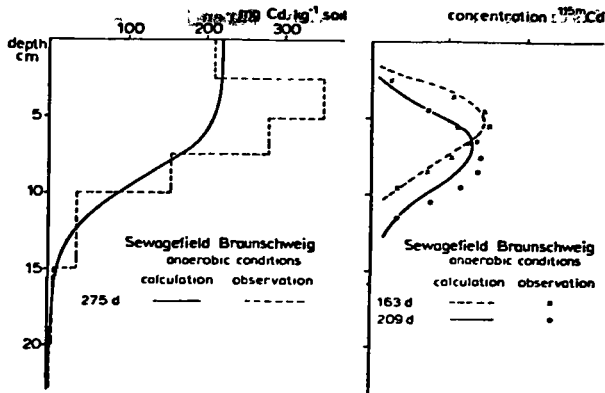


Fig. 3 - Calculated and observed distribution of stable and radioactive Cd in undisturbed soil columns. Cd concentration of influent  $5 \text{ mg} \cdot \text{l}^{-1}$ , influent rate approx.  $2 \text{ cm} \cdot \text{d}^{-1}$ ,  $K_D = 43$ .

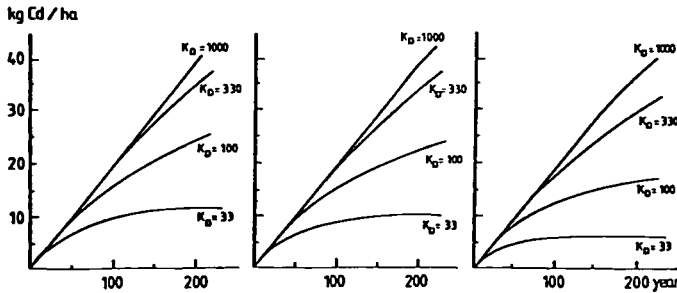


Fig. 4 - Accumulation of Cd on arable land.  $K_D$  values are indicated. From lefthand side to righthand side: selectivity coefficient for Cd uptake: 10%, 30% and 100%. (With a layer thickness of 30 cm and a density of 1,4, 5 ppm Cd equals about 20 kg Cd per ha).

Contractor : Institute for Soil Fertility, Haren, NL  
Contract no. : 274 - 78 - 1 Env-N  
Project leader : R.G. Gerritse  
Title of project : Biogeochemical aspects of organic heavy metal complexes

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### Objective of the research

Land application is frequently resorted to in disposing of sewage sludge. The beneficial effects of sewage sludge on soil fertility will, however, be more than offset by accumulation of toxic elements in the soil if land disposal is not carefully controlled. Present practice is leading to an accumulation in the soil of a number of trace elements. To estimate the effect of pollution of the soil with heavy metals and other elements from sewage sludge on surface and ground water quality, data on the physical and chemical parameters involved in the adsorption processes in soils are essential. Adsorption is affected by speciation of the elements in the soil solution and pH, Eh, ionic strength and composition of the soil solution as well as by the clay and organic matter content of the soil. However, most adsorption studies of elements in various types of soil pertain to concentration levels much higher than those to be expected under field conditions. Adsorption data for conditions prevailing in the soil are essential to the modelling of mobilities of elements. This paper gives basic information on the distribution of 26 elements between the solid and liquid phase in two types of soil and in sewage sludges.

### Materials and methods

Eighteen phase systems resulting from the combination of two soils with nine liquid phases were used to characterize the distribution between soil and soil solution of Be, V, Cr, Mn, Fe, Co, Ni, Zn, As, Se, Sr, Tc, Ag, Cd, Sn, Sb, Cs, Ba, Pb, Bi, Cu, Hg, Mo, B, F and P. Contamination and unwanted adsorption was kept to a minimum. Basic characteristics of the two soils, a sandy soil and a sandy loam soil, are given in Table I. The soils differ mainly in pH and in clay content. Organic-matter contents and cation exchange capacities (CEC) are similar. The soils were equilibrated with water, salt solutions or solution phases of sewage sludges. The ionic strength of

the salt solutions was adjusted at two levels: 0.005 N or 0.05 N CaCl<sub>2</sub> NaCl + KCl (3:1:1). Sludge solution phases were obtained after centrifugation at 40,000 g for 1 h. An industrial and a domestic type of sewage sludge, anaerobically digested, were used. In addition, 10-litre quantities of these sludges were subjected to intensive aeration for a few months.

TABLE I. Characteristics of the soils used in the adsorption experiments

soil type	pH(H <sub>2</sub> O)	moisture* (g/g)	organic matter* (g/g)	clay* (g/g)	CEC* (meq/g)
sandy	5	0 11	0 035	0	0 22
sandy loam	8	0 25	0 025	0 2	0 16

\* of 0.0 soil

During this period the total volume of the sludge was kept constant by adding distilled water. After centrifugation and decantation of the solution phase, the remaining sludge solids were made up with water to the same dry-matter content as before termination of the aeration. After another period of intensive aeration, sludge supernatants were again collected after centrifugation. The cationic composition of the solution phases obtained in this way is given in Table II. Dry-matter contents of the anaerobically digested sludges as well as the aerated sludges ranged from 1 to 3%. Organic-matter contents were between 50 and 60% of dry matter.

TABLE II Cationic (Ca, Mg, Na, K, NH<sub>4</sub>) composition of the aqueous solutions used in the adsorption experiments with soils.

	solution phase*									
		10	11	12	20	21	22	30	31	32
NH <sub>4</sub> <sup>+</sup>	(meq/l)	0	0	0	60	11	0 23	65	7	0 12
Ca <sup>2+</sup>	-	0	3	30	5	16	5	5 2	30	12 0
Mg <sup>2+</sup>	-	0	0	0	0 6	3 2	0 8	0 7	6	1 4
Na <sup>+</sup>	-	0	1	10	4 8	5 2	0 6	-8	7 3	1 1
K <sup>+</sup>	-	0	1	10	2 1	2 3	0 3	2	2	0 44
Total cations	-	0	5	50	72 5	37 7	6 9	87 8	52 3	15 9
pH	-	-	-	-	7 7**	6 4	5 2	7 6**	5 8	5 6
$\frac{Ca+Mg}{Na+K+NH_4}$	-	1 5	1 5	0 08	1 04	5 1	0 08	2 2	8 5	

\* Solution phase. 10 = deionised water, 11 and 12 = salt solutions, 20 = supernatant of industrial type anaerobically digested sludge, 21 = idem after aeration of the sludge, 22 = idem of sludge solids which, after aeration and centrifugation, were resuspended in water and aerated, 30, 31 and 32 = as 20, 21 and 22, but obtained from domestic type sludge

\*\* Before equilibration with the sandy soil the pH was adjusted to between 5 and 6 with HNO<sub>3</sub> to eliminate carbonate

The soil adsorption of an element was determined with a series of solutions in which the concentration of the element increased from 0 to 5 ppm above the concentration in the initial solution. To 25 ml of solution a radioactive tracer and 5 g soil were added. These soil/solution mixtures were equilibrated at 20 °C for three days on an orbital shaker after which the soil solutions were obtained by centrifuging at 40.000 g for 1 h. The radioactivity of the solution phase was measured and the distribution constant was calculated from:

$$K_d = \frac{(C_{tw}^* - C_w^*) \cdot V_w}{C_w^* \cdot G_s} = \frac{\Delta C_s}{\Delta C_w} \quad (\text{ml/g}) \quad (1)$$

in which:

$C_{tw}^*$  is the total concentration of added radioactive isotope in the phase system (cpm/ml)

$C_w^*$  is the concentration of the radioactive isotope in the soil solution after three days equilibration (cpm/ml)

$V_w$  is the volume of the solution phase (ml)

$G_s$  is the mass of the soil solid phase (g)

$\Delta C_s$  is the increase in concentration of the element in the soil solid phase ( $\mu\text{g/g}$ )

$\Delta C_w$  is the corresponding increase in concentration of the element in the solution phase ( $\mu\text{g/ml}$ ).

The specific radioactivity and amounts of radioisotopes added were such that no significant increase in the background concentration of the elements in the various soil solutions resulted.  $K_d$  could thus be determined at a  $\Delta C_w$  of almost zero. Assuming the  $K_d$  in this region ( $= K_0$ ) to be constant, a relation with the migration velocity of the elements in the soil can be given as shown in Fig. 1. This relation between  $K_0$  and the migration of elements in the soil ( $= V_{rel}$ ) is given by Eq.(2), in which  $\omega$  is the phase ratio in a volume of soil and  $V_{H_2O}$  is the velocity of water in the soil. The phase ratio is given by the ratio of volumetric weight of the soil and soil moisture content.

$$V_{rel}^{en} = \frac{V_{H_2O}}{1 + \omega \cdot K_o} \quad (2)$$

The distribution behaviour in soils of Cu, Hg, Mo, B, F and P was studied without radioactive tracers. These elements were determined directly by atomic absorption spectrophotometry (Cu and Hg), by colorimetry (Mo, B and P) and by potentiometry with a selective membrane electrode (F).  $K_d$  was calculated from:

$$K_d = \frac{(\Delta C_{tw} - \Delta C_w) \cdot V_w}{\Delta C_w \cdot G_s} = \frac{\Delta C_s}{\Delta C_w} \quad (\text{ml/g}) \quad (3)$$

in which  $\Delta C_{tw}$  is the total concentration of the element added to the phase system ( $\mu\text{g/ml}$ )

Working without a tracer usually entails a significant increase of the concentration of the element in solution before a measurement can be made. The distribution constant at the background concentration of the element in the soil solution is found by plotting  $\Delta C_s$  against  $\Delta C_w$  and extrapolating  $K_d$  at  $\Delta C_w = 0$ , giving  $K_o$ .

Distribution constants of the elements ( $K_o$ 's) were also measured in the anaerobically digested and the aerated sewage sludges. After addition of the isotopic tracer or element, the sludges were equilibrated for one month.

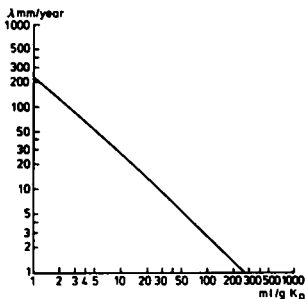


Fig. 1: Mobility of elements in the soil, expressed as the distance migrated in a year ( $= \lambda$  in mm), as a function of the distribution coefficient ( $K_o$ ). Surplus annual rainfall is put at 350 mm, soil moisture content at 30% and bulk density of the soil at  $1.2 \text{ g.cm}^{-3}$ . Dispersion effects are neglected.

### Results + discussion

Solution concentration ranges of the elements in the various sewage sludge supernatant solutions are given in Fig. 2. Cu, Hg, Mo, B, F and P were analysed directly in the sludge solution phase. The concentration ranges of the other elements were calculated from the distribution constants (Fig. 3) and the ranges of the total concentration of the elements in the sludges.

In Figs. 4 A and B, element mobilities are given for the sandy soil and sandy loam soil, respectively. The range of mobilities found with the water and salt solution phases is represented by white bars and the range found with the six sludge solution phases by dark bars. In most cases the sludge solution appears to increase the mobility of the elements. This is due to a combination of complexation, high background concentration (e.g. of Mn, Zn, Sr, B and P) and high ionic strength of the equilibrating solution, the relative effects of which will vary strongly among elements. Highly mobile elements (possible velocity relative to water =  $V_{rel} > 10\%$ ) are Tc, B, Mn, Sr, F and Sb in the sandy soil and Tc, Mo, B and F in the sandy loam soil. Mobility of most elements in the sandy loam soil is lower than in the sandy soil. Mo and P appear to be the only exceptions.

For most trace elements soil organic matter and pH are the dominant factors determining the extent of adsorption. Increasing either factor will usually increase adsorption. Anionic species, however, such as  $B(OH)_4^-$ ,  $TcO_4^-$ ,  $H_2AsO_4^-$ ,  $SeO_3^{2-}$ ,  $SeO_4^{2-}$ ,  $MoO_4^{2-}$ ,  $F^-$ , and cations that do not readily form complexes (e.g.  $Cs^+$ ) show much less affinity for organic matter. These species are strongly influenced in their adsorptive behaviour by the chemical and physical make-up of the soil.

### Additional comments

Work on the speciation of trace elements in soil and sludge solution phases is still in progress (Gerritse, 1980) and will be reported on soon.

Soil column studies on the leaching of trace elements are being carried out and will be terminated in 1982. Columns were taken from the same soils as were used for the batch experiments described in this report. In this way a comparison can be made between theoretical prediction and practice.

Gerritse R.G. (1980) in Environment and Quality of Life. CEC publ. EUR. 68388 EN.



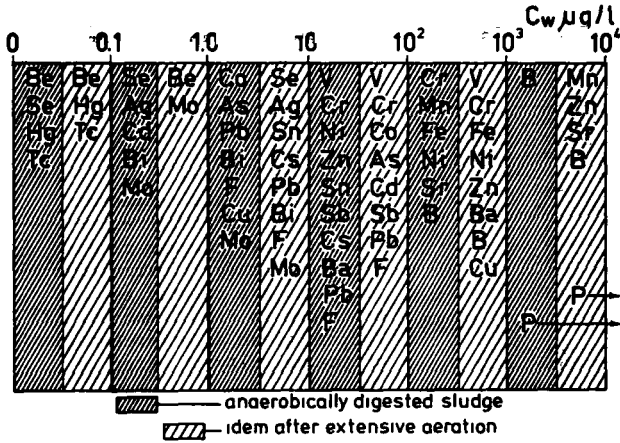


Fig. 2: Solution concentration ranges of elements in sewage sludge.

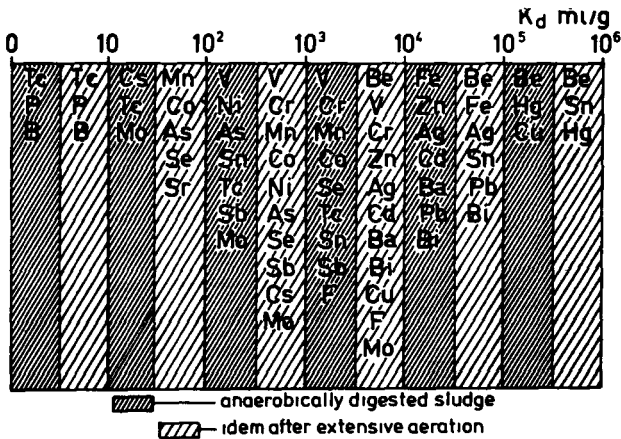


Fig. 3: Distribution constant ranges of elements in sewage sludge.

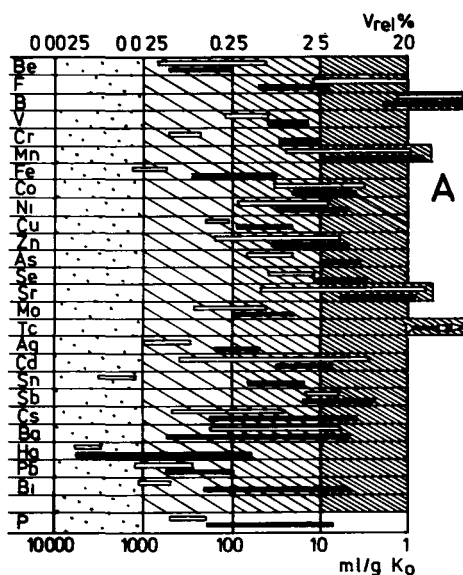
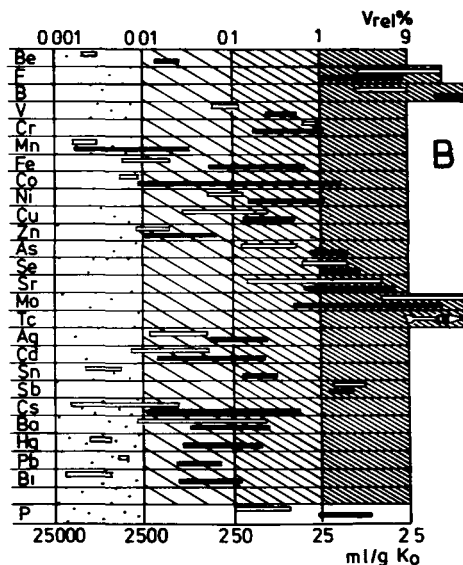


Fig. 4: Trace element mobility in a sandy top soil (A) and a sandy loam top soil (B). White bars represent the range of mobilities ( $V_{rel}$ ) or distribution constants ( $K_0$ ) found after equilibrating the soil with inorganic solution phases. Dark bars represent the range found after equilibrating with sewage sludge solution phases. Distribution constants are related as described in Fig. 1 and eq. (2).  $V_{rel}$  is given as a percentage of the velocity of water in the soil.



Contractor : Université de Liège, Liège, Belgique

Contract n° : 160-77-1 ENV B

Project leader : J. Remacle

Title of project : The influence of cadmium on the activities of saprophytic bacterial population in freshwaters

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#### Objective of the research

The influence of cadmium upon the saprophytic bacterial communities in freshwaters was analysed from the following points of view :

- a) taxonomical and physiological characteristics of bacteria colonizing aquatic systems contaminated with heavy metals specially with cadmium.
- b) genetic analysis of a wild cadmium resistant strain.
- c) behaviour of bacterial communities contaminated with cadmium and other heavy metals (zinc, copper) and elaboration of mathematical models describing the influence and fate of cadmium in microbial freshwater systems.

#### Materials and methods

- a) The bacterial communities of three aquatic systems, two contaminated sites and one control, were analysed in order to compare the influence of heavy metals specially cadmium. The taxonomical and physiological characteristics of the strains were examined by factorial analysis (reciprocal averaging method). Besides, their sensitivities to antibiotics and other toxic metals were checked in regard to their cadmium resistances.
- b) The genetic analyses were performed by curing wild cadmium-resistant strains isolated in a contaminated aquatic system.
- c) The influence of cadmium upon bacterial communities was mainly studied in continuous cultures where the microorganisms cultures reach a steady state in a wide range of submaximal growth rates as it occurs in natural systems. The bacterial inoculum was prepared from samples taken in the uncontaminated river "Ourthe". The analytical determinations (heavy metals, carbon, A.T.P.) and microbial methods were made by standard methods.

## Results

- a) The reciprocal averaging method showed that two sub-communities could be described in aquatic systems contaminated with cadmium. Some strains, the Cd-resistant ones, were able to develop in very high cadmium concentrations (up to 100 ppm) whereas other strains, the Cd-sensitive ones, were killed by cadmium (20 ppm). The physiological characteristics of the resistant strains were not the same as those of the sensitive ones. In addition, the characteristics of the resistant strains depended on the environment. Correlations between resistance to heavy metals and to antibiotics were observed but were not the same in all communities. The densities of resistant strains were roughly related to the level of toxicity in the aquatic system. Most of the Cd-resistant strains belonged to a Gram negative genus, *Pseudomonas*.
- b) Genetic analyses were performed with a wild Cd-resistant strain belonging to the genus *Pseudomonas*, by M. Mergeay (C.E.N., Mol, Belgium). It resulted that the R-factor was coded by a plasmid. Up to the present, extrachromosomal R-factors had been mainly observed in collection strains such as *Escherichia coli*.
- c) The composition of freshwater bacterial communities showed that Cd-resistant strains could be isolated even in rivers where cadmium was not detected by chemical analyses. Therefore, it was interesting to check whether Cd-resistant strains could compete successfully in uncontaminated systems. On the basis of the growth parameters of a Cd-sensitive and a Cd-resistant strain, mathematical simulations were performed by D. Dubois and G. Monfort (Inst. de Mathématique, Univ. de Liège). The results showed that the mathematical model appeared to agree with the experimental data when the selective attachment of bacteria on the walls of the chemostat was taken into account. So, it was concluded that, at steady state, Cd-resistant strains could develop in cadmium-free mixed cultures in spite of an expected loss of R-factor in these conditions. This conclusion corroborated the field observations.

The next step was to study how a bacterial community could develop when it was contaminated with cadmium. Bacterial communities were cultured in continuous cultures characterized by a gradient of productivities. When the cultures had reached a steady state, they were contaminated by a cadmium flux (1 and 0,5 ppm) for one month. These

concentrations were similar to cadmium concentrations in industrial effluents.

The results lead to the following conclusions. At 20°C, the cadmium contamination does not affect to a great extent the bacterial productivities. In fact, in cultures characterized by low productivities, between 2 and 5 mg bact. d. w. l<sup>-1</sup> h<sup>-1</sup>, the productivity is slightly altered and slows down, but they will reach soon their previous level. So, the microbial system exhibits a good homeostasis, therefore, the biodegradation processes seem to be not substantially influenced by cadmium. Cadmium resistant strains (resistant against 20, 100, 200 and 300 ppm cadmium) are quickly recovered in crowded populations. So, in some cases, nearly all strains become resistant against 20 ppm cadmium one month after contamination and their respiratory activities remain unchanged.

However, at 10°C, the bacterial productivities generally drop to 50% of the control. From these observations, it can be concluded that among others, two factors could control the response of bacterial cultures against cadmium: the productivity and the temperature.

Another interesting fact appears to be the cadmium uptake by free and adhering bacteria. The maximum concentration equals 1300 ppm at 20°C and 960 ppm at 10°C in free bacteria whereas adhering bacteria are able to accumulate up to 6100 ppm at 20°C. It must be mentioned that bacterial cultures incubated in a polluted river, can immobilize up to 600 ppm cadmium. A good correlation is noted between cadmium accumulation in cells and bacterial productivity. However, at productivities above 90 mg bact. d.w. l<sup>-1</sup> h<sup>-1</sup>, the cadmium accumulation is lowered owing to the bacterial aggregates and the lower cadmium concentration in the water phase. In any way, cadmium concentration in the water phase of continuous cultures depends on bacterial productivity and adhering bacteria. But, the part taken by adhering bacteria in cadmium budget diminishes as productivity is high.

Cadmium is mainly located in the cell wall-membrane fraction where 83-93% of cellular cadmium are detected; 33-60% of this fraction exhibits a good extractability into water. The resistant against cadmium could be due to the trapping of the heavy metal in the wall membrane fraction which would hinder its transfer to the cytoplasm.

When bacteria developed in cultures contaminated with several heavy metals (Cd, Zn, Cu), it would seem that copper impeded the cadmium accu-

mulation whereas zinc did not influence it.

Significant amounts of cadmium begin to be released from contaminated cells which have reached the stationary phase of growth. Two stages can be generally recognized. The cadmium loss is important during the first days as the cells decay. Afterwards, it becomes slower. At 20°C, in aerobic, the high decrease of cadmium content during the first days leads to its complete wash-out from the cells before the total lysis of bacteria. The pattern of cadmium evolution is similar at 10°C but at a slower rate. In anaerobiosis, cadmium is firmly trapped in cells until the end of the experiment. On the contrary, cadmium is released within a few days from cells incubated in acid aquatic system. The release rates of cadmium from cells incubated in a river (neutral pH, high oxygen concentration) are similar to the values observed in the same laboratory conditions, mainly during the first days incubation.

Substantial settling occurs in bacterial cultures seeded by a mixed microfauna (mainly protozoan) even in cultures poisoned with cadmium. The cadmium concentration in the aggregates, bacteria-microfauna, is as high as 1350 ppm. So, these aggregates can also enhance cadmium immobilization in the bottom of the river. Indeed, "in situ" experiments have proved that they trap it very efficiently because of the very slow release of the heavy metal.

It is now possible to build a simple mathematical model in order to simulate the fate of cadmium in bacterial sediment. Indeed, by growing in a polluted aquatic system, bacteria accumulate cadmium. After the growth phase, they die and settle and begin to release cadmium because of their lysis. The mathematical model takes into account these processes and fulfills two goals, first, checking and partially understanding the data observed "in vitro", secondly, extrapolating the results to a river polluted by cadmium. The model leads to the following main conclusions. Despite the release processes, the cadmium concentration increases in bacterial sediments, the accumulation being higher at 10°C than at 20°C. So, at 10°C, the concentration equals after ten days contamination, the one obtained after one year at 20°C. The system reaches a steady state, e.g., at 20°C, a pollution duration of three months has the same effect as a ten years pollution or more. The calculated concentrations rank in the same order of magnitude as the observed concentrations. Moreover, after cadmium contamination being stopped, the time of cadmium depollu-

tion is very long. Indeed, at 10°C, one day of pollution needs more than one year of depollution whereas at 20°C, the maximum depollution duration is approximately two months. This result appears to be important for the river management. Finally, it must be noted that temperature is a determinant factor, therefore, the long term simulation must be carefully interpreted.

### Conclusions

- Freshwater bacterial communities contaminated with cadmium are composed of two sub-populations defined by their resistance against cadmium and their physiological characteristics. Most of Cd-resistant strains belong to a Gram negative genus, *Pseudomonas*.
- Correlations between the resistance to heavy metals and to antibiotics are observed but are not the same in all bacterial communities. Genetic analyses of a wild Cd-resistant strain have showed that the R-factor is coded by a plasmid.
- Microbial systems developed in continuous cultures exhibit a good homeostasis, specially at 20°C. Therefore, they are not greatly damaged when contaminated with 0.5 and 1 ppm cadmium. In these cases, Cd-resistant strains are quickly recovered in crowded populations. At 10°C, the bacterial productivities generally drop to 50% of the control owing to the cadmium contamination.
- An important feature appears to be the cadmium accumulation by bacteria incubated at 20°C and 10°C. So, bacteria incubated in a polluted river are able to accumulate up to 600 ppm cadmium. The cell wall-membrane fraction contains 83-93% of cellular cadmium. The resistance against cadmium could result from the cadmium immobilization in the cell membrane fraction which would impede its transfer to the cytoplasm. The cadmium concentration in the water phase of continuous cultures depends on bacterial productivity and attached bacteria.
- When bacteria are cultured in media supplemented with several heavy metals (Cd, Zn, Cu), they are able to accumulate all the metals.
- When contaminated bacterial cells have reached the stationary phase of growth, they begin to release cadmium. The cadmium loss from the cells is controlled by temperature, oxygen concentration and pH.
- Significant settlings are enhanced in bacterial cultures seeded by a mixed microfauna even in cultures poisoned with cadmium. The cadmium

concentration in the aggregates, bacteria-microfauna, equals up to 1300 ppm. So, these aggregates can contribute to the cadmium immobilization in the bottom of river as proved by "in situ" experiments.

- By taking into account the above processes, it is possible to build a mathematical model in order to simulate the cadmium fate in bacterial sediments of a river. Despite the release mechanisms, the cadmium concentration increases in bacterial sediments mainly at 10°C until the system reaches a steady-state. Moreover, after the end of cadmium contamination, the time of depollution is very long. Indeed, at 10°C, one day of pollution needs more than one year of depollution. This result appears to be important for the river management.

#### Oral communications

- Freshwater contact group C.E.C. : Windermere 1977, Metz and Saarbrücken 1978, Ispra 1979, Dublin 1980.
- Management and Control of Heavy Metals in the Environment, London 1979 (poster session).
- Second international symposium on Microbial Ecology, Warwick (U.K.) 1980.

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- Mergeay M., Houba C. and Gerits J. 1978. *Archiv. Intern. Physiol. Bioch.* 86, 440-441.
- Mergeay M., Gerits J. and Houba C. 1978. *C.R. Séances Soc. Biol.* 172, 575.
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- Dubois D., Remacle J. and Monfort G. 1980. in "State-of-the-Art in Ecological Modelling" (in press).
- Remacle J., Dubois D. and Monfort G. 1980. in "State-of-the-Art in Ecological Modelling" (in press).
- Houba C. and Remacle J. 1981. in "Heavy metals in the environment", Amsterdam, 1981. (to be published).
- Houba C., Remacle J. and De Parmentier F. 1981. *European J. Appl. Microbiol.* 11, 179-182.



Contractor : Rijksuniversiteit Gent

Contract : 414 - ENV B

Project Leader : Prof. Dr. Ir. A. COTTENIE, Dr. Ir. M. VERLOO

Title of project : Distribution patterns of mineral elements in soils and surface water

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### Objectives of the research

Safe disposal of sludges and sediments, generally containing high amounts of heavy metals, requires a suitable methodology for estimating and evaluating its environmental impact. As the environmental effect of heavy metals is not only determined by their total content but also by a series of parameters like pH, redoxpotential and organic matter content, the influence of these parameters on the transfer of heavy metals from the solid to the liquid phase of a soil or sediment has been studied in more detail.

Experimentally the heavy metal distribution and effects of organic matter content and sediment granulometry have been elucidated in some dredged sediment disposals while also the influence of redoxpotential and pH on the behaviour of some heavy metals in a series of sediments has been studied in controlled conditions.

### Materials and methods

#### 1. Distribution of heavy metals in dredged sediment disposals

In two recently land disposed dredged sediments different layers were sampled. The samples were dried and analyzed for total carbon,  $\text{CaCO}_3$ , sulphate and heavy metal content using the methods of COTTENIE et al. (1979) while the particle size distribution was determined by mechanical analysis according to DE LEENHEER (1977).

#### 2. Study of the influence of pH and redoxpotential on the behaviour of some heavy metals in sediments

At 5 different locations sediments of heavy polluted rivers were collected ; part of each sample was dried and part was stored under nitrogen and kept in the refrigerator until analysis.

The dried samples were analyzed for total element content according to methods described by COTTENIE et al. (1979).

The freshly stored sediment sample was split up in two parts ; one part was analyzed directly and the other part was analyzed after oxidation of the sample by shaking during 148 hours. On the original and the oxidized samples the redoxpotential was measured by means of a platinum electrode against the standard calomel electrode, and adjusted to the zero potential of the standard hydrogen electrode using the equation  $E_h (mV) = E_{SCE} + 242$  in which  $E_h$  is the redoxpotential and  $E_{SCE}$  is the potential measured against the standard calomel electrode. The sulfide content was determined by the iodometric procedure recommended for sewage, in Standard Methods for the examination of Water and Waste Waters (1965) and an exchangeable fraction of the metal ions and sulphate was determined by extracting 10 g sediment with 50 ml 1 n  $NH_4AC$  pH 7. Heavy metal concentration was determined by atomic absorption spectrometry. The extraction of the original reduced sediments required special precautions as during shaking oxidation may occur thus falsificating all analytical results. To avoid this, special equipment can be used as described by GAMBRELL et al. (1977) but even then the avoidance of any oxidation is problematic. Therefore we preferred to add ascorbic acid (5 g/l) as an antioxidizing agent during the extraction as well for the reduced as for the oxidized samples. To study the influence of pH on heavy metal behaviour the sediments have been acidified and equilibrated at a fixed pH.

## Results

### 1. Distribution of heavy metals in dredged sediment disposals

The heavy metal contents in two dredged sediment disposals are given in table 1. Different layers were sampled according to differences in colour and or structure.

It is seen that the heavy metal contents in the sediment layers show a great variability.

Very significant correlations were found between the metal content and organic matter for all elements as well as between the metal content and the particle size fraction  $< 16 \mu m$ . It has to be noted that this  $< 16 \mu m$  fraction contained about 40 % of organic matter expressed on a dry matter basis.

### 2. Influence of pH and redoxpotential on the behaviour of some heavy metals in sediments

The total elementary composition of the sediments studied showed a great variability (Table 2).

Table 1. Heavy metals (mg/kg dry matter) in sediment disposals after extraction with 0.5 n HNO<sub>3</sub>

Sample + depth	mg/kg air dry sediment								
	Fe	Zn	Pb	Mn	Cu	Cr	Ni	Cd	Co
<b>BALGERHOEKE</b>									
0 - 3 cm	3885	3312	378	244	159	81	43.8	26.6	12.5
3 - 8	6385	2037	318	194	113	91	32.8	15.0	8.2
8 - 14	3437	769	155	88	39	36	14.1	4.4	4.0
14 - 24	6114	1657	331	150	84	77	28.1	10.6	5.3
24 - 34	5368	1431	280	137	72	67	24.2	9.1	5.3
34 - 44	4349	881	166	106	58	42	20.3	5.0	4.6
44 - 54	4336	960	171	92	52	47	18.7	6.2	4.7
<b>MENDONK</b>									
0 - 2 cm	4867	2387	158	920	150	125	31.2	17.2	10.0
2 - 13	285	13	3	27	0.6	0.5	0.7	0.2	0.6
13 - 23	786	90	20	46	3.5	3.2	1.6	0.8	1.1
23 - 25	696	80	16	41	4.6	12.5	1.9	0.5	1.1
25 - 35	1018	172	30	49	8.8	23.4	2.9	1.1	1.8

Table 2. Total analysis of elements in the sediment samples

Elements in mg/kg	Sample 1 Dijle	Sample 2 Demer	Sample 3 Grote Laak	Sample 4 Leuvense Vaart	Sample 5 Zenne
S	543	525	17292	473	775
Fe	18600	31600	51675	12700	12250
Mn	307	277	253	137	247
Zn	348	155	1169	99	353
Cu	38.3	15.3	155.3	14.8	46.4
Pb	222.0	42.5	406	68.1	69.4
Ni	52.7	22.6	37.1	15.6	18.9
Cr	62.0	6.7	14.0	23.0	31.0
Co	7.02	5.96	10.18	2.46	5.61
Cd	2.02	1.25	1.75	1.47	1.95
C in %	1.84	2.80	10.40	2.00	0.48

Especially sample 3 is heavily loaded with the metals Fe, Zn, Cu, Ni and Co but also the S and carbon content is very high.

The influence of redoxpotential seemed best reflected in the composition of the exchangeable fraction of the sediments.

Table 3 is giving the exchangeable metal ion contents in the reduced sediment, as it naturally occurred, while table 4 shows the same element fraction after oxidation of the sediments.

Sulfide ( $S^{2-}$ ), sulphate ( $SO_4^{2-}$ ) and redoxpotential (Eh) are given as parameters reflecting the redox situation of the sediment system.

Table 3. Exchangeable fraction of  $SO_4^{2-}$ , Fe, Mn, Zn, Cu, Pb and Cd in fresh sediments determined after extraction with neutral  $NH_4Ac$  pH 7 + 0.5 % ascorbic acid (results in mg/kg air dry sediment)

Elements in mg/kg	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
$SO_4^{2-}$	0.50	<0.50	158.00	0.50	0.75
Fe <sup>4</sup>	529.00	4049.00	4270.00	468.00	770.00
Mn	68.30	128.00	70.70	34.80	54.10
Zn	10.38	6.56	4.85	2.55	6.80
Cu	0.09	0.10	0.08	0.08	0.03
Pb	3.00	<0.43	0.43	0.22	0.21
Cd	0.02	0.02	0.06	0.02	0.02
Total $S^{2-}$	93.3	162.5	7894.0	168.0	209.0
Redox potential Eh (mV)	18	-80	-148	-51	-150

Table 4. Exchangeable fraction of  $SO_4^{2-}$ , Fe, Mn, Zn, Cu, Pb and Cd in the same sediments after oxidation and extraction with neutral  $NH_4Ac$  pH 7 + 0.5 % ascorbic acid (results in mg/kg air dry sediment)

Elements in mg/kg	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
$SO_4^{2-}$	18.00	36.90	7788.00	26.40	8315.00
Fe <sup>4</sup>	51.40	343.00	2144.00	74.20	156.00
Mn	52.30	87.10	173.00	36.00	101.00
Zn	18.50	16.30	9.71	15.90	118.00
Cu	0.14	0.10	0.10	0.08	0.14
Pb	4.89	<0.43	1.68	3.48	4.13
Cd	0.38	0.14	0.10	0.33	0.22
Total $S^{2-}$	16.7	13.6	5183.0	23.3	85.1
Eh (mV)	414	379	186	331	369

From these tables the effect of oxidation on the exchange properties of different elements in sediments can be summarized as follows :

- the sulphate content increases very strongly as the sulfide content decreases
- the iron concentration decreases strongly
- manganese concentration increases or decreases depending on the sample

- Zn concentration increases
- the Cu content is almost stable
- Pb and Cd concentrations increase.

The increase of exchangeable sulphate and the decrease of iron content after oxidation of the sediments are easily explained by direct oxidation.

The increase of exchangeable Zn, Pb and Cd is probably the effect of oxidation of sulfide-compounds resulting in a higher solubility or release of the cations  $Zn^{2+}$ ,  $Pb^{2+}$  and  $Cd^{2+}$  while the apparent neutral behaviour of Cu might be explained by the very high stability and insolubility of CuS.

The apparent aberrating behaviour of manganese of which the solubility should decrease in more oxidizing conditions can be a combined effect of oxidation to less soluble  $Mn^{4+}$ -oxides and release of  $Mn^{2+}$  from sulfides while also the role of organic matter and indirectly also of pH changes during these processes have to be considered. Indeed during oxidation an average pH decrease of 0.2 was noticed in the sediments.

The influence of pH on the behaviour of metal ions in soils or sediments has frequently been studied (COTTENIE & DHAESE, 1978 ; VERLOO et al., 1980).

It appears that as a direct effect of pH decrease the solubility of heavy metals increases (see table 5) besides that pH has an indirect effect on sorption reactions, complexation mechanisms and complex or colloid stability.

Table 5. Effect of pH decrease on the solubility of heavy metals in sediments (mean value for 5 sediments ; results expressed as percentage of total amount)

pH	Fe	Mn	Zn	Cu	Pb	Cd	Ni	Cr	Co
8.0	0.02	0.28	0.03	0.00	0.31	0.30	1.37	0.00	0.00
5.0	0.20	11.09	2.68	0.13	0.91	2.12	11.05	0.33	3.55
3.0	3.61	40.33	20.13	0.25	2.62	15.67	40.69	1.10	24.43
1.0	14.77	74.64	57.10	34.05	59.20	67.32	63.51	7.51	47.63
0.5	21.16	77.03	64.28	54.81	67.77	72.43	69.24	16.02	52.34

To give an idea about the mobility of the different elements in the sediments, the pH at which 10 % of the total amount is mobilized by acidification has been calculated (table 6).

Table 6. pH at which 10 % of the total element amount is mobilized

Element	Fe	Mn	Zn	Cu	Pb	Cd	Ni	Cr	Co
pH 10 %	1.5	5.1	3.8	2.0	2.7	3.3	5.1	0.8	4.3

### Discussion and conclusions

From these experiment it may be concluded that in dredged sediment disposals a considerable fraction of heavy metals is associated with small sized particles consisting mainly of organic matter.

This may imply a potential danger during dredging operation of polluted sediments as small particles are easily suspended, resulting in an increased oxygen demand of the water and the release of heavy metals.

In addition, oxidation of reduced sediments caused an increased exchangeability of Zn, Pb and Cd while also a pH decrease was noted, thus increasing the mobility of most heavy metals.

These observations also imply that chemical characterization of a sediment is to be done on the fresh material as any treatment e.g. drying or storage, results in alterations of redoxpotential and pH.

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### Remark :

Dr. M. Verloo gave an oral communication "Influence of redoxpotential and pH on the transfer of heavy metals from the solid to the liquid phase in soil water systems" at the Dublin meeting of the Freshwater Contact Group, October 1980.

Contractor: University of Cambridge  
Contract no. 165-76-12 ENV UK  
Project Leader: S.M. Haslam  
Title of Project: River macrophytes of the European Community

#### Objective of the research

To record and interpret the vegetation of watercourses (streams, dykes etc) throughout the EEC, to assess conservation value of different regions, and to develop macrophyte monitoring for the assessment of pollution.

#### Results and conclusions

During the 11½ years of this investigation, c. 27,500 site records have been collected. Although over half come from Britain, in the other countries, excluding the Alps and Appennines, few places are further from a surveyed area than 50 km in the larger countries, and 25 km in smaller ones. All widespread plant communities should have been recorded, though important local ones may not have been. Experimental work has been less important in the research programme.

Vegetation has, therefore, been recorded, and the main plant communities are known. For regions or, in some parts, individual rivers, enough information is available to assess conservation value.

A pollution index has been quantified for Britain, which proved satisfactory on test. Its performance after publication and general release is not yet known. For the rest of the (non-mountainous) parts of the EEC, indices can be obtained, except in The Netherlands, where an index (de Lange's) is already available. It is hoped that the non-British indices can be tested in 1981, although any publication is unlikely before 1983.

Macrophyte monitoring has three main uses:

- 1) to complement methods using other organisms, to give a more complete picture of biological river quality.
- 2) to assess macrophyte value for e.g. conservation, quality of irrigation water.
- 3) as a quick general monitoring method, when all that is required is an assessment of good or bad (on which basis the macrophyte index is well

correlated with other indices).

The survey records will take at least another year to interpret. The initial analysis confirms that, as in Britain, the most important factors are rock type, water force (as determined by precipitation, landscape, rock type and climate), upstream-downstream variation, and man's activities.

The subjects investigated range from the detailed, such as determining whether species distribution in relation to water depth is constant throughout the EEC, to the less obvious effects of cultural patterns, such as the effect of the German drainage tax on river patterns and channel shape, and consequently, on river vegetation. These can be summarised as:

Geology, landscape and flow patterns. These are the basic habitat factors.

Settlement patterns (villages, towns), river history and culture, recent changes due to losses of mills, water meadows etc, and Improvement schemes. Varying intensities of man's influence, from the little-affected Sardinia to the much-managed Netherlands. Effects on vegetation.

The multiple uses of watercourses, the advantages and disadvantages of vegetation for these uses, and the means of maintaining the optimum vegetation satisfying the divergent demands.

Responses of species to individual factors such as depth, flow, substrate texture.

Climatic and bioclimatic effects. Discharge, precipitation and water force.

Nutrient regimes, and their correlations and causal associations with species and plant communities.

Changes in vegetation from year to year, their type, and the causes of instability (weather, dredging, pollution, grazing etc).

Channel banks, their history and origin (natural or man-made). The variation in height, slope and vegetation, their effect on water force. Effect on channel vegetation.

Geographic variation, the distribution of species and communities throughout the EEC. Causes of the variation.

Vegetation description and classification. Key species, phytosociology. Downstream changes.

Maintenance management (dredging, cutting, herbicides etc). Variations in the EEC. Effects on plants.



Cultural management, transport patterns (formerly), irrigation and drainage patterns, farming pressures (drainage, fertiliser, access for machinery etc), industrial pressures (factory effluents, mill dams etc). Former patterns and recent changes. Variations in the EEC. Effects on plants.

Pollution, its effects, mode of action. Various kinds of pollution: Geographic variation in species patterns. Country-wide variations in severity and types of pollution (quarry, gravel, untreated and treated sewage effluents, small-scale and large-scale effluent outlets). Indices.

Most of this research will be incorporated into a book, "River Plants of Western Europe".

#### Publications and oral communications

(excluding those listed in previous Final Reports)

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Monitoring river pollution using macrophytes.

Death of water plants in a disused gravel pit at Hemingford Grey.

River vegetation in Sardinia, Sicily and S. Italy.

Macrophyte survey for Anglia Water Authority.

Indices for dyke (ditch) vegetation.

Macrophyte pollution in Luxembourg.

The Lough Neagh catchment basin: proposals for research.

Contractor: Water Research Centre  
Stevenage Laboratory  
Stevenage  
Hertfordshire SG1 1TH  
UK

Contract No: 216-77-10 ENV UK

Project Leaders: Mr J F Solbè, WRC  
Mr H A Hawkes, University of Aston

Title of project: Effects of an oxidised sewage effluent  
on freshwater fisheries

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Objectives of the research

- (a) To investigate the effects, on benthic communities and fish, of the presence in river waters of different proportions of a sewage works effluent containing low concentrations of heavy metals, including cadmium, with particular emphasis on survival, growth, and accumulation of metals in macro-invertebrates and in fish, in relation to water quality.
- (b) To contribute to the establishment of an EEC method of biological surveillance of water quality by comparing the community structure developed under the different water-quality regimes.

Materials and methods

The experimental facility, fully described previously<sup>(1 to 5)</sup>, consists of three artificial streams 300 m in length, and six 2500-litre tanks. The former carry flows of 4.5 Ml/d and represent a compromise between the controlled, but unnatural, environment of the laboratory and the natural, but inherently variable, conditions of a river. Both stream and tank systems receive mixtures of an unpolluted river water and an oxidised sewage-works effluent. In addition one tank and one stream receive only river water and act as controls while one tank has on occasion received only sewage-works effluent.

The site is in the grounds of a sewage works treating a mixture of domestic and industrial wastes, including heavy metals (cadmium, chromium, copper, lead, nickel and zinc). It has been assumed that these materials have caused the absence of brown trout (Salmo trutta fario) in the receiving river for 9 km downstream of the sewage works. (Brown trout flourish upstream of the site and the river is physically suitable for trout throughout.)

In an experiment the streams were allowed to acclimate to the chosen mixtures of effluent and river water (which three of the tanks were designed to mimic). The sediment patterns, algal and invertebrate communities could therefore adjust to the given water-quality regimes. Brown trout were then released in the streams (at the optimal carrying capacity of 3.5 kg live weight per stream) and, with roach (Rutilus rutilus), in the tanks.

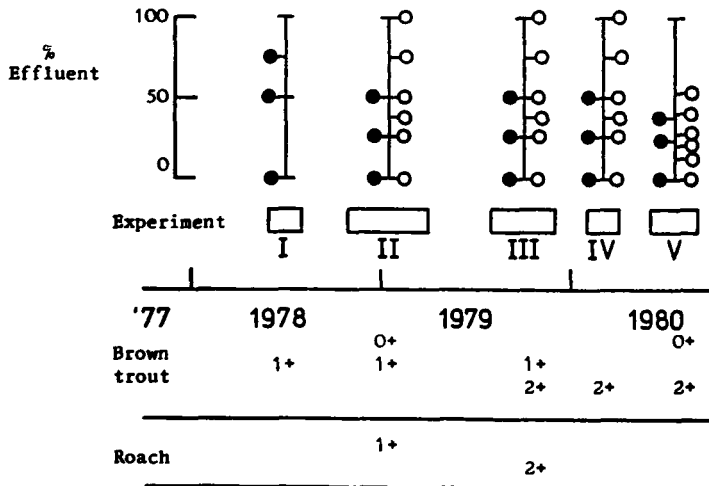
Organisms were removed from the streams or tanks at intervals and any effects of the water-quality were examined. Effects on fish included the accumulation in their tissues of heavy metals, changes in rates of growth and mortality. Changes in species composition and biomass of invertebrates were noted.

The artificial streams have been operated for 6 years, in the first 3 of which operational problems were identified and solved. The last 3 years to 31 December 1980 have constituted the period of this EEC contract. During the contract five separate experiments were completed. Figure 1 sets out the water mixtures used, the period of each experiment and the ages and species of fish used.

A summary of the principal findings is given below.

Fig. 1. Experimental programme

● STREAMS ○ TANKS



## Results

### Experiment I<sup>(2)</sup>

This 60-d experiment demonstrated that the carrying capacity of the streams for brown trout was correct and that the mortality and growth rate of trout were related to water quality. The macro-invertebrate communities in the 3 streams differed significantly. Some species (Gammarus pulex and Ancylus fluviatilis) were very intolerant of the presence of sewage works effluent, while others (Asellus aquaticus, Chironomus riparius and the alga Cladophora) were abundant in the polluted streams and absent or rare in clean water.

### Experiment II<sup>(5)</sup>

After 44 days exposure it could be concluded that roach were much more resistant than brown trout to the effects of the effluent. Mortality and growth rate data were obtained for brown trout and mortality data for roach. Fish were held in cages in the 2500-litre tanks and in smaller tanks but the cages caused fish to become diseased and aggressive and were therefore abandoned.

During the experiment a nickel-pollution incident of 38 days duration occurred, increasing the concentration of the metal in the most polluted stream for a few days from around 20 µg/l to more than 800 µg/l. This coincided with the rapid mortality of about 40% of yearling brown trout in the stream. In the tanks and the less-polluted stream the effect of the nickel was much less severe. These results may indicate that brown trout are rather more sensitive to nickel than are rainbow trout (Salmo gairdneri), for which the median lethal threshold concentration, observed after an exposure period of 8 weeks, was 1300 µg/l<sup>(6)</sup>.

Neither roach nor brown trout showed any clear relationship between water quality and the pattern of uptake of heavy metals over a period of 11 days. The metal content of fish given artificial food in the tanks did not differ from those feeding on natural food in the channels. The macro-invertebrates were affected by the change in water quality, as reported previously<sup>(5)</sup>.

### Experiment III<sup>(10)</sup>

The growth rates of fish were observed for 78 days in the tanks and streams and fish were finally removed from the streams for analysis of the

metal content of their tissues after 140 days of exposure. Apart from gradual decreases in concentration of nickel and zinc and the seasonal decrease in water temperature throughout the experiment the water quality appeared to vary little, with one exception. On four occasions, at intervals of about 19 days, the concentrations of chromium in the effluent-carrying streams rose to more than 70  $\mu\text{g}/\text{l}$  from a background level of about 15  $\mu\text{g}/\text{l}$ , but these events did not appear to affect the rate of mortality of roach or brown trout.

Placing the roach in the same tanks as the trout caused some problems of aggression and led to infection of damaged skin and to the death of some roach.

After 140 days exposure there was very little difference in metal content of trout in the various qualities of water. Indeed, the greatest concentrations of cadmium in liver and kidney were found in the fish from the clean-water stream. However, although the levels were low, there was a graded increase in cadmium in both organs in the fish from the tanks, related to ambient conditions and fish in the stream carrying 25% effluent contained higher concentrations than fish in any of the tanks.

The concentrations of heavy metals in the macro-invertebrate fish-food organisms gave a far clearer indication of water quality than the fish. The proportions of each metal in Simulium larvae almost exactly reflected the proportions dissolved in the polluted water. However this may be a chance result: the larvae in unpolluted water did not show the same pattern of metal content as the unpolluted water itself; instead they resembled the larvae from the other streams except for containing more zinc and less copper than the latter. The crustacea were particularly rich in copper (the metal in their respiratory pigment) and correspondingly poor in zinc and lead. None of the organisms contained high concentrations of cadmium (5-20  $\mu\text{g}/\text{g}$ ) or chromium (5-90  $\mu\text{g}/\text{g}$ ).

#### Experiment IV

The principal objective of this experiment was a study of metal accumulation in tissues and organs of 2-year old brown trout exposed in the streams and tanks for 35 days.

The data are still being processed but it is clear that  
(a) over short periods (days rather than weeks) brown trout did not accumulate heavy metals. It is reassuring that at ambient concentrations of around 10 µg/l Cd trout muscle showed no tendency to accumulate cadmium.  
(b) The metal content of the faeces of fish was much higher in fish feeding on a natural diet, whether or not it originated in a polluted stream, than in fish fed a proprietary pelleted food. 'Enrichment' of the metal content of fish faeces supports, but does not prove the opinion that the fish gut is an effective barrier to the entry of heavy metals into fish tissues.

#### Experiment V

In this 90-day experiment the proportions of effluent in the tanks and streams were reduced (Fig. 1) and a larger biomass (8.1 kg/stream, 30 brown trout each of about 270 g) was used. Despite favourable temperatures (10-16°C), growth was still largely determined by water quality<sup>(10)</sup>.

The time-course of mortality in the streams and tanks was not a smooth ogive but proceeded in a series of steps. The relationship of these steps and water quality is now being investigated. They are generally associated with peaks in concentration of metals occurring at the time of the mortality, or a day or so before. There was no clear evidence of a delayed mortality in the fish exposed to less polluted water.

A full analysis of the effects of water quality on the growth and mortality of brown trout will be presented in the final report of the contract, with details of the accumulation of heavy metals by these larger trout.

#### Conclusions

Although data from work using complex mixtures of pollutants are exceedingly difficult to handle, the use of artificial streams has cut out one source of variation in the experiment and full analytical data on water quality, fish and benthic invertebrates are now available for detailed study. Already, as well as confirming and describing the rôle of water quality in controlling fish growth rate and mortality and the species composition of the benthos, it is possible to state the percentage of a population of brown trout or roach which will survive a given number of days exposure to a given concentration of, say, cadmium despite the

presence of other pollutants. Such information will help to validate and modify EEC Directives for the protection of the fresh waters of Europe.

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Contractor: Justus-Liebig-Universität Giessen

Contract No: 137-76-10-ENVD

Project Leader: Dr. Anna Barbara Fischer

Title of project: Acute and chronic effects of heavy metals on mammalian cells cultivated in vitro

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#### Objective of the research

It was the aim of these studies to investigate the effects of heavy metals on cellular functions following acute and chronic exposure of mammalian cells cultivated in vitro. Special consideration was to be given to cadmium and mercury, but other metals of environmental significance should also be included. Different cell types should be used to determine representative cellular reactions. Comparative studies with several heavy metals should lead to a toxicological characterization and classification of these pollutants. Potential synergisms and combination effects of the heavy metals among themselves and with other environmental pollutants, should be investigated later. With chronic exposure resistance towards lead, mercury, cadmium and arsenic was observed, which is based on adaptive processes. The kinetics and conditions of these cellular responses were of special interest.

#### Material and methods

Most of the materials and methods have been described in detail (Fischer 1975, 1976, 1979). The following cell cultures were employed: (a) Human diploid fibroblasts (strain FH<sub>31</sub>); (b) Human portiocarcinoma line HeLa; (c) Mouse fibroblasts L 929, subline L-A, growing in stationary suspension culture. Analytical grade solutions of heavy metal salts, mostly chlorides of known molarity were used. Thirteen metal compounds were studied. Microscopical examinations of morphology and mitotic rate were carried out with living cells and on preparations stained with hemalum-eosin. Viability was tested by the trypan blue exclusion test. Membrane damage was determined by measuring the release of the enzyme lactic acid dehydrogenase (LDH) into the culture supernatant. As an important parameter of carbohydrate metabolism lactic acid production was monitored. Cell growth and proliferation were followed by cell counting and protein determination. The incorporation of

labelled precursors into DNA, RNA and protein was studied by liquid scintillation counting, and  $^3\text{H}$ -thymidine uptake was also investigated by autoradiography. Cell cycle phases were determined by cytophotometry (collaboration with Dr. F. Otto, Institut für Aerobiologie, Grafschaft, FRG). Cadmium uptake was studied with the aid of  $^{115}\text{Cd}$ .

## Results

### a) Short-term tests

Cell cultures were treated with graded concentration of heavy metals for up to 7 days. The following results were obtained:

1. The sensitivity of all the cell types employed was generally very similar. No consistent differences could be observed between human and mouse or normal and cancer-like cells.
2. The following rank order of toxicity was found for lethal effects:  
Methyl-Hg > CrVI > Cd > Ni  $\cong$  AsIII > CoII  $\cong$  Hg > Zn > TII  $\cong$  MnII > Pb  $\cong$  AsV > Cu (Fig. 1).
3. Inhibition of proliferation was noted in the following order:  
Methyl-Hg > CoII  $\cong$  CrVI > Cd > Ni > AsIII > MnII > Hg > AsV > Pb  $\cong$  TII  $\cong$  TlIII > Zn > Cu (Fig. 2).
4. Some of the metal compounds studied exhibit cellular effects over a wide concentration range. They include  $\text{NiCl}_2$ ,  $\text{CoCl}_2$ ,  $\text{MnCl}_2$ ,  $\text{Na}_2\text{HAsO}_4$ , and  $\text{PbCl}_2$ . Others show cytotoxicity in extremely narrow ranges, e. g.  $\text{Na}_2\text{CrO}_4$ ,  $\text{CdCl}_2$ ,  $\text{HgCl}_2$ , and  $\text{ZnCl}_2$ .
5. The ranges of concentrations leading to 50 % cell death ( $\text{LC}_{50}$ ) and causing a 50 % reduction of cell proliferation ( $\text{IC}_{50}$ ) are relatively far apart for the following compounds:  $\text{Cu}_2\text{SO}_4$ ,  $\text{PbCl}_2$ ,  $\text{TlCO}_2\text{CH}_3$ ,  $\text{Na}_2\text{HAsO}_4$ ,  $\text{MnCl}_2$ ,  $\text{CoCl}_2$ , and  $\text{AsCl}_3$ , i. e. growth inhibition results at concentrations which have only a small effect on viability.  $\text{LC}_{50}$  and  $\text{IC}_{50}$  ranges overlap in the case of  $\text{ZnCl}_2$  and  $\text{HgCl}_2$ , while they touch in the case of  $\text{NiCl}_2$ ,  $\text{CdCl}_2$ ,  $\text{Na}_2\text{CrO}_4$  and  $\text{CH}_3\text{HgCl}$ . Here inhibition of proliferation is accompanied by a marked depression of viability. These differences are interpreted as intrinsic properties of the metals and their compounds which characterize their cytotoxic potential.
6. The uptake of labelled precursors into DNA, RNA and protein was depressed in all the metals studied so far.

7. LDH release was inversely related to lethality indicating that intracellular enzymes are set free concomitant to cell death, whereas gross membrane damage resulting in significant enzyme leakage preceding death could not be demonstrated.

8. The production of lactic acid was increased after exposure to all metals except nickel. The enhanced glycolysis was interpreted to represent a mechanism to compensate for the impairment of cellular oxidative respiration.

Fig. 1.  
Effect of heavy metals on the viability of L-A cells exposed for 7 days

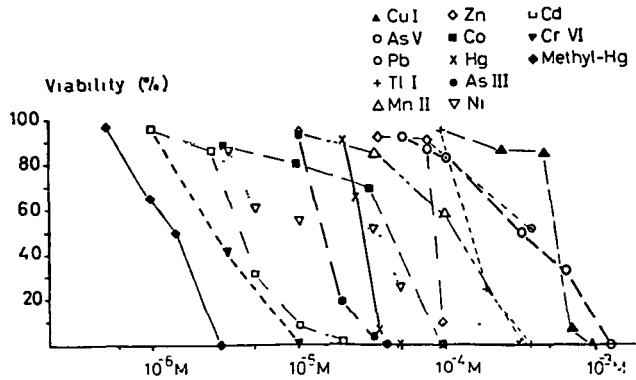
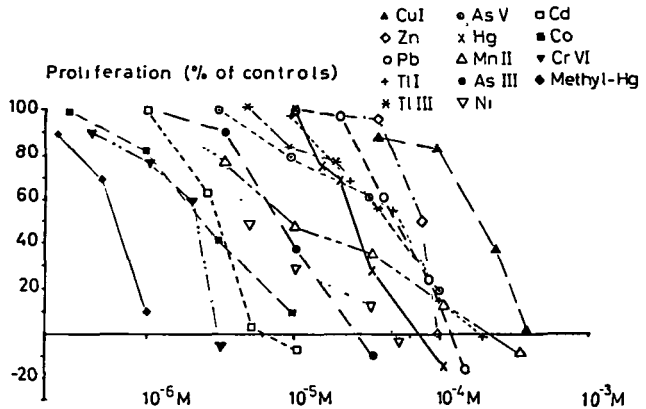


Fig. 2.  
Effect of heavy metals on the proliferation of L-A cells exposed for 7 days



b) Chronic exposure

In long-term tests cells were passaged for weeks in the presence of gradually increasing metal concentrations. Increasing resistance was observed to lead, mercury, and cadmium. The concentrations finally tolerated by L-A cells for periods up to several months were: Pb -  $2 \times 10^{-4}$  M, Hg -  $4 \times 10^{-5}$  M, Cd -  $3 \times 10^{-5}$  M. These levels approached the  $LC_{50}$  in the case of lead and even exceeded the respective  $LC_{50}$  in the case of cadmium and mercury. Tolerance

Pretreatment	Challenge concentration	Cell number (x 10 <sup>6</sup> )	Viability (%)	LDH (mU/10 <sup>6</sup> cells)	Lactic acid (mg/10 <sup>6</sup> cells)
None	8 x 10 <sup>-6</sup> M CdCl <sub>2</sub>	2,257 ± 0,123	68,7 ± 8,62	59,9 ± 1,79	5,395 ± 1,21
4 x 10 <sup>-6</sup> M CdCl <sub>2</sub> (7 days)	8 x 10 <sup>-6</sup> M CdCl <sub>2</sub>	2,623 ± 0,123	86,25 ± 1,77	27,9 ± 1,21	3,97 ± 0,1

Table 1. Effect of the pretreatment of human diploid fibroblasts with a low concentration of CdCl<sub>2</sub> on their response to a challenge concentration applied for 7 days

to these heavy metals was interpreted to be caused by adaptation rather than mutation and selection for the following reasons: (1) lead resistance was again lost after only a few generations in a lead-free environment; (2) in the case of cadmium and mercury a protective effect could be observed after only a few days; (3) heavy metal adaptation could also be demonstrated in diploid human fibroblasts which have an extremely low mutation rate (Table 1). In the pretreated cells not only viability and proliferation (cell number) are improved, but also the values of LDH and lactic acid have normalised.

Cadmium tolerance is not due to reduced metal uptake, but pretreated and control cells incorporate approximately the same amounts of CdCl<sub>2</sub> (Table 2).

Pretreatment	CdCl <sub>2</sub> concentration	Cd taken up (µg/10 <sup>6</sup> cells)	
		24 h	48 h
None	2,5 x 10 <sup>-6</sup> M = 1,2 µg/culture	--	0,42 ± 0,002
	5 x 10 <sup>-6</sup> M = 2,4 " "	0,42 ± 0,004	0,58 ± 0,010
	10 <sup>-5</sup> M = 4,8 " "	0,52 ± 0,021	0,86 ± 0,070
Cd-pretreated (10 <sup>-6</sup> M for 6 days)	2,5 x 10 <sup>-6</sup> M = 1,2 µg/culture	--	0,53 ± 0,017
	5 x 10 <sup>-6</sup> M = 2,4 " "	0,48 ± 0,041	0,60 ± 0,055
	10 <sup>-5</sup> M = 4,8 " "	0,64 ± 0,029	0,86 ± 0,003

Table 2. CdCl<sub>2</sub> uptake of HeLa cells with or without 6 days pretreatment with 10<sup>-6</sup> M CdCl<sub>2</sub>

The adaptation to cadmium is probably caused by the induction of a metal-binding protein, metallothionein. This protein, which consists of approximately 30% cysteine could be demonstrated indirectly by the increased uptake of <sup>3</sup>H-cysteine in cadmium-treated cells (Table 3).

CdCl <sub>2</sub> concentration:	Cell number/ml.	cpm	cpm/10 <sup>5</sup> cells
0	547 261	1820 ± 82,72	332,57
3,3 x 10 <sup>-6</sup> M	474 978	2110 ± 53,57	444,23
5 x 10 <sup>-6</sup> M	498 943	2128 ± 72,37	426,50
10 <sup>-5</sup> M	377 685	1934 ± 56,3	512,07

Table 3. Incorporation of <sup>35</sup>S-cystein into L-A cells treated for 24 h with different CdCl<sub>2</sub> concentrations

Other authors have previously shown the presence of metallothionein-like proteins in tissue culture cells, so far no mercury tolerance has been reported in vitro, though metallothionein induction in vivo by mercury has been proved. In experiments with Hg-tolerant L-A cells no cross-resistance to Cd could be demonstrated.

Chronic exposure of L-A cells to AsCl<sub>3</sub> for 10 weeks did not lead to improved growth. However, cells treated for 1 or 6 weeks with 10<sup>-5</sup> M AsCl<sub>3</sub> were significantly less damaged by 2 x 10<sup>-5</sup> M (Table 4).

Pretreatment	Challenge concentration	Cell number (x 10 <sup>5</sup> )	Viability (%)	Lactic acid (mg/10 <sup>6</sup> cells)
None	0	14,8	96,2	1,2
	2 x 10 <sup>-5</sup> M AsCl <sub>3</sub>	1,0	12,4	104,2
10 <sup>-5</sup> M AsCl <sub>3</sub> ( 7 days)	0	14,2	96,3	0,7
	2 x 10 <sup>-5</sup> M AsCl <sub>3</sub>	12,4	94,3	1,0

Table 4. Effect of the pretreatment of L-A cells with 10<sup>-5</sup> M AsCl<sub>3</sub> on their response to 7 days exposure to 2 x 10<sup>-5</sup> M AsCl<sub>3</sub>

## Conclusions

Cell cultures have proved to be a valuable test system for the evaluation of heavy metal effects at the cellular level. With this system a rank order of toxicity as regards lethal and growth inhibiting effects could be established. The sensitivity of all tested cell lines (human - animal; cancerous - noncancerous; fibroblast - epithelial-like) was similar, and similar biochemical reactions as well as adaptative processes could be demonstrated. Cell cultures

seem to be especially suitable for the study of heavy metal uptake, points of attack, and combination effects as well as cellular reactions including mechanisms of adaptation (e.g. formation of metal-binding proteins).

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**Contractor** : Institut für Arbeits- und Sozialmedizin  
und Poliklinik für Berufskrankheiten der  
Universität Erlangen-Nürnberg

**Contract** : 147-77-4 ENV D

**Project Leader** : Prof.Dr.med. H. Valentin

**Title of project** : External/internal dose-response relationship for  
mercury and vanadium in occupationally exposed  
and normal persons. Effects on kidney function

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### Objective of study

In the past years subclinical kidney disorders in man caused by mercury were reported. These were already found in the lower exposure range at the workplace. The objective of our studies was to test the frequency of early kidney damages in different Hg exposed groups at the workplace.

### Materials and Methods

The examined groups are described in table 1.

In all the factories morning urine samples as well as blood samples were received. The mercury was determined by cold vapour technique, albumin in urine by laser nephelometry, B-2-MG by RIA and B-galactosidase activity fluorimetrically. The urine parameters were relativated to 1 g creatinine.

group	N	Age (a) (range)	Exp.-T.(a) (range)	Hg-air ( $\mu\text{g}/\text{m}^3$ )
1. Chloralkali Plant A	25	47 (31-60)	20 (11-34)	75
2. Chloralkali Plant B	38	38 (20-56)	11 ( 2-20)	55
3. Reference group to 2.	33	38 (22-56)	11 ( 2-22)	not measured
4. Factory for Hg- compounds	16	43 (21-55)	9 ( 1-30)	100-650
5. Thermometer Manu- factory	99	35 (15-59)	11 ( 1-38)	5,0-180
6. Reference Group (general)	268	(15-65)	-	



## Results

The medians and the central 66.6 % ranges are given in table 2. As expected the mercury levels in urine and blood differed in the various collectives and correlated to the external exposure.

The medians for the albumin-levels of the higher exposed groups 1, 4 and 5 are a little bit above the levels in the other groups. The B-2-MG was highest in the reference groups and in the highly exposed collective 4.

The B-galactosidase activities are above the values in the ref. groups in collective 1 and 3. In the second chloralkali-collective the results are wrong. The activity of the enzyme has decreased by insufficient storage. The statistic comparison with the H-Test of Kruskal-Wallis showed significant differences for the mercury levels between all groups. The albumin excretion was significantly higher in the persons from the chloralkali-plant 1. No significant differences could be evaluated for the B-2-MG. For the highly exposed group there were no differences in B-2-MG and albumin to the ref. collective.

Table 3 shows the measured ranges for the different groups and the percentage of levels higher than normal. Only the B-galactosidase activity shows parallel behaviour to the exposure.

The exposure data show partly a high mercury load. This could be seen by the Hg-levels in air, urine and blood. The B-galactosidase activity is very often above the normal upper limit.

The correlation analysis demonstrated in no case a significant correlation between the exposure parameter and the protein excretion. In some cases there were significant correlations between Hg-U and Hg-B and the proteins one with another.

## Conclusions

The collectives examined showed no evidence for proteinuria in relationships with an exposure to metallic mercury or its compounds. A relationship is only observed for the B-galactosidase activity. No correlations were found between the exposure and the renal protein excretion. From the results we concluded that no significant kidney damages by Hg will arise at least in the exposure range till 200 µg/l urine or 50 µg/l blood.

Group	Hg-B (µg/l)	Hg-U (µg/g cr.)	Alb. (mg/g cr.)	β-2-MG (µg/g cr.)	β-gal'ase (U/g cr.)
1. Chloralkali Plant A	11 ( 7-24)	45 (20-78)	6 ( 3-19)	26 (10-78)	1,0 (0,2-2,2)
2. Chloralkali Plant B	4 ( 1-7 )	22 ( 9-39)	4 ( 3-11)	30 (10-65)	0,03 (0,00-0,5)
3. Reference group	not measured	0,2 (0,1-0,7)	4 ( 3- 8)	59 (31-103)	0,7 (0,55-1,2)
4. Factory for Hg-compounds	44 (19-101)	235 (100-400)	6 ( 3-20)	59 (11-123)	1,3 (0,5-2,1)
5. Thermometer manufactory	14 ( 8-24)	32 (13-82)	8 ( 4-30)	65 (12-140)	1 (0,4-1,8)
6. Reference group (general)	0,6 ( < 3 *)	0,8 ( < 8 *)			

\* upper limit

Table 2: Results of the measured parameters (medians; 66.6 % - Ranges)

Parameter	1.Chlor- alkali Plant A	2.Chlor- alkali Plant B	3. Reference group	4.Factory for Hg- compounds	5.Thermome- ter manu- factory
Hg-B range	4-61	1-14	-	8-268	4-116
% above the limit	100 %	60 %		100 %	95 %
Hg-U range	11-148	5-100	0,5-2	28-891	4-670
% above the limit	100 %	71 %	0 %	100 %	100 %
Alb.-U range	10-912	2-41	2-17	3-24	2-52
% above the limit	12 %	0 %	0 %	0 %	0 %
β-2-MG range	7-250	5-265	10-724	5-315	7-494
% above the limit	8 %	2,5 %	2 %	6 %	2 %
β-gal.-U range	0,2-5,7	0,2-1,6	-	0,3-4,0	0,03-21,3
% above the limit	28 %	2,5 %		44 %	34 %

Table 3: Ranges of the measured values and percentage of levels higher than normal for the different groups.

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Contractor : Institut für Arbeits- und Sozialmedizin  
und Poliklinik für Berufskrankheiten der  
Universität Erlangen-Nürnberg

Contract n° : 147-77-4 ENV D

Project Leader : Prof.Dr.med. H. Valentin

Title of project : External/internal dose-response relationship for  
mercury and vanadium in occupationally exposed  
and normal persons. Effects on nervous system

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### Objective of the research

The objective of the research was to investigate the neurotoxic effects of mercury exposures at the work-place.

### Materials and Methods

We investigated 21 workers, who had contact with metallic mercury and/or its inorganic or organic compounds between 5 months and 30 years (mean 93 months) and 18 workers, which were exposed to metallic mercury only in a thermometer-factory between 1 month and 40 years (mean 107 months).

### Results

The workers of the first collective were strong exposed because of the analytical results by means of 1. actual, 2. maximum, and 3. "time weighted average" mercury levels in urine and blood (range 12-720 µg Hg/l urine, 28-86 µg Hg/l blood).

In the case of the second collective the Hg-levels ranged from 58 to 380 µg/l in urine and from 13 to 165 µg/l in blood.

The median sensory conduction velocities of both nerves are decreased in case of the Hg-workers (N. ulnaris 47.9 m/s (normal > 50.6 m/s), N. medianus 46.0 m/s (normal > 50.0 m/s)). This reduction is not significant.

A dose-effect-relationship between the internal Hg-dose and the various nerve conduction velocities could not be demonstrated in the present study (coefficients of correlation between -0.19 and -0.49).

### Conclusion

In some cases there are hints, that the decreased nerve conduction velocities are caused by the exposure to mercury. These hints are based on individual evaluation of the toxicological and neurophysiological data.

Our findings indicate, that an impairment of nervous function does not occur, if the present biological threshold limit values for blood (50 µg Hg/l) and/or urine (200 µg Hg/l) are not exceeded.

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**Contract Nr.:** 265-77-i ENV D  
**Project Leaders:** Dr. H. Oldiges, Prof. Dr. W. Stöber  
**Title of project:** Influence and effects of NiO inhalation

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### Introduction

Since nickel is to a minor degree only a constituent of urban air, its main importance as air contaminant occurs at the workplace. The main routes of entry into body are via ingestion and inhalation, and a very limited route may be percutaneous absorption. In the course of our inhalation studies with heavy metals we investigated the effects of subchronic NiO inhalation. NiO was chosen as it constitutes the major component of nickel contaminants in air. If NiO is applied via inhalation the lung will be the target organ and therefore influences of NiO on lung had to be studied carefully. From the various studies being performed at our institute the following experiments are discussed:

The first experiment deals with the effects of NiO-inhalation on the elimination of NiO from the lung. Here the clearance of NiO itself was determined after deposition by inhalation. In the next experiment the effect of continuous NiO-inhalation on the lung clearance was established by measuring the half life of inert  $Fe_2O_3$ -particles in the lung. The alveolar macrophages contribute to the lung clearance, too, in phagocytizing foreign cells or particles which have entered into the lung. Therefore they are investigated in the third experiment. Alveolarmacrophages together with granulocytes and lymphocytes which were received by lung lavage, too, indicate

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inflammatory processes in lung. These cells are important cell-systems within the cellular immune-system. Therefore the effects on the humoral immune-system were studied in addition in the last experiment.

#### Materials and methods

NiO aerosols were generated by adopting the same methods which we used for CdO aerosols generation (1): A 1 % nickelacetate solution was nebulized and the produced aerosol was introduced into a tube furnace heated to 500 °C. This temperature was found to oxidize nickelacetate completely to NiO-particles. After deionizing these particles by a <sup>85</sup>Kr source, concentrations of NiO ranging from 25-800 µg/m<sup>3</sup> were established by diluting these aerosols with air.

For determination of lung clearance of NiO, 72 Wistar rats (strain TNO W 74) were exposed to NiO-aerosols at a concentration of 750 µg/m<sup>3</sup> for 1,5 hours. At day 0, 1, 3, 6, 16, 24, 55 and 100, respectively, 8 animals each were sacrificed. The lungs were excised and the Ni content determined by AAS.

To show the influence of chronic NiO inhalation on lung clearance, 40 male Wistar rats of 210-225 g body weight were continuously exposed to a NiO-aerosol of 50 µg/m<sup>3</sup> in whole body exposure chambers. 40 control rats were kept in Ni-free air. After 6 weeks all animals were exposed to a <sup>59</sup>Fe<sub>2</sub>O<sub>3</sub>-aerosol for 1 hour in nose-only-chambers. During the following days the lung field was measured using a NaJ-detector. At day 0, 1, 2, 7, 14, 24, 42 and 63 respectively, 5 animals were sacrificed, their lungs were excised and the activity was measured in a well crystal.

For investigating the immune-system and humoral response groups of 12 wistar rats were continuously exposed to NiO-concentrations of 12.5, 25, 50, 100, 200, 400 and 800 µg/m<sup>3</sup> for 4 weeks and of 25 and 150 µg/m<sup>3</sup> for 4 months, respectively, in whole body exposure chambers. Control rats were kept in Ni-free air.

At the end of the inhalation period,  $1.8 \times 10^9$  sheep red blood cells were given i.p.. After 5 days the rats were sacrificed, the blood was collected and the spleen taken out. The antiserum titers in the blood were determined according to the ambocepter-titration method described by Zwilling (2). The portion of antibody forming cells in the spleen was obtained by a modified plaque-test (3). The alveolar macrophages were isolated by a lung lavage method modified from that used by Brain (4). Dead and living cells

were determined by trypan blue exclusion test. The number, size and phagocytosis-activity of macrophages were measured with freshly prepared cells, differentiation of cell types and morphological investigations were done on slides stained according to Pappenheim.

## Results

### 1. Effects of NiO-inhalation on the elimination of NiO from the lung

The results of the elimination on inhaled NiO from the lung are shown in figure 1. In this experiment the nickel-content of control animals was below the detection limit of our method; that means below 30 ng/g.

You can see that the elimination of nickeloxide from the lung obeyed a bi-exponential way. After an initial deposition of 1.185 µg NiO/lung about 30 % of the deposited particles were cleared very quickly with a half life of about 0.7 days. However the half life of the second clearance mechanism, the clearance from the alveoli, was much longer. The clearance of these 70 % of the deposited particles last about 36 days. You can express the clearance by the following formula:

$$y = 0,27 \cdot \exp\left(-\frac{0.693}{0.7} t\right) + 0,73 \cdot \exp\left(-\frac{0.693}{36} t\right)$$

### 2. Effects of NiO-inhalation on lung clearance

The results are shown in figure 2. After the  $^{59}\text{Fe}_2\text{O}_3$ -exposure the determined radioactivity was set to 1.0. So it was much easier to compare the controls and NiO-exposed animals in the graph. You can see that in the first time of the lung clearance until day 7 there was no significant difference in the clearance of  $\text{Fe}_2\text{O}_3$  between controls and NiO-exposed animals. In the following days an increasing discrepancy between the two curves can be recognized. The controls eliminated the test-aerosol much quicker than the NiO-exposed animals. Therefore we could calculate the following half lives of the test-aerosol: controls 58 days, exposed animals 520 days.

This strongly delayed clearance of ironoxide demonstrated a significant impairment of alveolar clearance mechanisms by NiO-inhalation. The long lasting clearance could be expressed by the following formula:

$$\text{controls: } y = 0,67 \cdot \exp\left(-\frac{0.693}{58} t\right)$$



exposed rats:  $y = 0,68 \cdot \exp\left(-\frac{0,693}{520} t\right)$

The two coefficients: 0,67 and 0,68 indicated that the alveolar deposition did not differ significantly by both groups. Therefore the different clearance was not due to a different deposition of the particles.

The results from the thoracic counts were confirmed by measuring the activity of the excised lungs.

### 3. Effects of NiO-inhalation on alveolar macrophages

The results of the 4 weeks exposure experiments are summarized in figure 3. The number of alveolar macrophages was increased at an exposure concentration of 25  $\mu\text{g}/\text{m}^3$ . At 50, 100 and 200  $\mu\text{g}/\text{m}^3$ , there were no significant differences compared with controls. Above and at 400  $\mu\text{g}/\text{m}^3$  the number of alveolar macrophages decreased significantly. At 800  $\mu\text{g}/\text{m}^3$  there was only a small number of intact macrophages left in the lung; about half of the cells had lost their function. Above 100  $\mu\text{g}/\text{m}^3$ , the number of granulocytes increased quickly while the number of lymphocytes began to increase above 50  $\mu\text{g}/\text{m}^3$ . At 200  $\mu\text{g}/\text{m}^3$ , when the number of macrophages began to decrease, their ability to phagocytize was increased. At 400  $\mu\text{g}/\text{m}^3$  phagocytosis was strongly stimulated to 141 %; at 800  $\mu\text{g}/\text{m}^3$  the few intact macrophages showed an increased phagocytosis, too, however most of the others had lost their function.

In the 4 months experiment (figure 4), 25  $\mu\text{g}/\text{m}^3$  caused an increase in number of macrophages, granulocytes and lymphocytes. At 150  $\mu\text{g}/\text{m}^3$  granulocytes and lymphocytes had further increased, while the number of macrophages had decreased. The phagocytosis was strongly stimulated to 232 %.

To summarize this, in our 4 weeks experiments the number of alveolar macrophages was decreased above 200  $\mu\text{g}/\text{m}^3$ . In the chronic 4 months experiments both concentrations show significant effects on the alveolar macrophages. This is in good agreement with the experiments on the humoral immune system, which will be shown next.

### 4. Effects of NiO-inhalation on the humoral immune system

After 4 weeks of exposure, the immune response to injected sheep red blood cells showed a decrease dependent on the NiO-concentration, which was significant at a concentration higher than 200  $\mu\text{g}/\text{m}^3$  (figure 5). The observed effect was more pronounced in spleen than in serum. The trend towards a

slight increase at a concentration of 50  $\mu\text{g}/\text{m}^3$  was not significant. At this low concentration the number of macrophages was clearly stimulated, as you may remember.

After 4 months of exposure, the immune response to injected sheep red blood cells, did not significantly differ from controls at 25  $\mu\text{g}/\text{m}^3$  (figure 6). However at 150  $\mu\text{g}/\text{m}^3$  there was a significant decrease of the immune response. In both cases the antibody titer and the antibody formation dropped down to about 55 %. The 4 fold longer inhalation period caused a two fold higher effect. So, we could show, that subacute (4 weeks) and subchronic (4 months) inhalation of NiO caused a significant decrease of the antibody formation against sheep red blood cells.

#### Discussion

In our experiments we could demonstrate that about 30 % of inhaled NiO particles were cleared by a half life of 0.7 days. The rest was cleared by a half life of 36 days. This was significantly shorter compared to the determined half life for CdO (5).

Using these values for elimination you can calculate that a steady-state of NiO-concentrations in the lung will be reached after an inhalation period of about 200 days; that means thereafter deposition and elimination will be at equilibrium, provided that a chronic NiO-exposure does not impair lung clearance mechanisms. So we had to study the effects of NiO-inhalation on these mechanisms in the following experiment:

The experiment on lung clearance showed that continuous exposure of 50  $\mu\text{g}/\text{m}^3$  NiO caused a significant effect on lung clearance. The half life for eliminating inert particles of iron-oxide was ten-fold increased compared to controls. The clearance at the beginning of the experiment at day 1 to 7 indicated that the ciliar clearance-mechanism was not affected at this level; therefore the decreased clearance may be caused by an impairment of alveolar macrophages as it is pointed out by authors performing in vitro experiments (6,7). So in the next experiments the effects of NiO-inhalation on alveolar macrophages will be discussed.

In 4 weeks experiments the number of alveolar macrophages was decreased above 200  $\mu\text{g}/\text{m}^3$  NiO and in the 4 months experiments at 150  $\mu\text{g}/\text{m}^3$ , respectively. However, when the number of macrophages began to decrease, phagocytosis was

stimulated. The increase of granulocytes and lymphocytes indicated inflammatory processes in the lung. This damage to the lung was significant at  $200 \mu\text{g}/\text{m}^3$  in the acute 4 weeks experiments and at  $150 \mu\text{g}/\text{m}^3$  in the chronic 4 months experiments. These results were confirmed by increased IgG concentrations in serum of exposed rats. In addition, distinct foci of macrophages and emphysema could be observed in the lungs histologically.

In our last experiment we could show that subacute (4 weeks) and subchronic (4 months) inhalation of NiO caused a significant decrease of the antibody formation against sheep red blood cells. This is in good agreement with the experiments on alveolarmacrophages. Both systems, the number of alveolar macrophages and the humoral response, began to decrease at about the same concentrations of NiO. Effects of NiO on the humoral immune-system were also described by authors (8,9), however performing no inhalation experiments. The results indicated that animals may become more prone to infections caused by bacteria or viruses after NiO-inhalation.

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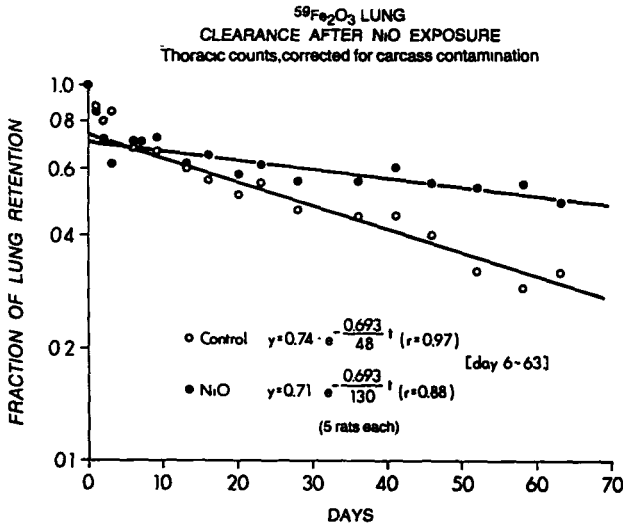
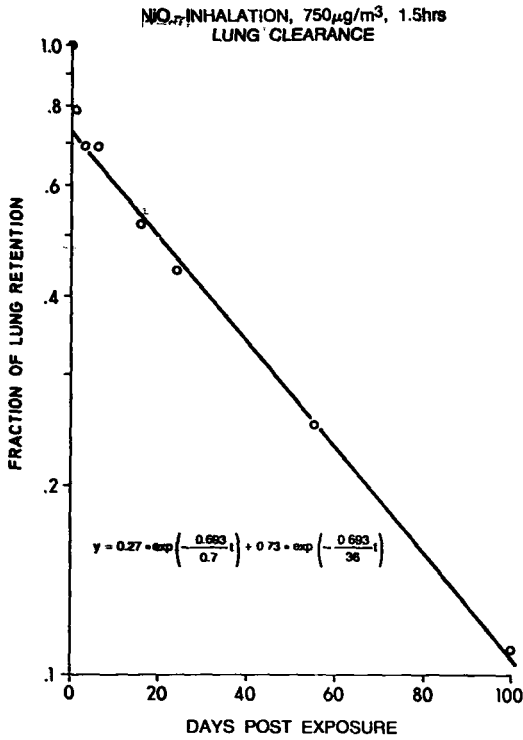
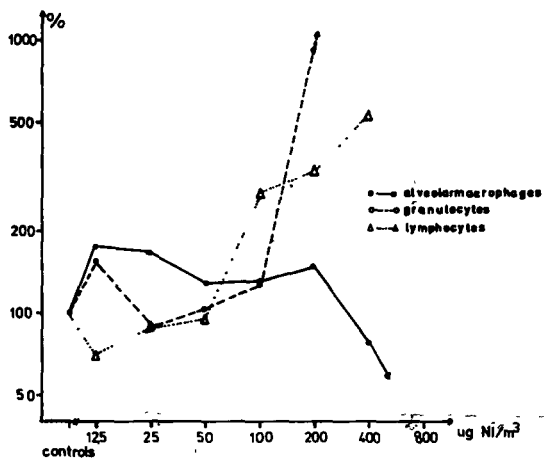


fig. 2

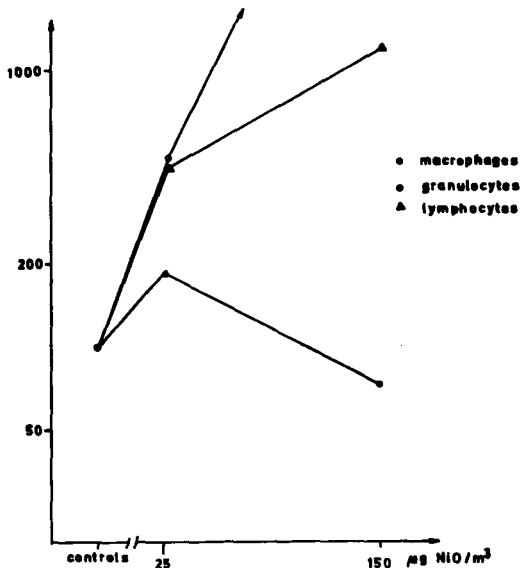
Fig. 3 Effects of 4 weeks NiO-inhalation on the number of macrophages, granulocytes and lymphocytes

Fig. 3



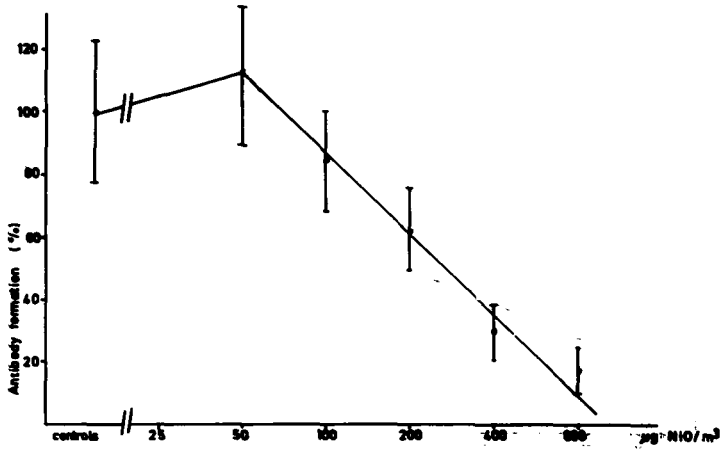
Effects of a 4 months NiO-inhalation on the number of macrophages, granulocytes and lymphocytes

Fig. 4



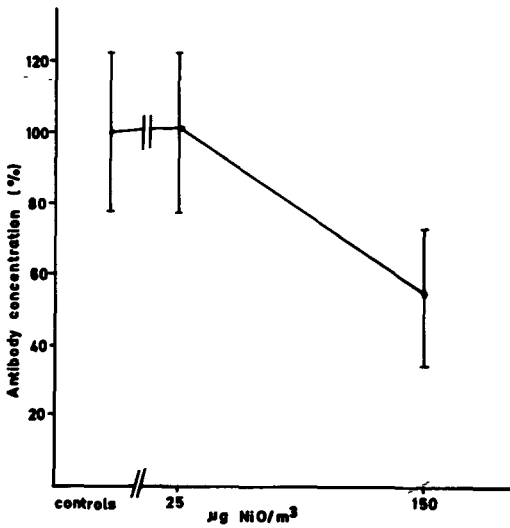
Effects of a 4 weeks NiO-inhalation on the antibody formation by spleen cells.

fig. 5



Effects of a 4 months NiO-inhalation on the antibody titer in serum

fig. 6





**Contractor:** Gesellschaft zur Förderung der Lufthygiene  
und Silikoseforschung e.V., Medizinisches  
Institut für Umwelthygiene an der Uni-  
versität Düsseldorf

**Contract No.:** 289-76-10 ENVD, Project 3

**Project leader:** Prof.Dr.med. H.-W. Schlipkötter

**Title of project:** Local effect of lead on lungs after  
inhalation of lead aerosols

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Objective of the research

The objective of our previous study carried out in 1977/78 was to investigate the effects of lead aerosols on the clearance of inhaled bacteria in the lungs of mice. In this study the lead-containing aerosol was administered in the form of lead chloride ( $PbCl_2$ ). It could be shown that lung clearance as measured by the number of retained bacteria as well as the number of macrophages was reduced after 15 days of exposure (5d/week, 3h/d) to a concentration of  $15 \mu g \text{ lead}/m^3$  air.

It has been pointed out that the lead aerosol administered in our previous study is different from the Pb-containing particulates in the ambient air which contain a variety of lead compounds adsorbed to other materials. In order to overcome

these shortcomings we have tried to improve the inhalation technique. Three methods were tested, one of which was selected for the subsequent inhalation experiments in rats. In the following paper recent results concerning the improvement of aerosol generation and first results on dust retention in rats are reported.

#### Part I: Development of inhalation technique

##### a) Preliminary experiments

These experiments were designed to produce a lead aerosol by evaporation of lead oxide and subsequent condensation. An air stream (500 l/min) was blown through a clay-pipe, where lead oxide was heated to 500°C. This air stream was then cooled to 25°C, and the resulting lead aerosol formed by condensation was blown into an inhalation chamber. It turned out, however, that it was impossible to produce stable Pb-concentrations over a prolonged period of time. This approach was therefore discarded.

##### b) Experience and results with grinding disc

With this method there was already some experience in the field of silicosis research, inhalation of quartz and clay dusts in animal experiments. Dusts of different origin and composition are pressed by a special apparatus to specimens of the dimensions 20x20x100 mm. By means of a grinding disc rotating at 2800 rpm these specimens are pulverized and the resulting lead-containing dust is added to a stream of air which is blown into the inhala-

tion chamber. The concentration of lead in the inhalation chamber can be controlled by varying the air stream, changing the grinding speed, and by using the generator in an discontinuous manner. Preliminary experiments with dust consisting of pure, unprepared titan dioxide showed that it was possible to make stable specimens, which could be used in combination with the grinding disc. There were many more difficulties involved in making specimens with low contents of lead. In view of the composition of particulate matter in the ambient air it seemed not very useful to make a mixture of titan dioxide-dust and lead oxide particles, but to adsorb lead ions onto the fundamental dust.

c) Method used in animal experiments

Three rotating disc generators were used in three different, but similar inhalation chambers. Titan dioxide dust (P 25 Fa. Degussa, Frankfurt) was suspended in distilled water with different content of lead salts. After reaching adsorption equilibrium the sample was filtered, dried and ground. Dust specimens of given dimensions were made by a mechanical device, described in detail by Polley (Polley: Tierbestäubungsanlagen Inhalationstest. In: Ergebnisse von Untersuchungen auf dem Gebiet der Staub- und Silikosebekämpfung im Steinkohlenbergbau, Bd 5. - Detmold: H. Bösmann GmbH. 1965, S. 41-48). Results of test experiments without animals showed that dust concentrations with little day to day variance could effectively be made.

## Part II: Animal experiments

### Materials and methods

#### a) Inhalation boxes

Inhalation boxes (2 m<sup>3</sup>) coated inside with V2A steel were used for all experiments. Each box was equipped with an aerosol generator, an air sampling system, and a tyndalloscope.

#### b) Aerosol generation and aerosol measurement

The aerosol was generated as described above by a grinding disc rotating at 2800 rpm, which dusted a pressed specimen consisting of titan dioxide, titan dioxide + 1% lead (adsorbed), and titan dioxide + 5% lead (adsorbed), respectively. The mean aerosol concentration of a period of 6 hours was determined by using a filter device for low volume sampling. Air was drawn by a piston pump with a constant speed of 76 l/h through a glass filter, and a membrane filter, respectively (filter area 17.32 cm<sup>2</sup>). Before reaching the filter the air had to pass an inlet tube with a diameter of 50 mm and a length of 250 mm. Particles with a Stokes diameter  $> 20 \mu\text{m}$  were precipitated by this system and did not reach the filter surface. The total dust concentration was determined by gravimetric analysis using glass fiber filters. The lead concentration was determined by sampling the dust on membrane filter. For analysis a defined section of the membrane filter was ashed in concentrated sulphuric acid/hydrogen peroxide. Lead was determined by electrothermal atomic absorption spectro-photometry (Perkin Elmer, Model 400,

equipped with a graphite furnace HGA 500 and an auto sampling system AS 1). Short time alterations of the dust concentration in the inhalation boxes were determined by measuring the scattering of light by the aerosol with a tyndalloscope.

c) Animals

Female Wistar rats (body weight 180-210 g) were obtained from S. Ivanovas, Kisslegg, W.-Germany. The animals were received when approximately 8 weeks of age and were maintained on RMH-TM diet (Hope, Woerden, Holland) and water ad libitum.

The animals were divided into three groups each group consisting of 78 rats. Each group (I - III) was further subdivided into three subgroups each consisting of 26 rats. The composition and the exposure times were as follows:

	Subgroup	Exposure time	Composition of the inhaled dust
Group I	A	113 h	
	B	338 h	titan dioxide
	C	Exposure time not yet terminated	
Group II	A	113 h	
	B	338 h	titan dioxide
	C	Exposure time not yet terminated	+ 1% lead
Group III	A	113 h	
	B	338 h	titan dioxide
	C	Exposure time not yet terminated	+ 5% lead

d) Determination of titan dioxide in the lungs

After termination of exposure 20 animals per subgroup were kept in clean air for 6 days. They were killed by an overdose of ether inhalation. Blood was removed by heart puncture, and the lungs were excised. After cleaning with Krebs-Ringer solution the wet weight of the lungs were determined. Until chemical analysis the samples were preserved in acetone. For analysis the lungs were dried (120°C, 12 h) and solubilized with concentrated sulphuric acid/hydrogen peroxide. The content of titan dioxide was determined photometrically at 436 nm as  $\text{TiO}_2(\text{SO}_4)_2^{2-}$ -complex (Koch/Koch-Dedic: Handbuch der Spurenanalyse, 2. Edition. - Berlin, Heidelberg, New York: Springer 1974, p. 1210).

e) Morphological analyses

Six animals per subgroup, which had been kept in clean air for 6 days after termination of exposure, were killed by an overdose of Nembutal (i.p.). The lungs were fixed by intratracheal instillation of glutaraldehyde (2%) dissolved in cacodylate buffer (320 Osmol; pH 7.2). Tissue samples of the left lung lobe were prepared for ultrastructural investigations by embedding in Araldit. Semithin and ultrathin sections were prepared from this material.

Results

At time of report, exposure of animals of subgroups C (6 months exposure) was continuing. Chemical and histological analysis

for subgroups A and B (exposure times of one and three months) are only partly completed. Therefore the results of animal experiments are preliminary and give an initial view of the possible biological effects.

For comparison of the groups and subgroups, control of the dust concentration is most critical. Results of the measurements in the inhalation chambers are summarized in table 1. As can be seen there are only small differences in dust concentration between the three groups. By combination of dust concentration and inhalation time it is possible to get a relative measure of the inhaled dose. The dust offered, calculated on the basis of a minute volume of 100 ml amounted to 6.8 - 7.6 mg for subgroups A and 20.6 - 22.2 mg for subgroups B. General data about body and lung weights are given in table 2 in combination with the data for dust content of the lungs. Increase in body weight is very slight, lung weights are constant during inhalation time and no significant differences exist between the groups. In figure 1 increase of dust content of the lungs with exposure time is shown. As can be seen there are significant differences between the control on one hand and the two groups with different lead content of the inhaled dust on the other hand. Dust retention in percent of total dust offered is summarized in table 3.

Retention is lowest for group I (control) and highest for group II. Differences are more pronounced after 1 month than after 3 months of exposure. Evaluation of the histopathomorphology by light and electromicroscopy is not finished. First results

of qualitative histological examination after inhalation time of one month revealed no remarkable differences between the groups I - III. It seems however, that lead dusted animals showed some enhancement of cellularity in comparison to the controls. This will be further checked by quantitative measurements on lungs from animals after 3 and 6 months of exposure time.

### Discussion

The objective of our previous studies on the acute and chronic effects of lead aerosols on the lung was to examine the effect of inhaled lead aerosols on the clearance of inhaled bacteria. This study was carried out in 1976-78. The aim of our subsequent studies carried out in 1979/80 was primarily to improve the aerosol generation. The experiments could not be terminated successfully until 1980. For this reason it was not possible to terminate the inhalation experiments up to now. The subgroups C which consist of animals that will be exposed for six months (6 h/d, 5 days/week) are still inhaling the aerosols as described above. The last part of the experiment will be finished in April/May 1981. Our preliminary results show, however, that over a period of 1-3 months relatively stable aerosol and lead concentrations can be generated in the inhalation boxes by the aerosol generation technique described above. As shown in table the variations of the dust concentration are rather low. It is therefore possible to compare the retention rates of the inhaled dust at various air lead levels. Tables 2 and 3 show that the lung clearance of titan dioxide is decreased in animals that



inhaled lead containing dust. This effect might be due to an impairment of alveolar macrophages. It is unclear, however why this effect decreases with increasing lead content of the inhaled dust.

From basic theoretical considerations a decrease of the relative retention rate of dust has to be expected for longer exposure times. While this is the case for groups II and III the expected effect could not be verified for group I. A more detailed analysis of these findings should be possible at the end of the experiment. Preliminary morphological investigations which are yet to be completed, do not show any gross effects that might be caused by the lead content of the inhaled dust.

#### Summary

The inhalation technique for lead aerosols was improved. It is now possible to use aerosols which more closely represent exposure via ambient air than in our previous study. Moreover stable dust and lead concentrations for long periods could be reached. In inhalation experiments with rats only slight differences of lung dust retention at dust concentration of  $10 \text{ mg/m}^3$  could be shown between animals inhaling dust with different lead content. Initial qualitative histological examination of the lungs was unable to show any difference between control and lead-exposed groups. Experiment and evaluation are yet to be completed.

Table 1 : Dust exposure data. Average dust concentration, inhalation time and dust dose for group I - III with subgroups A and B. ( \* dust dose calculated on the basis of the minute volume 100 ml/min ).

	dust concentration		inhalation time h	dust dose $\frac{\text{c.t}}{\text{m}^3}$	dust dose* mg
	$\bar{x}$	n			
Group I TiO <sub>2</sub>	A	19	9.83 - 10.2	1133	6.8
	B	57	10.1 - 10.5	3483	20.9
Group II TiO <sub>2</sub> + 1% Pb	A	18	9.82 - 11.7	1210	7.3
	B	55	9.62 - 10.7	3426	20.6
Group III TiO <sub>2</sub> + 5% Pb	A	17	10.2 - 11.5	1266	7.6
	B	54	10.2 - 11.1	3698	22.2

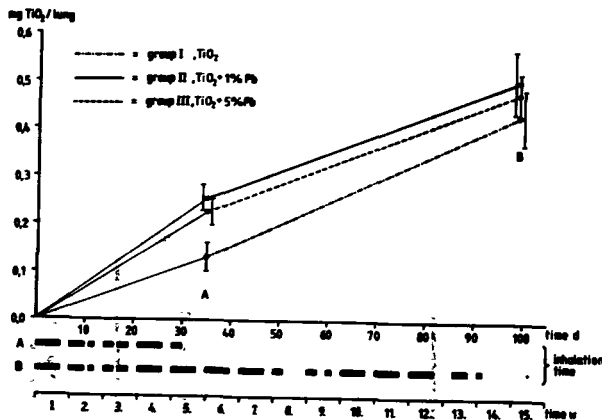
Table 2 : Mean body weight, wet and dry weight of lungs and dust content of lungs ( n = 10 )  
in relation to the lead content of the inhaled dust and in relation to the inhalation  
time ( A = 113 h, B = 338 h ).

	body weight g		wet weight lung g		dry weight lung g		dust content lung mg TiO <sub>2</sub>	
	$\bar{x}$	95%-confidence limits	$\bar{x}$	95%-confidence limits	$\bar{x}$	95%-confidence limits	$\bar{x}$	95%-confidence limits
group I TiO <sub>2</sub>	A	197 - 204	1.07	1.02 - 1.13	0.174	0.167 - 0.180	0.132	0.102 - 0.162
	B	226 - 241	0.979	0.926 - 1.03	0.174	0.166 - 0.181	0.430	0.373 - 0.487
group II TiO <sub>2</sub> + 1% Pb	A	186 - 197	1.10	1.03 - 1.17	0.176	0.165 - 0.188	0.255	0.228 - 0.283
	B	220 - 233	0.993	0.938 - 1.05	0.170	0.159 - 0.181	0.504	0.438 - 0.569
group III TiO <sub>2</sub> + 5% Pb	A	191 - 201	1.11	1.06 - 1.15	0.172	0.163 - 0.181	0.226	0.198 - 0.254
	B	209 - 229	1.00	0.968 - 1.04	0.169	0.165 - 0.174	0.478	0.434 - 0.522

Table 3: Final retention in percent of total dust after inhalation time of 113 hours (A) and 338 hours (B).

	TiO <sub>2</sub> -retention (%)	
	A	B
group I TiO <sub>2</sub> (control)	1.94	2.06
group II TiO <sub>2</sub> + 1% Pb	3.49	2.45
group III TiO <sub>2</sub> + 5% Pb	2.97	2.15

Fig. 1: Dust content of lungs in relation to inhalation time for lead groups and control.



Contractor: Medizinisches Institut für Umwelthygiene an  
der Universität Düsseldorf

Contract no.: 289 - 76 - 10, Project 4

Project leaders: Prof. Dr. med. H.-W. Schlipkötter  
Dr. rer. nat. G. Winneke

Title of project: Neurobehavioral studies on the effects of  
lead and cadmium on the developing central  
nervous system

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#### Objective of the research

Subsequent to earlier work on neurobehavioral deficit in lead-exposed rats, namely impairment of learning and activity-increase, the research carried out under this contract should answer the following questions: (1) Can the findings observed in small laboratory animals at blood lead-levels (PbB) between about 20 and 40  $\mu\text{g}/\text{dl}$  be extended to non-human primates, pre- and postnatally exposed to comparable PbBs?; (2) Do the adverse behavioral effects observed subsequent to early exposure to inorganic lead occur after exposure to other heavy metals as well? The intention behind the 1st objective was to narrow the gap between lower animals and man with respect to Pb-induced neuropsychological dysfunction. The 2nd objective touches the important problem of the specificity of Pb-induced neurobehavioral deficit. This problem was added to the project when it became clear, that, due to the inherent behavioral complexity of non-human primates the progress of experimental work was slower than expected.

#### Materials and methods

##### 1) Behavioral studies in Pb-exposed primates

16 infant Rhesus monkeys (*M. mulatta*) represent the sample, 6 (3♀, 3♂) being controls, 5 (2♀, 3♂) belonging to the low-lead-group (A-Pb), and 6 (3♀, 3♂) being classified as high-lead (B-Pb). These animals were born in 1978 to females having received either non-leaded control-diet, or diets containing either 350 ppm lead as lead-acetate (A-Pb) or 600 ppm lead. These diets, fed for a duration of 150 days before mating and continued afterwards, gave rise to average PbBs below 1  $\mu\text{g}/\text{dl}$  for controls and of  $23.6 \pm 3.5$   $\mu\text{g}/\text{dl}$  ( $\bar{x} \pm \text{SEM}$ ) for the low lead

group (A-Pb) and of 40:8±7.7 µg/dl for group B-Pb. After separation from their mothers after 6 to 9 months these infant monkeys were transferred to our laboratory and raised in groups of 2 or 3 animals.

Behavioral testing could not be done until 1980. Spontaneous activity of the animals in their home-cages was measured over four consecutive 24-hour-intervals by means of piezo-ceramic transducers placed below a sitting board. Discrimination-learning is being tested in a modified "Wisconsin General Test-Apparatus" (HARLOW, 1949): The animal is offered a choice of objects on a tray, one of which covers a food-well, while the other covers an empty well. The animal is given one discrimination-problem/day for a specified number of trials; number of trials-to-criterion and errors are recorded for each problem. The sequence of problem-trial-blocks is patterned according to a "learn-to-learn" paradigm (HARLOW, 1949). 90 problems have to be solved altogether, before "higher-order-learning" is tested in final learning-blocks.

## 2) Testing the specificity of Pb-induced CNS-damage in rats

Two experiments were performed under this heading, using cadmium as a model. In experiment I (WINNEKE et al., 1979a,b) three groups of rats were formed as follows (n=10 each): Controls (saline treatment), Cd-A (only maternal exposure until weaning), Cd-B (maternal plus subsequent direct postnatal Cd-treatment until testing). Cd was given as CdCl<sub>2</sub> by repeated s.c.-injections (0.15 mg Cd/d for 50 - A - or 150 days - B). In experiment II Cd was given as CdCl<sub>2</sub> in the diet (100 ppm as Cd) for 60 d prior to mating and afterwards until weaning or until testing. Three groups of animals (n=15) were formed as before. Cd-concentrations were measured by AAS in liver, kidney and brain at different times during the experiments. Behavioral testing was done in adult animals (100-150 d) according to our standard procedure (WINNEKE et al., 1977), using discrimination learning performance and activity (open field) as measures of an effect. In experiment II spontaneous homecage-activity over 24 h, recorded by means of piezo-ceramic transducers, was measured also.

## Results

### 1) Behavioral studies in Rhesus monkeys (M.mulatta)

The results of the activity-studies are given in table 1 for night-activity and in table 2 for daytime-activity. As far as night-activity is concerned there is a clear tendency for the high-lead group to exhibit reduced motor activity as compared to the control-group, with the low lead-group in between. For daytime-activity the situation is less clearcut: On day I, when the animals are not yet habituated to the new surroundings the high lead-group exhibits increased activity as compared to the controls with a complete reversal later on. These preliminary

findings need confirmation by subsequent animal-by-animal-measures, however.

	1st night	2nd night	3rd night	4th night
Control	0.47±0.25	0.42±0.0.4	0.55±0.09	0.62±0.39
A - Pb <sup>*)</sup>	0.36	0.15	0.54	0.45
B - Pb	0.15±0.11	0.09±0.04	0.09±0.06	0.18±0.14

\*) one cage only

Table 1: Total night-activity-counts in relative units ( $\bar{x} \pm S.E.M.$ ) for the three groups (n=2 cages for controls and group B-Pb).

	1st day	2nd day	3rd day	4th day
Control	13.6 ± 1	21.6±2.6	20.2±0.3	22.5±1.3
A - Pb <sup>*)</sup>	14.1	14.9	16.5	15.5
B - Pb	17.2±1.9	15.6 ± 1	17.8 ± 1	18.2±0.4

\*) one cage only

Table 2: Total daytime activity-counts in relative units ( $\bar{x} \pm S.E.M.$ ) for the three groups (see above).

Discrimination-learning has not yet progressed very far: 5 animals (2 controls, 2 from group B and 1 from group A) are in different phases of the complete learning-cycle. Table 3 summarizes the present situation. It is clear that the two high lead-animals are lagging behind the two controls, which is obviously due to the difficulties in getting these animals prepared for learning in the training-sessions; both groups are, however, inferior to the one low lead-animal.

	duration of pretrain. (days)	problems solved until March 1, 1981
Controls	14.5±2.5	31.5±2.5
A-Pb <sup>*)</sup>	8.0	75.0
B-Pb	25.0±7.1	13.0±6.0

\*) one animal only

Table 3: Present status of learning-experiments in terms of days of necessary pretraining and no. of problems solved.

As for actual learning-data fig. 1 illustrates the present situation for these five animals.

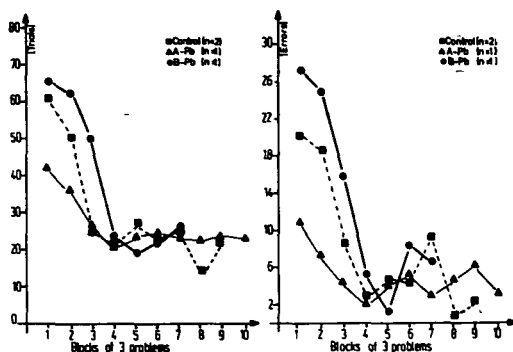


Figure 1: Discrimination-learning for consecutive blocks of three trials, with "trials-to-criterion" (left) and "errors" (right) as performance-measures

## 2) Neurobehavioral effects of cadmium in rats

The Cd-concentrations in different tissues and for both experiments are given in table 4. The data from both experiments are roughly comparable, at least for the adult animals. From these data some, albeit small, placental transfer of Cd, als well as some, albeit small, Cd-accumulation in the brain can be inferred. By comparing perfused and non-perfused brains it was shown that the presence of blood in the tissue does not influence the Cd-concentration.

As for discrimination learning-performance the outcome of both experiments was largely the same: Both Cd-groups were significantly inferior to their controls, but did not differ from each other. It is remarkable that even after only maternal Cd-exposure learning-performance is clearly disturbed in the adult animal with largely normal Cd-tissue-levels. As for open-field-behavior the outcome of both experiments is slightly different: Whereas in experiment I significant hypoactivity occurred in group Cd-B-animals only, both Cd-groups displayed reduced activity in experiment II. Spontaneous homecage-activity revealed interesting



differences between both Cd-groups, however, since Cd-B-animals appeared hypoactive but Cd-A-animals hyperactive, relative to the activity of the control-animals.

		Experiment I			Experiment II		
		1-7d	21-30d	160-200d	1-7d	21-30d	160-200d
brain	Control	-	7	-	1.8	1	1
	Cd-A	16	2	2	4.7	8.3	1.8
	Cd-B			63			82
liver	Control	-	18	-	3.5	1.5	2.8
	Cd-A	88	35	6	15.3	19	19.8
	Cd-B			63640			30500
kidney	Control	-	21	-	5.3	4.5	38
	Cd-A	128	108	23	26	14.7	85.9
	Cd-B			48640			46167

Table 4: Average Cd-concentrations (ng/g wet weight) in different tissues at different ages in both experiments.

### Conclusions

With average PbBs of 23 (group A) and 41,ug/dl (group B) our behavioral studies with Rhesus monkeys have a direct bearing for environmental exposure-conditions. These experiments have not yet progressed far enough, however, to allow definite conclusions concerning the effects of low-level lead-exposure on the developing CNS of non-human primates. The following remarks would seem justified, however: (1) As for spontaneous motor-activity hypoactivity does seem to be the prevailing action of lead in these animals; (2) the course of daytime-activity with initial hyper- and subsequent hypoactivity seems consistent with the "reactivity"-hypothesis put forward earlier in connection with open field-activity of rats (WINNEKE et al., 1977); (3) the apparent performance-deficit in discrimination-learning of the high-lead group as compared to the controls is seemingly more related to the difficulties in getting these animals prepared for learning than to impaired learning-capacity as such.

As for cadmium the results of both experiments with either parental or dietary exposure are very consistent: They demonstrate quite clearly that Cd has some neurotoxic properties at body-burdens below those levels, which are usually associated with kidney dysfunction in rats; this neurobehavioral deficit is clearly longlasting after early developmental exposure, and the behavioral pattern is somewhat different from lead, because the impairment of learning-performance is associated either with no activity-change or with hypoactivity in the open field.

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Contractor : Ecole Nationale Vétérinaire - 23, chemin des Capellès  
31076 TOULOUSE CEDEX - FRANCE

Contrat n° : 148-77-1 ENV F

Project leaders : Professeur RICO André et Professeur BURGAT-SACAZE Viviane

Title of project: Medium and long-term. Toxicity of the cadmium ion in rats :  
Biochemical and metabolic studies : Cold stress

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Previous studies have shown that in rats slightly contamination by cadmium (2.5 ppm in drinking water for a year) cold stress could reveal a latent renal dysfunction. The origin of this phenomenon is the subject of the present research ; biochemical results and metabolic data are reported.

## A - BIOCHEMICAL ASPECTS

### Material and methods

The experiments were carried out in 8 groups of 20 Sprague Dawley rats (10 ♂, 10 ♀), given a standard diet and drinking tap water (TT and TF groups) or tap water supplemented with 2.5 ppm Cd (CdT and CdF groups) for 6 or 12 months. At the end of this period, groups TF and CdF underwent a cold-stress (+1°C, 48 hr); they were then immediately sacrificed and compared with TT and CdT groups housed at room temperature.

The following biological parameters were measured :

- urine : sodium, potassium, urea, creatinin, catecholamines, aldosterone, cortisol.
- plasma : sodium, potassium, calcium, chloride, inorganic phosphates, glucose, urea, alkaline phosphatase, plasma renin activity (PRA).

### Results

#### 1 - Six months experiment

- Few differences could be observed between non-stressed animals i.e. TT and CdT groups. Daily urinary sodium elimination is greater in CdT rats than in TT.
- In cold stressed rats, cadmium interact with phosphatemia which increases in CdF group and not in TF group.

## 2 - One year experiment

• Non stressed-animals are similar ; no significant difference could be observed for any of the parameters between T T and T Cd groups.

• In cold stressed groups (TF, CdF) urine and plasma differences could be noted :

- urine : first, the biochemical profile is similar to that previously reported (RICO and al, 1978) ; more particularly, in cadmium treated animals, polyuria and daily sodium and potassium elimination are less intense (fig 1)

Moreover, urine catecholamines excretion is more increased in CdF group than in TF (fig 2) ; but cortisol and aldosterone increases did not differ between groups .

- serum : main results are the stability of kaliemia in cadmium stressed rats and of PRA in TF and CdF groups. Cadmium interactions with calcium, phosphates, glucose and creatinin observed in previous studie are not find.

## Discussion

This results demonstrate that low dose long term (12 months) cadmium ingestion decreases the polyuria and increases the urine catecholamines excretion produced by a cold-stress. On the contrary, serum PRA, urinary cortisol and aldosterone no differ between controls and cadmium groups.

The difference in catecholamines excretion could be related to the less important polyuria noted in Cd treated animals. Indeed, previous observations stated that for the first 48hr of cold-exposure, the main catecholamines excreted is Norepinephrine (LEDUC 1961), which produces high renal arteriolar vasoconstriction and diminution of GFR.

Moreover these data support cadmium interaction with the adrenergic system or with adrenals. Low doses of cadmium have been reported to inhibit MAO and COMT and thus increase the half-life of NE (REVIS 1978). Cadmium was also reported to enhance the vascular response to adrenergic stimulation (NECHAY 1978).

Adrenal direct effect could be also involved ; cadmium increases the weight of adrenals (DER 1977) and the catecholamines release by this glands (HART 1974) (SHANBAKY 1978), in vitro.

Experiences now performed aim to determine which catecholamines are involved in the cold response.

## **B - METABOLIC ASPECTS**

### **Material and methods**

The kinetics of cadmium - 109 have been studied in particular by macroscopic autoradiography according to the ULLBERG technique in mice and rats and in the case of the latter, by oral route before and after cold stress. Informations were also obtained by pulmonary route. Measurements in liquid scintillation have also been carried out.

### **Results and discussion**

#### **1 - Oral route :**

Distribution in rats shows that intestinal absorption is low : the target organs, although not very marked, are the kidney, the liver and the pancreas. The kinetics do not seem to be changed by the cold stress.

#### **2 - Pulmonary route :**

It was interesting to note an absorption more important than by oral route. The distribution is the same. The target organs are the kidney, the liver and the lung. We can see also cadmium in the guts.

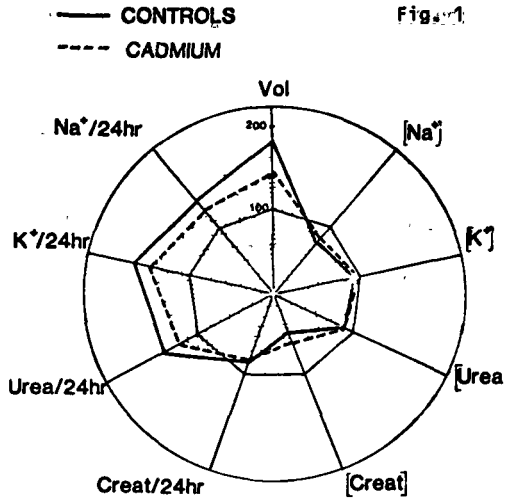
But the most important information is the presence of metal in pituitary and thyroid glands, 24 hours after administration.

BIBLIOGRAPHIE

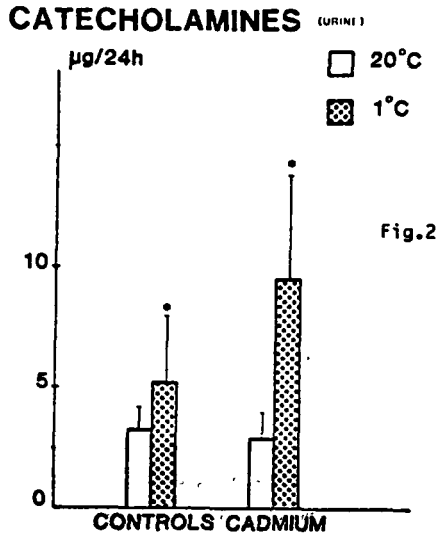
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- Second International Congress of Toxicology - Bruxelles, Juillet 1980.



URINARY PARAMETERS IN COLD-STRESSED RATS (48hr, -1°C)  
MEANS OF 20 RATS (10 ♀, 10 ♂) AS PERCENTAGE OF  
CONTROL GROUPS



Contractor : Institut Pasteur de Lyon, France  
Contract n° : 269-77-1 ENV F  
Project Leader : Professor M. Carraz, Doctor P. Tachon  
Collaborators : Miss A. Laschi (CEC stagiaire), Doctor J.P. Briffaux (Vet.)  
Title of Project : Experimental saturnism in the *Macaccus irus* monkey and immunoreactivity of lead and nickel

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## EXPERIMENTAL SATURNISM IN THE MACACCUS IRUS MONKEY

### I. OBJECTIVE OF THE RESEARCH

We have been working on the experimental saturnism in the primate for several years. After having precised the alterations caused by a chronical intoxication of the monkeys, we now work upon the "mother-infant" relationships in the intoxicated *Macaccus irus*.

### II. MATERIALS AND METHODS

#### II.1. Chronical intoxication of the monkey

During 1978-1979, we carried out an intoxication by Pb acetate (oral route) on 11 *Macaccus irus* monkeys at the following doses : 1 and 5 mg/animal/day. At the end of the intoxication period, which lasted 16 months, the animals were sacrificed, and the liver, the kidneys, the testicules were examined in electronic microscopy.

#### II.2. "Mother-infant" relation in the experimental saturnism in the primate

25 pregnant *Macaccus cynomolgus* female monkeys are distributed in three groups, among which only two are treated with Pb acetate, administered by intramuscular route at the dose 1 and 5 mg Pb<sup>++</sup>/kg/day.

The treatment starts as soon as the diagnostica of pregnancy is confirmed by post-implantatory haemorrhage (about the 21st day). This treatment is maintained during the gestating period and the lactation (for the females which are not sacrificed before delivery).

### III. RESULTS

#### III.1. Chronical intoxication of the monkey

. The kidneys : we did not find the intranuclear inclusions observed in a work carried out before, when using 5 mg/animal/24 hours. However, we noted important signs of desquamation and fibrosis on the proximal tubules.

These observations confirm those already effectued on rodents, which



show that the presence of these intranuclear inclusions is an early phenomena, and non constant. The inclusions disappear with time.

. The liver and the testicules: on these organs, we have not observed any ultrastructural modification due to the treatment.

No tumor was found.

### III.2. "Mother-infant" relation in the experimental saturnism in the primate

#### - Experimental results

a) clinical : . the dose 5 mg/kg/day is lethal for the pregnant females within 3 months of treatment. The mean gestating time was then 109 days, the mean weights of the foetuses 118 grams.

The obtained foetuses were normal, we did not observe any macroscopical abnormality.

. the dose 1 mg/kg/day is well tolerated during the entire gestating period, however, during the lactation period, all of the females that fed their new-borns presented intense intoxication signs at about the second month, and had to be sacrificed.

	control group	treated group 1 mg/kg/day
gestating period	156,0 $\pm$ 4,1 days	157,6 $\pm$ 4,8 days
weight of new-borns	339 $\pm$ 31 grams	315 $\pm$ 48 grams

b) biology : Pb dosage : the day of the delivery, the mothers present a plumbemia of 63,8  $\pm$  12,7  $\mu$ g/l and 1513  $\pm$  537,5  $\mu$ g/l.

The difference is therefore distinct between the new-born of an intoxicated mother, and that of a healthy mother. It appears clear to us that the Pb passes the placenta barrier.

During the lactation period, we have also looked for the passage of Pb into the mother's milk. The rates found were of 39,1  $\pm$  24,4  $\mu$ g/l for the control group, and of 2220  $\pm$  623  $\mu$ g/l for the group treated at 1 mg/kg/day. Thus Pb passes very easily into the mother's milk ; but the digestive absorption is it important in the new-born ?

We used new-borns, issued from mothers intoxicated from the first day of lactation and on. We observe that the plumbemia of the mother increases.

progressively, as well as in the milk and in the new-born.

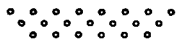
Pb ug/l

	on birth day	30 days after	60 days after
in the mother	63	785	1719
in the milk	24	647	771
in the new-born	56	130	224

We have therefore demonstrated that in the primate,  $Pb^{++}$  passes through the placenta barrier, and passes in quite important quantities into the mother's milk, and then into the new-born's blood

c) final examinations : . 50 % of the foetuses were excised by caesarian, than autopsied, and the different tissus were analysed. We have not noted any bone malformation. The only thing noted was an evident cumul of Pb in these tissus (control group : 21 mg/kg - group 5 mg/kg/day : 241 mg/kg).

. the placentas examined revealed no modification due to the treatment.



IMMUNOREACTIVITY OF Pb AND Ni

I. OBJECTIVE OF THE RESEARCH

We proposed the influence of the lead and nickel metallic ions in different animal immunological models, for our study.

II. MATERIALS AND METHODS

II.1. Effect in the non specific immunity

We used 60 day-old mice, treated during 6 weeks with a weekly subcutaneous injection of 24 mg  $Pb^{++}$ /kg in acetate form, or 4 mg  $Ni^{++}$ /kg in chloride form. Two days after the last injection, we carried out the Biozzi test,

which consists in evaluating the phagocytic index according to time.

### II.2. Delayed hypersensitivity

We analysed the sensitizing effect of Pb and Ni and their association in a delayed hypersensitivity reaction in the guinea pig, after having determined the doses of Pb and Ni which do not cause orthoergic reactions.

### II.3. Immediate hypersensitivity

Two experimentations were carried out in order to bring into evidence the effects of both metals on the trigger point of the immediate hypersensitivity reaction in the rat : the passive cutaneous anaphylaxis (PCA) and the mastocyte degranulation.

- PCA : the experimentation was carried out on rats weighing 200 g, and treated with Pb and Ni by subcutaneous route during 3 days at different doses. The second day, we effected the transfert of the sensitization by injecting a serum rich in IgE antiovalbumin, and the third day, we carried out the trigger point of the reaction by injecting ovalbumin associated to a colouring agent.

- mastocyte degranulation : several different cases have been studied : treatment of the rats during 3 days by subcutaneous route and degranulation test in vitro included by chemical or immunological way, and addition of the two metals in vitro to the studied cells and the degranulation test.

## III. RESULTS

### III.1. Effect in the non specific immunity

The results obtained do not show any modification of the phagocytic index, according to time, for the treated groups compared to the control group. The index according to time follows a exponential diagram, identical for all the groups.

### III.2. Delayed hypersensitivity

- irritation : the Pb in acetate form reveals to be slightly irritating for a concentration of 320 mg Pb<sup>++</sup>/rabbit, whereas the Ni (60 mg and 120 mg Ni<sup>++</sup>/animal) and the association Pb-Ni (160 mg Pb<sup>++</sup> + 30 mg Ni<sup>++</sup>/animal) are moderately irritating, when administered by transcutaneous route.

When the animals are treated during several weeks with Pb and/or Ni, we observe the appearing of an irritation which disappears along the treatment, when administered by transcutaneous route.

- sensitization : the Pb does not cause a delayed hypersensitivity in the guinea pig at the dose 500 mg Pb<sup>++</sup>/animal. The Ni seems to be more active at small doses (6 µg Ni<sup>++</sup>/animal) than at high doses (6 mg Ni<sup>++</sup>/animal). The association Pb-Ni has no synergia nor antagonizing effect.

### III.3. Immediate hypersensitivity

. Passive cutaneous anaphylaxis (local reaction) : the Pb influences in a significant way the trigger point of a mediation by IgE immediate hypersensitivity ; the anaphylactic activity increases according to a diagram which has as a maximum [Pb<sup>++</sup>] = 6 mg/kg, but then decreases and stabilizes at about 270 %.

The increase of this activity is relatively less important for Ni than for Pb, but the diagram also presents a maximum (at 0,25 mg Ni<sup>++</sup>/kg), and stabilizes at higher doses.

For the Pb-Ni association, the final effect approaches that of Ni.

. Mastocyte degranulation : the degranulation included in a non-immunological (compound 48/80) or immunological way (ovalbumin-antiovalbumin system) is inhibited by the contact in vitro of the examined cells (mastocytes) with the Pb and/or Ni (contact by simple washing of the cells with a solution containing both metals, or by incubation during several minutes). The in vivo treatment does not modify the degranulation rate included by the compound 48/80, nor the system Ag-Ac



## CONCLUSIONS

### I. CHRONICAL INTOXICATION OF THE MONKEY WITH LEAD

We note that after 16 months of intoxication, the animals tolerated very well the treatment. The observed lesions on the kidneys are doubtlessly of toxic origin.

### II. "MOTHER-INFANT" RELATION IN THE EXPERIMENTAL SATURNISM IN THE PRIMATE

In the pregnant female, the Pb<sup>++</sup> diffuses very well through the placenta, causing a distinct intoxication of the foetus. The passage through the milk during the lactation lengthens this intoxication.

### III. IMMUNOREACTIVITY OF Pb AND Ni

No action was brought into evidence by the phagocytic activity. The Ni causes delayed hypersensitivity, whereas the Pb does not show any activity.

For what concerns the immediate hypersensitivity models, we noted opposite responses according to the models used in vivo or in vitro.

Contractor: CÉPBEPE - LABORATOIRE D'ÉTONOLOGIE,  
Hôpital Boucicaut, 75015 Paris

Contract N°: 318-79 ENV F

Project leader: Henri LABORIT

Title of project: Changes in cadmium (Cd) toxicity in relation to possibilities of controlling environmental situations by motor activity in rats: behaviour patterns: 1. escape, 2. combat, 3. inhibition.

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#### OBJECTIVE OF THE RESEARCH

We observed in previous experiments that it is possible to produce permanent arterial hypertensions in a rat by subjecting it daily for ten minutes and for seven consecutive days to plantar electric shocks (PES) at the same time preventing it from escaping or fighting an opponent. We have given this type of hypertension the name of "Neurotic Hypertension" (Kunz, Valette and Laborit, 1974 a).

We attribute the occurrence of these stable hypertensions to the involvement of what we call the "action inhibiting system" (AIS) which comprises in particular, the medial septal area, the dorsal hippocampus, the lateral amygdala and the ventromedial hypothalamus. Synaptic transmission in this system is mainly cholinergic, whereas active avoidance ensuring the reinforcement of escape from punishment is facilitated by MFB (Olds and Milner, 1954), in which synaptic transmission is predominantly catecholaminergic. It involves the learning of gratification which follows the effectiveness of the action. We have been able to show that inhibition also results from learning of the ineffectiveness of the action (Kunz, Valette and Laborit, 1974 b).

A certain number of experimental studies have shown us (Laborit, 1975) that the action inhibiting system should be held responsible for increases of plasmatic noreadrenalin concentration, in the adrenalectomised animal and for the involvement of hypothalamic-pituitary-adrenal system, responsible of alarm reaction (Seyle, 1936). Thus, we can observe the hypercortisolemia in these animals.

It is therefore certain that the way the animal can or cannot cope with the problem, set by the environment, may profoundly change its neuro<sup>2</sup> endocrin, vascular and metabolic balance. In the animal, as in the man, the reaction to social environment, which are the main point of its behaviour, may dominate what we used to call health or illness.

Several years ago, in our experiments, we schematized these behaviours in three patterns: 1) Control animal satisfies its fundamental needs without any problem. It also satisfies its acquired needs by learning without any opposition. It is taken as control. 2) The animal is subjected to PES; we inform it by a loud or light signal, four seconds before they operate. It learns very quickly how to prevent them, the operating conditioning cage gives it an escape opening. This active avoidance (operating) looks like gratification; it is reinforced by the escape from punishment. If there is any possibility of escaping from punishment, the learning of the ineffectiveness of its action brings into play its central system for inhibiting the action the physio-pathological consequences of which we have described.

3) Finally, if the animals are placed in pairs and can fight, i.e. even ineffectively since they cannot avoid the PES, the physiological disorders (neurotic hypertension, gastric ulcers) do not appear, in spite of the punishment they have failed to avoid.

Taking into account the role which the main central neuromodulators appear to perform in these various patterns of behaviour: acetylcholine (ACh), catecholamines (CA), in particular, norepinephrine (NE) and dopamine (DA), and serotonin (5-HT), on the one hand, and the hypothalamic-pituitary-adrenal hormones (CRF ACTH Cortisone), on the other hand, we have been prompted to measure changes in the central concentration of the former and changes in enzymatic activity influenced by the latter.

Thus it is known that the adrenal glucocorticoids initiate synthesis de novo of tyrosine aminotransferase (TAT).

Under the action of glucocorticoids, the rise in the activity of hepatic TAT must result in a reduction in the synthesis of cerebral CA and an increase in that of ACh. We have in fact observed in animals subjected to hydrocortisone a major reduction in cerebral CA (Laborit and Thuret, 1977).

It was therefore interesting to study, under the effect of a combination of Cd administration with a particular environmental situation, 1. changes in certain cerebral biological values, 2. changes in arterial pressure (AP) and 3. changes in weight gain.

#### MATERIAL AND METHODS

Male rats of race Wistar-Vag, adults of weight 250-300 g, subjected to the standard laboratory diet.

##### A. On tissue sections

Effect of Cd on consumption of O<sub>2</sub> and production of lactate with the conventional Warburg technique in vitro (liver, brain) at doses of 60 ug/ml, 30 ug/ml, and 15 ug/ml in a volume of 0.2 ml.

##### B. On homogenates

1. Consumption of O<sub>2</sub> and lactic production on homogenates with or without Cd at doses identical to those above.

2. In the presence of reduced glutathione (GSH) same procedure as described previously, with the addition of 1 umole or 2 umoles of GSH for 60 ug/ml of Cd.

3. Study of the activity of glucose-6-phosphate dehydrogenase (G-6-PDH) (Boehringer) at the same Cd doses as described above.

C. Effect of Cd in vitro on hepatic and cerebral tyrosine amino-transferase\* on homogenates in vitro. The TAT is dosed in accordance with the method of Lin et al (1958) as modified.

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\*TAT

#### D. Physiological controls

- (a) Weight curves
- (b) Evolution of learning
- (c) Arterial pressure by the non-surgical method
- (d) Lactacidaemia (rabbit), IV perfusion, Cd: 500 ug/kg and 2 mg/kg.

For (a), (b), and (c), the measurements were carried out on the control animals and on the experimental animals placed in the behavioural conditions described:

1. avoidable plantar shocks (PES),
  2. combat,
  3. unavoidable PES,
- whether or not combined with chronic daily Cd doses: 3.7 or 7.5 mg/kg/day/6wks.

#### E. Cerebral biological studies under different experimental conditions

(a) Environmental situations produced in a conditioning cage with two compartments separated by a partition having an escape opening which can be opened or closed. The PES are delivered according to a set programme.

(b) Route of introduction: oral by daily gastric intubation. First experiment 3.75 mg/kg/day/4 wks (192 rats). Second experiment: 7.5 mg/kg/day/6wks, (144 rats).

(c) Dosage after separation on resin column.

- tyrosine (Tyr) (Udenfriend method modified by Rapin).
- dopa (Kehr, Carlsson and Lindquist technique).
- 5-hydroxytryptophan (5-HTP) (Tachiki and Aprison method).
- tryptophan (TP) (Bédard, Carlsson and Lindquist method).
- TAT (Butler technique).
- Noradrenalin (NE).
- dopamine (DA). (modified Shellenberger and Gordon method)
- 5-hydroxytryptamine (5-HT).

### RESULTS

#### A. On cerebral tissue sections

Reduction  $O_2$  consumption and lactate production as a function of dose.

#### B. On homogenates

1. More significant effects than on sections (70% for 60 ug/ml as distinct from 20% on section for the reduction in lactic production compared with controls).

2. But in chronic administration in vivo on the liver homogenates, an increase in  $O_2$  consumption and lactic production is observed.

3. After the addition of GSH, the inhibitions described above are partially antagonised.

4. Effect on G-6-PDH activity: inhibition as a function of Cd doses.

#### C. On hepatic and cerebral TAT (in vitro)

40% inhibition for 60 ug/ml of Cd on cytosolic hepatic TAT. On cerebral (mitochondrial) TAT, 37% activation for 60 ug/ml and 15% for 30 ug/ml.

#### D. Physiological controls

1st Experiment: 3.75 mg/kg/day/4wks: Cd.

(a) No significant influence either on the control animals or on the animals subjected to the three behavioural conditions; only the unavoidable PES significantly retarded the taking on of weight.

(b) Arterial pressure: Cd alone slightly increases AP (10 mm Hg on average): effect antagonised by avoidable PES and combat. Cd does not aggravate arterial hypertension (AH) due to unavoidable PES.

(c) At these doses, Cd does not seem to influence learning.

2nd Experiment: 7.5 mg/kg/day/6wks: Cd.

(a) At this dose, Cd alone increases AP and inhibits weight gain. This effect is maintained during avoidable PES. Cd accelerates AH at the fourth week after unavoidable PES. Cd no longer influences AP at the fifth week after PES and combat.

(b) Distinct improvement in learning (three times fewer errors at the ninth day).

#### 3. Changes in AP, glycaemia and lactacidaemia in rabbit (500 µg/kg and 2 µg/kg, IV)

Slight increase in AP at the 60th minute at 500 µg/kg.

Drop in AP at 2 mg/kg at the 5th minute.

Hyperglycaemia from the 5th to the 30th minute.

Hypolactacidaemia at 500 µg/kg.

#### E. Biological studies in the different behavioural situations

(a) O<sub>2</sub> consumption and lactate production *in vivo* on liver homogenates: Cd increases these two values. PES with combat return these values to normal. Unavoidable PES do not inhibit the effect of Cd in raising O<sub>2</sub> consumption.

(b) On the brain:

1st Experiment: Cd: 3.7 mg/kg/day/4wks.

Cd causes a drop in the concentration of Tyr, NE, DA and an increase in 5-HT and in TAT activity. Avoidable PES give rise to: a drop in NE and DA and a greater increase in 5-HT than that brought about by Cd alone. Unavoidable PES give rise to an increase in 5-HT, which Cd suppresses, and a reduction in the concentration of Tyr. PES with combat increase NE and DA; 5-HT undergoes little influence but is strongly reduced when Cd is added.

2nd Experiment: Cd: 7.5 mg/kg/day/6wks.

Cd does not affect the concentration of Tyr or that of Dopa, DA, TP, 5-HT and 5-HTP; avoidable PES reduce concentrations of Tyr and Dopa and increase those of TP and 5-HTP, without influencing those of NE, DA and 5-HT. They reduce the activity of cerebral TAT. The administration of Cd restores values generally close to normal. However DA is reduced and 5-HT is heavily increased. Unavoidable PES increase the concentration of Tyr without changing that of TP, 5-HTP and biogenetic amines. The administration of Cd reduces Tyr concentration, TAT activity and DA concentration without influencing concentrations of TP and 5-HTP. PES with combat increase Tyr, cerebral and hepatic TAT activity and concentrations of Dopa and 5-HTP. The administration of Cd restores the values to normal.



### CONCLUSIONS

The inhibition of respiration and glycolysis shown on tissue sections and homogenates by the Warburg technique prompted us to ascertain its effect with GSH in combination on homogenates. The partial antagonism observed led us to study its effect on G-6-PDH activity and to ascertain its inhibiting effect on this enzyme as a function of dose. In fact it inhibits the pentose phosphate pathway and shows a pro-oxidant capacity. It also inhibits hepatic and cerebral TAT in vitro on homogenates. It thus blocks the movement of Tyr on the homogentisic acid pathway and directs its utilisation towards synthesis of catecholamines (CA).

In the whole animal, doses of 7.5 mg/kg/day/6 wks by the oral route (gastric intubations) reduce weight gain and raise arterial pressure. On the other hand, they favour the learning of active two-way avoidance.

With chronic administration to rabbits (by IV perfusion), the pro-oxidant action of Cd suggests that it can labilise CA granule membranes which would explain the arterial hypertension and hyperglycaemia, whereas the blocking of glycolysis would explain the paradoxal reduction in lactacidaemia.

Under the conditions of chronic administration doses of 3.7 mg/kg/day/4 wks and 7.5 mg/kg/day/6wks in the different behavioural situations that we are dealing with (avoidable PES, PES with combat, unavoidable PES), the changes observed in concentrations of cerebral biogenic amines and their precursors lead us to think that, when these concentrations are raised, among the many possible interpretations, the primary cause would be a reduction in their synaptic release and vice versa. In this case, Cd would influence behaviour patterns and cerebral biochemistry in the direction of an anti-depressant effect of a new type, facilitating the activity of catecholaminergic systems and the inhibition of the cerebral serotonergic systems.

PUBLICATION IN AGRESSOLOGIE, 1980, 21, 6: 269-290.

Contractor: Institute of Occupational Health "Clinica del Lavoro L. Devoto",  
University of Milan (Italy)

Contract n° ENV/384 I

Project Leader: prof. Vito Foà

Title of project: Study of the metabolic forms of the arsenical compounds in human biological fluids and optimization of the analytical methodologies for their determination

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#### OBJECTIVE OF THE RESEARCH

Of the chemical forms to which man can be exposed, trivalent arsenicals are believed to be the most toxic. Little information is available on the mechanism of arsenic toxicity and on the changes in the chemical forms of arsenic that occur within the body: current knowledge suggests that arsenic which enters the blood stream is excreted mainly in the urine in several forms, including arsenite (AsIII), arsenate (AsV), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA) and other unidentified organically bound arsenic compounds.

The aim of this research has been to develop analytical procedures whereby the various chemical forms of arsenic present in human fluids can be distinguished and further data on the biotransformation of absorbed arsenic can be acquired. In particular, the studies reported here were directed at: (I) optimization of the analytical procedures for determining total arsenic content in blood and urine and for distinguishing the various forms of arsenic in urine; (II) evaluation of arsenic presence in human blood and urine as a result of dietary intake, industrial exposure to  $As_2O_3$  and oral ingestion of arsenite.

The results should permit a more correct evaluation of the external/internal dose-effect relationship and improve the possibility of biological monitoring of exposure to arsenical compounds in various living and working environments.

#### ANALYTICAL METHODS

The measurement of arsenic was performed by atomic absorption spectrophotometry, after the reduction of arsenic to the correspondent arsine.

In order to determine total blood and urine content of arsenic, blood and urine samples were treated with  $HNO_3$  and  $H_2O_2$  at  $100^\circ C$  and then calcined at  $600^\circ C$  with  $MgO$  and  $Mg(NO_3)_2$ .

The separation of inorganic arsenic (IA), monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) in urine was performed by ion-exchange chromato-

graphy on XAD-2 resin. Arsenic was then measured directly on the eluted fractions.

All the determinations were performed on spot urine samples collected from workers before the morning shift.

RESULTS

Optimization of analytical determination of arsenic

The measurement of total arsenic in blood (BAs) and urine (UAs) with the optimized method gave the following results: Cv: BAs = 4.0%, UAs = 3-6%; recovery: BAs = 96-101%, UAs = 95-102%; sensitivity: BAs = 1 mcg/l, UAs = 0.5 mcg/l.

The measurement of inorganic arsenic (IA), monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) in urine gave the following results: Cv: IA = 3.6%, MMA = 3.5%, DMA = 5.3%; recovery: IA = 106%, MMA = 89%, DMA = 104%; sensitivity: 0.5 mcg/l for every arsenic form.

Arsenic in blood and urine for people without occupational exposure to arsenical compounds.

Blood and urine arsenic concentration was measured in 27 healthy subjects (21 males and 6 females) with no occupational exposure to arsenic compounds. The results are given in Table 1. As regards the forms of arsenic in urine, IA accounted for 10%, MMA for 10% and DMA for 19% of the total arsenic urinary excretion, thus indicating that in the normal population over 50% of arsenic in urine (about 10 mcg/l) is present in other organic forms that cannot be measured directly by chromatographic separation but only after calcination of the samples. The arsenic concentration in blood poorly correlated with the correspondent concentration of total arsenic in urine in single subjects, Pearson's coefficient being 0.55.

Table 1. Blood and urine arsenic concentration in 27 subjects with no occupational exposure to arsenic.

	BLOOD (mcg/l)	IA	URINE (mcg/l)		TOTAL As
			MMA	DMA	
Mean ± SD	4.9 ± 6.2	1.6 ± 1.1	1.6 ± 1.3	3.1 ± 2.7	16.6 ± 10.3
Range	<1 - 25.0	<0.5 - 4.0	<0.5 - 6.2	<0.5 - 11.0	2.5 - 48.0

### Arsenic in blood and urine of workers with occupational exposure to $As_2O_3$

In the glass industry, workers are exposed to  $As_2O_3$ , which is used as an ingredient in the glass mixture. During our routine check of arsenic urinary concentration of these workers, values exceeding the limit of 100 mcg/l have been found very often even where investigations in the working environment failed to reveal excessive exposure to arsenic. Since arsenic may be also taken in through the diet, particularly through marine food ingestion, a volunteer was investigated measuring arsenic urine excretion before and after the ingestion of crabs. This experiment confirmed that, after eating 100 grams of crabs, there is a marked increase of urinary output of arsenic (from 29 to 552 mcg/l), which reaches its peak value 5-10 hours after ingestion and re-attains the pre-ingestion basal value 25-30 hours after administration. It is interesting to note that no increase was observed in IA, MMA and DMA urinary excretion, so that the difference in results between the total arsenic measurable in urine after calcination of the sample and that obtainable as IA, MMA and DMA by means of chromatographic measurement, seemed to be an indicator of the dietary source of arsenic. To confirm this hypothesis, the various forms of arsenic in urine were determined in 9 workers exposed to  $As_2O_3$  showing the total arsenic urinary concentration above 100 mcg/l (Figure 1).

The results clearly indicate that these workers can be divided into two groups: the former including those subjects whose high urinary arsenic excretion is due to an increased excretion of IA, MMA and DMA as a result of industrial exposure (subjects D, E, F, G and H), and the latter including the subjects whose high arsenic urinary excretion is not accompanied by an increased output of IA, MMA and DMA, thus revealing the alimentary - and non-industrial - source of arsenic. From these observations, we can conclude that in the biological monitoring of workers exposed to arsenite, particularly in the case of high urinary excretion values, the differentiation of the forms of arsenic is necessary to establish with certainty the source (industrial or alimentary) of arsenic.

This method was applied in a detailed survey of 36 workers exposed to  $As_2O_3$  in a glass artisan factory. The workers were divided according to job in groups of increasing exposure, namely packagers and storemen, glass blowers and foundrymen, and glass mix makers. Urine and blood samples were collected two months after resumption of work after the summer holidays. The results, having excluded two workers whose arsenic urinary output was clearly due to dietary intake, are collected in Table 2.

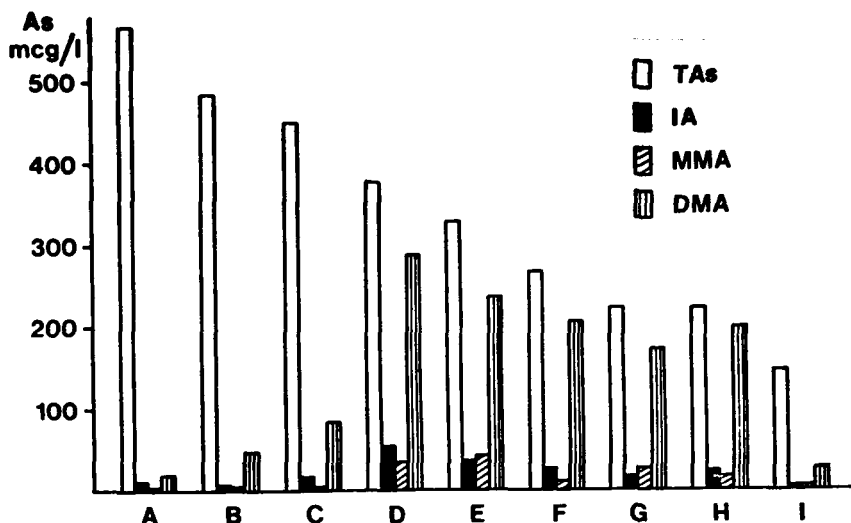


Figure 1. Urine concentration of inorganic arsenic (IA), monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) in nine workers exposed to  $As_2O_3$ , showing the total arsenic (TAs) concentration above 100 mcg/l.

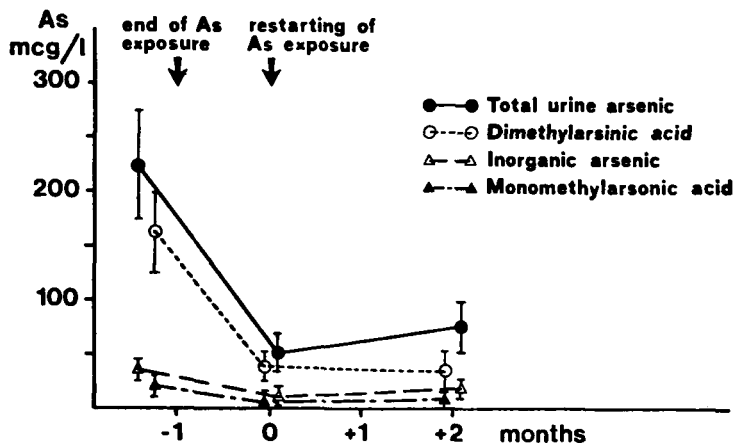


Figure 2. Change in urinary excretion of arsenic in workers exposed to  $As_2O_3$  after ceasing and resuming work. Values are means  $\pm$  S.E. of five subjects.

Table 2. Blood and urine arsenic concentration in workers exposed to  $As_2O_3$  in a glass factory according to job.

	BLOOD (mcg/l)		URINE (mcg/l)							
	$\bar{x}$	SD	IA		MMA		DMA		Total As	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
Glass mix makers (no. 4)	30.7±16.8		17.5±10.5		13.7±8.9		50.0±34.2		88.7±	34.0
Glass blowers and foundrymen (no. 23)	12.5±10.5		6.3± 4.3		4.1±3.0		15.9±13.4		33.9±	23.4
Packagers and storemen (no. 7)	13.4±15.0		4.9± 5.1		2.2±1.1		13.4± 7.9		25.7±	19.8
Total (no. 34)	15.7±13.2		7.3± 6.5		6.0±8.4		19.4±19.2		38.7±	29.9

Blood as well as urine arsenic concentration in these workers resulted significantly higher than that observed in non-exposed people.

The urinary output of arsenic was represented mainly by IA, MMA and DMA, which all together accounted for 80-90% of excreted arsenic.

This confirms that inorganic arsenic is excreted in great amounts as methylated forms. Moreover, the total arsenic as well as IA, MMA and DMA average urinary concentration was related to the intensity of exposure, showing an increasing trend parallel to the exposure level trend.

As already seen in non-exposed subjects, in these workers too the blood arsenic concentration correlated very poorly with urine values, since Pearson's coefficient is 0.01 for blood versus urine total arsenic, and 0.24 for blood versus respectively IA, MMA and DMA.

In order to investigate the rate of elimination and replacement of arsenic in the body, we selected from a glass-factory five workers having a high level of urinary arsenic excretion: they were taken away from the working environment for a month and then rechecked again two months after resuming work. The change in urinary excretion of arsenic in these workers demonstrated a higher rate of elimination than replacement of arsenic (Figure 2), but if

the change after resuming work is expressed as a percentage of value at the end of the period of absence, the values obtained are +55% for total arsenic, +124% for IA, +225% for MMA and -11% for DMA.

#### Arsenic in blood and urine after massive oral ingestion of arsenite

Blood and urine arsenic concentration was monitored in an 18 year old subject who ingested about 3 g  $As_2O_3$  in a suicide attempt. Three days after ingestion he had 450 mcg/l arsenic in the blood and 12.500 mcg/l arsenic in the urine. On the 8th day after ingestion the blood concentration was 155 mcg/l arsenic and the dominant form excreted in urine was 3.200 mcg/l IA, being MMA 960 mcg/l and DMA 1.760 mcg/l, with 6.600 mcg/l total arsenic. Correspondent concentrations on the 13th day were 10 mcg/l in the blood and 100 mcg/l IA, 50 mcg/l MMA, 250 mcg/l DMA and 310 mcg/l total arsenic in urine.

#### CONCLUSIONS

The analytical determination of the various forms of arsenic in urine can be performed by an ion-exchange chromatographic method coupled with atomic absorption spectrophotometry. In workers exposed to inorganic arsenic, this measurement is essential to distinguish the absorption of arsenic from either a dietary or an industrial source, so allowing an accurate estimation of industrial exposure to be made.

Trivalent arsenic ( $As_2O_3$ ) is excreted in urine as IA, MMA and DMA, the latter being the prevalent form. In workers exposed to  $As_2O_3$ , all these fractions increase according to the intensity of exposure, but after ceasing and resuming exposure to arsenite, the most sensitive indicators of dose are urinary IA and MMA.

In the case of oral ingestion of high doses of arsenite, inorganic arsenic is the prevalent form excreted in urine in the first few days, whereas DMA prevails only later.

Blood arsenic concentration poorly correlated with urinary excretion of arsenic and its utility for the biological monitoring of workers seems to be limited.

Contractor : Centre d'Etude de l'Energie Nucléaire, Mol, Belgium  
Contract n° : 140-76-12 ENV B Project 1  
Project leader : J.R. Maisin, H. Reyners  
Title of project : MORPHOLOGICAL STUDY OF THE INJURIES INDUCED BY SOME  
HEAVY METALS IN THE CENTRAL NERVOUS SYSTEM OF DEVELO-  
PING MAMMALS

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#### OBJECTIVE OF THE RESEARCH

The elucidation of the mechanisms by which encephalopathies are induced by heavy metals with particular emphasis on changes in the CNS vascular system and glia.

#### MATERIALS and METHODS

The various brain structures were analyzed by microscopic morphometry. In particular, the distribution of the different glial cells (astroglia, oligodendroglia and microglia) and the density of blood vessels were followed in the cerebral cortex. Animals (rats, monkeys) are given the heavy metals (lead, mercury, cadmium, thallium or zinc) chronically either via drinking water or diet.

#### RESULTS

##### Changes in glial populations in lead treated rats

As suggested in our previous reports on this subject, our investigations show a different picture for the saturnine encephalopathy after intoxication with doses in the range of environmental contamination from that described after large doses. Vascular damage, so prominent and characteristic after acute high level intoxication is not seen morphologically after very low dose levels of poisoning but a statistical evaluation of various quantitative brain parameters reveals that the relation between non-neuronal cell populations (the glial cells) is modified. These modifications still reach a significant level in female rats intoxicated from their conception with only 100 ppm in food although blood lead levels do not exceed 30 micrograms/100 ml blood even after extended periods of contamination ranging up to 27 months (data presented in the complete final report). The most important alterations found were a decrease in oligodendrocytes noted already at 3 months and persisting for at least 6 months during which treatment was continued. Data for later periods will be available soon. The astrocyte population increased slightly, but not significantly at the 100 ppm dose level. No change



was seen in the microglia at dose levels below 5000 ppm lead in the diet. The introduction of the Abercrombie correction for cell size still increased the sensitivity of the test so that modifications in the glia cell pattern may provide a valuable test to monitor delicate pathological changes in the central nervous system.

#### Glial alterations in other situations of contamination with heavy metals (cooperative projects)

Two projects of cooperation were started under the auspices of the CEE.

Brain from cynomolgus monkeys was obtained from the group of P. Tachon at Lyon and studied for morphological changes in the cerebral cortex. These monkeys had been treated with 1 or 5 mg of lead acetate per day during one year. The most important changes found in these monkeys were a reduction of oligodendrocytes which were satellites to neurons or blood vessels in the auditive cortex.

Cooperation with the group of G. Winneke (Dusseldorf) was started in 1980 and is to be extended in the future. Brain from rats poisoned with cadmium or thallium were sent to Mol for morphological assay. Due to the fact that the experiments were not initially designed for such a joint evaluation of both microscopical and behavioral modifications, their statistical evaluation still needs supplementary material to provide definitive conclusions concerning the particular metals involved, but it already appears that cadmium caused a decrease in beta astrocytes in particular of those in satellite position to neurons. In thallium treated rats, the cell volume of the alpha astrocytes and the number of microglia cells were reduced. It is a principal advantage of such multidisciplinary experiments, that the very same material is studied by different approaches.

#### Biochemical tests for glial function and brain maturation (Stephens and Gerber)

Formation of myelin and its characteristic lipids is an important task of the glia population. Myelin is first laid down during the first weeks of life, and biochemical assays thus can follow the pattern of brain maturation. Rats were given 100 or 1000 ppm of lead in the diet from conception. Animals which had received 1000 ppm of lead displayed a marked reduction in the onset and a reduction in the final level (at 32 days) of the lipids characteristic for the myelination process (cerebrosides and sulfatides). A slight decrease was also seen in ganglio-

sides at 32 days. The cellular changes in glia observed by the microscope thus find their counterpart in biochemical alterations although the sensitivity of the biochemical assay seems to be slightly lower than morphological observations. As oligodendrocytes elaborate and maintain the myelin sheets around axons, the reported disturbances in nerve conduction velocity in lead poisoned persons may be explained on similar grounds.

#### Studies on interaction of heavy metals

Most recently, we have directed our attention to the interaction of heavy metals as it appears that hazards may be greatly affected by simultaneous contamination with several metals. Two different models were used, one combining mercury and lead (10 ppm mercuric chloride and 100 ppm lead acetate in the diet), another one combining lead and zinc (1000 ppm Zinc chloride and 1000 ppm lead acetate in the diet). Addition of inorganic mercury did not modify the changes in glia population caused by lead administration. These experiments will have to be repeated with higher doses of mercury.

In the lead plus zinc studies, blood lead levels were found to be significantly increased by the simultaneous presence of zinc (see table) in contrast to recent reports which claimed a reduction of blood lead by zinc administration. On the other hand, dietary zinc, but not zinc in the drinking water, reduced significantly the changes induced by lead in the glia population, and oligodendrocytes diminished much less and eventually reached control levels in the zinc protected rats. The theory proposed by Abdullah, that zinc protects against toxic effects by lead thus receives support from our present studies.

#### CONCLUSIONS

Our data demonstrate that the glia and the myelination process which depends on the glia is profoundly affected by toxic levels of lead (1000 ppm in diet), and that changes in the glia population can already occur at lead levels (100 ppm) comparable to those encountered in environmental contamination. Glial monitoring has been extended to other models of intoxication and shows that certain metals (Cd,Tl,Zn) are also able to induce specific changes in the non-neuronal cellular populations of the cerebral cortex.

Z I N C      L E A D      I N T E R A C T I O N      S T U D I E S  
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MALE RATS.

Dose level ppm	Capill Dens.	Cortex Thk. mm	Pb-B level ug/dl	Rat Wgh. gm	Ast A	Ast B	Oli	Mic
O/Co	39.4	1.98	5.0	197		96.2	56.2	23.6
SE	1.3	.05	.9	13		3.0	6.6	2.4
1000 Zn wa	37.4	1.98	12.0	170				
	.1	.04	3.0	10				
1000 Zn fd			4.5	195				
			.9	9				
1000 Pb	37.1	2.00	145.0	160	85.0		57.2	22.6
	1.0	.03	9.0		2.6		15.2	3.0
1000 Z+P wa	42.5	1.95	188.0	117				
	1.8	.05	9.0	13				
1000 Z+P fd			173.3	161				
			23.5	9				

FEMALE RATS.

O/Co	38.3	1.99	4.0	153	85.4	25.4	80.6	28.0
SE	1.4	.06	2.8	1	3.2	3.0	5.9	2.8
1000 Zn wa	39.0	1.94	5.3	140	73.2	23.4	67.4	19.6
	.8	.03	.3	8	5.1	3.0	3.2	0.4
1000 Zn fd	38.4	2.00	5.0	131	67.0	23.4	73.0	26.8
	1.5	.03	4.8	3	2.4	3.2	5.0	2.4
1000 Pb	37.2	2.02	101.0	98	81.0	20.6	70.0	28.0
	2.2	.06	8.7	4	1.9	0.5	4.2	2.7
1000 Z+P wa	42.0	1.91	170.2	101	81.0	24.2	66.0	22.0
	1.0	.01	15.9	3	4.0	2.5	1.6	0.5
1000 Z+P fo	37.8	2.04	165.7	130	72.8	25.6	80.6	25.2
	.5	.02	30.9	3	4.9	3.7	2.7	2.6

RECENT PUBLICATIONS AND COMMUNICATIONS

Reyners H., E. Gianfelici de Reyners & J.R. Maisin, Alterations du cortex cérébral du rat causées par certains facteurs de l'environnement. Ann. Soc. Roy. Zool. Belg. 108, 215, 1978.

Reyners H., E. Gianfelici de Reyners & J.R. Maisin, An ultrastructural study of the effects of lead in the central nervous system. Int. Conference Management & Control of Heavy Metals in the Environment. London, CEP consultants Publ. Edinburg, p.58-61, 1979. (with oral presentation in London, november 79).

Reyners,H., E. Gianfelici de Reyners & J.R. Maisin, Modifications des populations de cellules astrocytaires dans le cortex cérébral du rat par divers agents expérimentaux. Biol.Cell. 38, 20a, 1980. (with oral presentation in Poitiers/France - June 80).

Reyners,H., E. Gianfelici de Reyners,P. Tachon, A. Laschi & J.R. Maisin, Lead encephalopathy in the adult monkey: an ultrastructural approach. Tox. Lett. S.I.1,76, 1980. (with oral presentation in Brussels, July 80).

Reyners,H., E. Gianfelici de Reyners & J.R. Maisin, Study of the effects of heavy metals on the CNS. (Project 1). 2d Env.Res.Prog.Eur.6388 EN, 152-156, 1980.

Stephens,M.C. & Gerber G., Development of glycolipids and gangliosides in lead treated neonatal rats. Accepted for publication by: Toxicology Letters.

To be presented shortly

Lilienthal,H., H. Reyners, M. Csicsaki and G. Winneke, Verhaltensbiologische und hirmmorphologische untersuchungen an Ratten nach kombinierter Blei-Kohlenmonoxic Exposition. Deutschen Neurobiologen-Tagung. 12-14 Juni 1981 in Gottingen. (with poster presentation in Gottingen, June 81).

Reyners,H., E. Gianfelici de Reyners, J.R. Maisin, M. Csicsaky and G. Winneke, Effects of different heavy metals (Cd,Tl,Zn, & Pb) in the central nervous system: a morphological assay. Int. Conference Heavy metals in the Environment. (with oral presentation in Amsterdam, Sept.81).

Contractor : Centre d'Etude de l'Energie Nucléaire, Mol, Belgium

Contract N° : 140-76-12 ENV B Project 2

Project leaders : A. Léonard, P. Jacquet

Title of project : STUDIES INTO THE TERATOGENIC AND GENETIC EFFECTS OF DIFFERENT HEAVY METALS IN THE MAMMALS

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The objective of the research was to study the teratogenic as well as the mutagenic effects of different heavy metals, by the simultaneous use of various embryological, cytogenetic and biochemical technics.

## Material and Methods

### I. Lead

#### Transfer of lead to the fetus :

Lead transfer during pregnancy was investigated by

- the uptake of (radioactive and non-radioactive) lead given in the diet from conception until day 7.
- the uptake and distribution of lead ( $^{210}\text{Pb}$ ) injected into the mothers during organogenesis (day 12).
- the concentrations of lead in the fetuses and placentas at day 19 of pregnancy when dietary lead was given from conception.

As calcium influences markedly the toxicologic effects of lead, including those on the developing organism, these investigations were carried out on animals on a normal and on a low calcium diet.

#### Teratogenic effects of lead :

Since vertebral anomalies were observed in mouse fetuses whose mothers had been injected with lead during organogenesis, we have now followed the effects of lead when it was given to the mothers into the diet, from the first day of pregnancy. Influence of a deficiency in dietary calcium on these effects of lead was also examined. Treated females (doses of lead : 200 - 1000 ppm in diet) were sacrificed on day 19 of pregnancy, and embryos were removed, cleared with KOH and stained with red alizarin. After dehydration, the embryos were microscopically examined for the presence of skeletal anomalies.

## II. Chromium

### Genetic effects of chromium

Since very few observations are reported on animals treated in vivo, experiments were performed to assess the clastogenic properties of hexavalent chromium in the somatic and germ cells of the mouse. For this study, two compounds were selected : potassium dichromate, a known powerful mutagen, and calcium chromate, a proved carcinogen in human beings professionally exposed to chromium.

- Chromosome aberrations in mammalian somatic cells were evaluated by the micronucleus test. Chromium nitrate, a trivalent salt, was also studied by this test. Animals were injected i.p. with the tested compounds, and dissected 30 hours thereafter or later. Bone-marrow cells were spread on grease-free slides, stained with May-Grünwald-Giemsa, and the numbers of micronucleated polychromatic erythrocytes were scored.
- Chromosome aberrations in mammalian germ cells were evaluated by the dominant lethal test. For this test, each male was caged with 3 virgin females after i.p. injection of 40 mg/kg of  $\text{CaCrO}_4$  or 20 mg/kg of  $\text{K}_2\text{Cr}_2\text{O}_7$ . The females were replaced by fresh ones 7, 14, 21 and 28 days later. On the seventeenth day after the beginning of the mating, all the females were dissected and the incidence of pre- and post-implantation losses in treated and control groups was calculated.

### Teratogenic effects of chromium

The incidence of chromium on the embryonic development was examined with the in vitro culture. Embryos at the 2-cell stage were cultured for 3 days in Brinster's medium in the presence of chromium. At the end of the culture, they were examined to determine their stage of development, and the embryos at the blastocyst stage were transferred into modified Eagle medium for a further period of 4 days. Blastocysts were then examined for the loss of the zona pellucida and their implantation on the bottom of the culture vessel. For this study, the 2 hexavalent salts  $\text{K}_2\text{Cr}_2\text{O}_7$  and  $\text{CaCrO}_4$ , and the trivalent one  $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$  were used at 3 concentrations, and their effects were compared.

### III. Nickel

#### Genetic effects of nickel

Up to now, very few studies had been performed on the in vivo cytogenetic effects of nickel in mammals. As for chromium, chromosome aberrations in somatic and germ cells were respectively evaluated by the micronucleus test and the dominant lethal test.

### IV. Varia

Studies are in course and not yet completed. They are concerned with

#### Toxic effects of chromium and nickel

Groups of 50 animals have been injected by a single sublethal dose of either  $K_2Cr_2O_7$  or  $CaCrO_4$  in the course of 1979. From this time, survival of the mice is studied. In addition, each animal dying is autopsied and histological sections are made from many organs.

#### Teratogenic effects of chromium on fetuses

Female mice are injected with 2 doses of  $K_2Cr_2O_7$  at different days during the fetal organogenesis period. The doses chosen are 10 mg or 20 mg/kg, since higher ones result in the death of the pregnant females. Animals are killed on day 19 of pregnancy, and the youngs are prepared by the red alizarin S method and examined for the presence of skeletal anomalies.

#### Genetic effects of nickel

Since the dominant lethal test gave interesting results, in that injection of the males seems to increase the preimplantation mortality in the pregnant females mated with such treated males, studies are now in course in vitro, to determine if the preimplantation loss is the consequence of a reduced rate of fertilization of the treated males, or the consequence of embryonic death during the preimplantation period or at the implantation period.

#### Genetic effects of mercury

In collaboration with Doctors Lauwerys and Roels from Louvain, a study is in course on people professionally exposed to mercury in the

metallurgy. Samples of blood were taken weekly from a total of 60 men (30 controls and 30 mercury-exposed) working at Overpelt or Tessenderloo, in the north region of Belgium. The blood samples were incubated at 37°C for 48 h in Ham's F-10 medium supplemented with bovine serum, phytohaemagglutinin and antibiotics. Arresting solution (colchicin) was added 2 1/2 hours before stopping the culture, and after hypotonic treatment and fixation, the cells were spread on clean slides, left to dry and stained with lacto-orcein. Examination of the slides is in course.

## Results and Conclusions

### I. Lead

#### Transfer of lead to the fetus :

Our results demonstrate that the amount transferred to the embryo when lead is given in the diet from the beginning of pregnancy is low during the first week. However, towards the end of pregnancy, concentration of lead in the fetus is only slightly inferior to that in parenchymal organs of the mother. Calcium deficiency increases the uptake of dietary lead by the embryo markedly. Experiments with injections at day 12 show a rapid and substantial uptake of lead in uterus and ovaries, fetuses and placenta.

#### Teratogenic effects of lead :

In contrast with the results obtained after injection of lead during embryonic organogenesis, negative results were found when lead was administered to the mothers into a normal diet as well as into a calcium deficient one, even at high doses. All our actual results lead to the conclusion that lead does not seem to represent a serious teratogenic hazard to man in the normal conditions of exposure.

### II. Chromium

#### Genetic effects of chromium :

- The results of the micronucleus showed that the trivalent salt  $\text{Cr}(\text{NO}_3)_3$  as well as the hexavalent one  $\text{CaCrO}_4$  were ineffective to produce an increase of micronuclei in the polychromatic erythrocytes, while the hexavalent salt  $\text{K}_2\text{Cr}_2\text{O}_7$  was highly effective in that respect, confirming the results obtained with this compound in vitro.



- Investigations with the dominant lethal test support the view that neither  $K_2Cr_2O_7$  nor  $CaCr_4$  are able to provoke chromosome anomalies in the postmeiotic germ cells.

Teratogenic effects of chromium :

The main conclusion of our work with cultures of preimplantation embryos is that the trivalent salt  $Cr(NO_3)_3$  should be less toxic than the hexavalent salts  $CaCrO_4$  and  $K_2Cr_2O_7$  at high doses, but more toxic for the embryo at low doses, when the duration of exposure is sufficiently long. However, a new experiment will be performed to confirm this interesting finding.

III. Nickel

Genetic effects of nickel :

While results of the micronucleus test seem to show that nickel is unable to provoke chromosomal breakage in the somatic cells, investigations with the dominant lethal test suggest an action of the metal on the postmeiotic male germ cells, mainly the spermatocytes. However, the dose required to provoke such effect is high (56 mg/kg) and toxic for the animals.

IV. Varia

As all these experiments are in course, the results of them will not be presented in this report.

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- P. Jacquet and P. Tachon, *Effects of a long term lead exposure in monkey leukocyte chromosomes.* in press in *Toxicology Letters*
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Contractor : Université Catholique de Louvain  
Contract n° : 229-76-12 ENV B  
Project Leader : Professor R. Lauwerys  
Title of project : Renal toxicity of metals

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#### Objectives of the research

The main objectives of the research project are :

1. Assessment of renal function of workers exposed to inorganic lead, cadmium or mercury vapor with the aim of defining biological threshold limit values for these metals.
2. Evaluation of possible interactions between lead and cadmium on renal function of workers simultaneously exposed to lead and to cadmium.
3. Evaluation of the critical level of cadmium in human cortex.
4. Study of the metabolism of arsenic in human.

#### Materials and methods

- The renal function of workers exposed to cadmium (n = 148), to mercury vapor (n = 63), to inorganic lead (n = 25) or to lead and cadmium simultaneously (n = 62) has been compared with that of workers with no occupational exposure to heavy metals (n = 88).
- The cadmium concentration in liver (CdL) and in kidney (CdK) was measured in vivo by neutron capture  $\gamma$ -ray analysis in 309 male workers occupied in two Belgian zinc-cadmium plants. At the same time, their renal function was assessed.
- The metabolism of arsenic was investigated in human volunteers receiving one or five daily doses of arsenic orally.

#### Results

A moderate exposure to lead (Pb-B < 62  $\mu\text{g}/100\text{ ml}$ ) does not seem to alter renal function. Excessive exposure to cadmium increases the urinary excretion of both low- and high-molecular-weight proteins and of tubular enzymes. These changes are mainly observed in workers excreting more than 10  $\mu\text{g Cd/g creatinine}$  or with Cd-B above 1  $\mu\text{g Cd}/100\text{ ml}$  whole blood.

Occupational exposure to mercury vapor induces glomerular dysfunction as evidenced by an increased urinary excretion of high-molecular-weight proteins and a slightly increased prevalence of higher  $\beta_2$ -microglobulin concentration in plasma without concomitant change in urinary  $\beta_2$ -microglobulin concentration.  $\beta$ -Galactosidase activity in blood and in urine is also increased. The likelihood of these findings is greater in workers with Hg-B and Hg-U exceeding 3  $\mu\text{g}/100$  ml whole blood and 50  $\mu\text{g}/\text{g}$  creatinine respectively.

No interaction between lead and cadmium on renal function was evidenced. The signs of renal dysfunction found in the group exposed simultaneously to lead and to cadmium can be ascribed to cadmium only.

The measurement of cadmium concentration in liver and in kidney in vivo by neutron activation suggests that there exists a range of critical CdKc-levels, i.e. approximately from 160 to 285 ppm. Beyond a CdKc of 285 ppm the probability is very high that all persons will show signs of renal dysfunction. It has been found that kidney dysfunction is likely to develop in workers with CdL between 30 and 60 ppm and that almost all the Cd-workers with CdL above 60 ppm evidence renal dysfunction. This study also demonstrates that in the absence of kidney dysfunction, CdU is correlated with the body's burden of cadmium but that CdB is not. On the basis of the interrelationships between CdL, CdKc, CdU and the indicators of renal function, it can be concluded that the probability of developing Cd-induced renal dysfunction in male Cd-workers appears to be very low when the critical CdU level of 10  $\mu\text{g}/\text{g}$  creatinine is not regularly exceeded. This CdU level corresponds to an average cadmium body burden of 160-170 mg.

A very sensitive and specific method for determining inorganic arsenic and its two organic metabolites, monomethylarsonate and dimethylarsinate in urine has been developed. The urinary elimination of the metabolites of arsenic has been followed up as a function of time in volunteers who ingested a single oral

dose of arsenic (500 µg) either as sodium arsenite (Asi), monomethylarsonate (MMA) or cacodylate (DMA). The excretion rate increased in the order Asi < DMA < MMA. After 4 days, the amount of arsenic excreted in urine represents 46, 78 and 75 % of the ingested dose in the case of Asi, MMA and DMA respectively. With regard to the in vivo biotransformations, it is concluded that DMA is excreted unchanged; MMA is slightly (13%) methylated into DMA while roughly 75 % of the arsenic excreted after ingestion of Asi is methylated arsenic (about 1/3 as MMA and about 2/3 as DMA). Determination of inorganic arsenic, MMA and DMA in urine appears the method of choice for the biological monitoring of workers exposed to inorganic arsenic since these measurements are not influenced by the presence of organoarsenicals from marine origin. In another study, arsenic (125, 250, 500 or 1000 µg as NaAsO<sub>2</sub>) was administered orally once a day for five consecutive days to volunteers who refrain from eating marine organisms during the experiment. From the linear relationship between arsenic administered and that excreted in urine, it has been estimated that a time weighted average exposure of 50 µg As/m<sup>3</sup> would lead to an average urinary excretion of 220 µg As (sum of Asi, MMA and DMA) per g creatinine.

### Conclusions

These studies have allowed

- 1) to propose biological threshold limit values for Cd in urine (10 µg/g creatinine), Cd in blood (1 µg/100 ml), Hg in urine (50 µg/g creatinine), Hg in blood (3 µg/100 ml) applicable to workers exposed to these heavy metals;
- 2) to evaluate the critical levels of Cd in human kidney cortex and to estimate the significance of Cd in urine and blood;
- 3) to demonstrate that a moderate exposure to lead (PbB < 62 µg/100 ml) does not exacerbate the renal toxicity of cadmium;
- 4) to propose a biological monitoring method for evaluating the intensity of exposure to inorganic arsenic.

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**Contractor :** The Victoria University of Manchester  
Department of Occupational Health

**Contract No.** 143-77-7 ENV UK

**Project Leader:** Professor W.R.Lee

**Title of Project:** The relationship between nerve conduction velocity and biochemical changes in lead poisoning.

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#### Objective of the research

It has been shown that nerve conduction velocity is reduced in lead poisoning but that such changes are not clearly related to biochemical indices of increased lead exposure measured at the same time. As changes in nerve conduction velocity do not therefore reflect such biochemical indices measured simultaneously in the blood and urine it is necessary to search for a relationship between development of altered nerve conduction velocity and corresponding changes in these biochemical indices. This would perhaps give a lead to the further understanding of the mechanism of development of such nerve conduction velocity changes.

Thus the objectives of the project were twofold:-

- (1) To search for a relationship between changes in nerve conduction velocities and corresponding changes in biochemical indices.
- (2) To establish and clarify the mechanisms of the development of these nervous system and biochemical changes.

#### Materials and Methods

A longitudinal study was set up using an experimental group of 30 rats (15 males and 15 females) which had lead introduced into their diet in the form of lead chloride solution (initially at 100p.p.m) as drinking water. After 6 weeks lead was introduced into the rats diet at 1000 p.p.m.

Before the introduction of lead the experimental animals were submitted to the tests listed below, in order to establish base-line levels, and after introduction of lead into the diet, they underwent these measurements on a regular basis, i.e. once every 14 days. The longitudinal study was continued for 28 weeks.

#### Biochemical Tests

Blood Lead (PbB)  
Erythrocyte Aminolaevulinic acid dehydrase (ALAD)  
Erythrocyte Zinc Protoporphyrin (ZnPP)  
Haemoglobin (Hb)  
Haematocrit (Hct)  
Urine Lead (PbU)  
Urinary Aminolaevulinic acid (ALA-U)

#### Neurophysiological Tests

Maximum Motor Conduction Velocity (M.M.C.V.)		Median Nerve (M)
Slow Fibre Conduction Velocity (% Amplitude)	of	Ulnar Nerve (U)
Distal Latency (D.L.)		Sciatic Nerve(S)



A control group consisting of 30 rats, matched for age and sex to the leaded group, on the same diet but with no lead added was similarly investigated.

All of the neurophysiological tests were carried out, using a Medelec Electro-physiological Recording system in a warm laboratory, with a Medelec NS6 Stimulator delivering rectangular pulses of variable voltage, duration and rate. Subcutaneous needle electrodes were used for both recording and stimulating purposes.

### Results

All the biochemical parameters studied displayed the expected behaviour after the beginning of exposure. ALAD activity fell significantly in the leaded groups compared to the controls. Blood lead, Urine lead, Zinc Protoporphyrin and Urinary Aminolaevulinic acid rose in the leaded groups compared to the controls. However, some interesting differences were evident. Blood lead and ALAD changed within the first two weeks of exposure. The values attained a steady state after some two months, ALAD a little sooner than PbB. The rapid reaction of ALAD upon increased lead absorption confirms that this parameter is the most sensitive one.

In contrast to PbB and ALAD, it was 8 weeks before a measurable increase became apparent in ALA-U and ZnPP. This was two weeks after the dose of lead in the exposed groups diet had been increased to 1000 p.p.m. At 8 weeks the corresponding PbB had risen to between 50-60ug/100ml blood. PbU followed PbB more closely. There was some fall in the mean Haemoglobin level and haematocrit in the lead exposed groups.

A Manova trend analysis was performed to test if there were any significant differences between the mean values of the various biochemical parameters in the leaded groups and the control groups (Table 1). Calculation of the correlations between PbB and the other biochemical parameters, both within the pooled material and within the same subject, were performed. Of all the relationships tested, ZnPP displayed the highest correlation with PbB. Its within subject correlation coefficients were all markedly positive and had a fairly narrow range in both males and females. The lowest correlations were found between PbB and haematocrit.

In addition, a multiple linear regression analysis was performed for selection of the best combination of tests to predict PbB. That analysis picked ZnPP as the most powerful variable in the model. ALAD and then ALA-U were next and improved the regression for both males and females.

By way of summary, it may be said that all the statistical procedures employed selected ZnPP from the various parameters indicating this response as being the most reliable reflector of PbB.

The graphs of the maximum motor conduction velocities with time show a decrease in MMCV in both male and female leaded groups in all the nerves measured. The first measurable decrease in MMCV became apparent after 16 weeks of exposure for the Sciatic nerve and after 18 weeks of exposure for the Median and Ulnar nerves. The values attained a steady state after 22-24 weeks of exposure. The conduction velocity changes occur therefore, after changes in all the biochemical parameters measured.

The results for the slow fibre conduction velocity are less consistent. There were no significant differences between the mean % amplitude measurement, which is an indication of slow fibre function, between the leaded and control groups for the Ulnar nerve. However, there was a small but significant decrease in the % amplitude between the male leaded group and its control group, but not in the females for the Sciatic and the Median nerves.

The slow fibre conduction velocity was introduced as a more sensitive method for detecting early or partial damage to peripheral nerves since it is assumed that the maximum motor conduction velocity would remain normal as long as a portion of the fastest fibres remain intact. The results obtained indicate that the MMCV is however, more sensitive than the % amplitude measurement of slow fibre function in detecting peripheral nerve damage.

The distal latency, the time between the distal stimulus and the beginning of the muscle action potential, was recorded for each nerve. The distal latency was increased significantly in the male and female leaded groups, compared to their respective control groups, for the Sciatic nerve. This suggests some slowing in the distal portion of the nerve and/or a delay at the neuromuscular junction. In the other two nerves studied the distal latency was increased in the female leaded group but not in the male leaded group.

Calculation of the correlations between the MMCV and the biochemical parameters, both within the same subject and within the pooled material, was performed (Table 2). In addition, a multiple linear regression analysis was performed for the selection of the best combination of the biochemical indices of exposure to predict the MMCV of the three nerves studied (Table 3).

Of all the relationships tested, the erythrocyte ZnPP displayed the highest significant correlation with all three maximum motor conduction velocities in both male and female leaded groups. The next best correlation was between ALA-U and MMCV of each of the nerves followed by PbB and MMCV.

When all the biochemical indices of increased lead exposure were analysed for the nerve conduction velocities by multiple regression analysis, the ZnPP and ALA-U level combined significantly correlated with the maximum motor conduction velocity of the Ulnar, Median and Sciatic nerves in both males and females.

#### Conclusions and additional comments

The results raise the question of the role of Zinc Protoporphyrin and Amino-laevulinic acid in the still unknown mechanism of depressed nerve conduction velocity in lead poisoning.

The strongest variable in the regression analysis, and the biochemical variable which showed the highest correlation with all of the maximum motor conduction velocities, was ZnPP. The increase in ZnPP seen in lead poisoning is due to a partial inhibition of the enzyme haem synthetase. This enzyme aids Protoporphyrin IX to accumulate in the developing red blood cells where it chelates Zinc Protoporphyrin. This lesion in haem synthesis has also been demonstrated to occur in neural cells, this could prevent the nervous system from maintaining adequate amounts of haem containing proteins. As a consequence this might lead, for example, to reduction of mitochondrial cytochromes required for oxidative phosphorylation, or of microsomal cytochromes that catalyse mixed function oxidations. A factor in favor of such a theory is that a hypochromic anemia was observed in the lead poisoned rats at the time of the development of depressed nerve conduction velocities.

A moderate but significant correlation was also exhibited by ALA-U with the maximum motor conduction velocity of each of the three nerves studied. In addition ALA-U and ZnPP combined significantly correlated with MMCV in the multiple linear regression analysis. It is possible that this porphyrin precursor, which is produced in excess in lead poisoning due to an inhibition of the enzyme ALAD in several tissues including bone marrow, kidney and liver, may gain access to the nervous system and exert direct neurotoxic effects. It is interesting that ALA has been shown to exhibit various pharmacological effects in mammalian central nervous tissue preparations. It has also been shown that ALA can inhibit the (Na/K) ATPase isolated from rabbit brain and red blood cells.

In conclusion, the neurological disturbance seen in the lead poisoned rats could conceivably be due to a combination of a direct deficit of haem in the nervous system and a toxic effect from the overproduction of ALA. In view of the changes in nerve conduction velocities associated with increases of urinary ALA, further research should be devoted to measuring the concentrations of ALA in blood. Such measures would provide further information on the alleged neurotoxic effects of ALA.

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TABLE I  
MANOVA TREND ANALYSIS FOR BIOCHEMICAL PARAMETERS

Biochemical Variable	MEANS FOR MALE GROUPS				MEANS FOR FEMALE GROUPS			
	Loaded	Control	N	Sig.	Loaded	Control	N	Sig.
Urea	48.00 +1.47	48.39 +1.07	209	< 0.05	47.71 +1.54	48.31 +1.10	209	< 0.0005
Urea/24 hours	1871.62 +501.2	1255.62 +215.5	209	< 0.0001	1935.72 +686.7	1091.90 +201.2	209	< 0.0001
Urea/24 hours	1.62 +1.34	4.14 +1.29	209	< 0.0001	2.54 +1.48	4.73 +1.45	209	< 0.0001
Urea/24 hours	12.99 +1.07	13.32 +0.76	209	< 0.05	12.86 +0.92	13.34 +0.77	209	< 0.0005
Urea/24 hours	0.32 +0.31	0.17 +0.07	209	< 0.0005	0.29 +0.22	0.15 +0.07	209	< 0.0001
Urea/24 hours	0.084 +0.04	0.086 +0.04	209	NS	0.082 +0.04	0.085 +0.04	209	NS
100ml	40.38 +23.3	6.08 +3.24	209	< 0.0001	46.20 +27.7	6.05 +3.31	209	< 0.0001
Urea/litre	796.07 +576.9	74.97 +50.1	209	< 0.0001	992.77 +913.4	73.97 +50.1	209	< 0.0001

TABLE 2

PEARSON CORRELATION COEFFICIENTS FOR THE MMCV'S WITH THE BIOCHEMICAL PARAMETERS IN THE LEAD TREATED GROUPS

FEMALE LEADED GROUP

Variable	N	SMCV		MMCV		UMMCV	
		r	Sig.	r	Sig.	r	Sig.
Hct	192	0.03	0.632	0.13	0.065	0.18	0.012
ZnPP	192	-0.40	0.001	-0.41	0.001	-0.41	0.001
ALA-D	192	0.10	0.164	0.04	0.622	0.01	0.972
Hb	192	0.18	0.030	0.12	0.099	0.13	0.062
ALA-U	192	-0.31	0.001	-0.36	0.001	-0.38	0.001
PBG-U	192	-0.12	0.095	0.01	0.953	-0.01	0.963
Pb-B	192	-0.34	0.001	-0.29	0.001	-0.36	0.001
Pb-U	192	-0.08	0.201	-0.23	0.001	-0.23	0.001

MALE LEADED GROUP

Variable	N	SMCV		MMCV		UMMCV	
		r	Sig.	r	Sig.	r	Sig.
Hct	192	-0.05	0.477	0.01	0.941	0.03	0.705
ZnPP	192	-0.51	0.001	-0.38	0.001	-0.49	0.001
ALA-D	192	0.26	0.001	0.01	0.900	0.19	0.009
Hb	192	0.22	0.003	0.16	0.029	0.20	0.008
ALA-U	192	-0.34	0.001	-0.33	0.001	-0.37	0.001
PBG-U	192	-0.13	0.075	-0.02	0.837	-0.09	0.090
Pb-3	192	-0.28	0.001	-0.22	0.008	-0.24	0.001
Pb-U	192	-0.17	0.020	-0.09	0.206	-0.28	0.001

TABLE 3

FORWARD STEPWISE MULTIPLE REGRESSION ANALYSIS: MMCV'S OF THE SCIATIC, MEDIAN AND ULNAR NERVES WITH THE BIOCHEMICAL PARAMETERS IN THE LEAD TREATED GROUPS

FEMALE LEADED GROUP

Variable	N	SMMCV		MMMCV		UMMCV	
		Sig.	R <sup>2</sup> change	Sig.	R <sup>2</sup> change	Sig.	R <sup>2</sup> change
ZnPP	192	<.001	.180	<.001	.255	<.001	.189
ALA-U	192	.005	.094	<.001	.165	<.001	.099
Pb-B	192	.059	.029	.639	.001	.271	.005
Pb-U	192	.257	.006	.050	.041	.128	.009
ALA-D	192	.735	.001	.636	.001	.268	.005
Hb	192	.205	.007	.279	.004	.351	.004
Hct	192	.622	.001	.813	.001	.567	.001

MALE LEADED GROUP

Variable	N	SMMCV		MMMCV		UMMCV	
		Sig.	R <sup>2</sup> change	Sig.	R <sup>2</sup> change	Sig.	R <sup>2</sup> change
ZnPP	192	<.001	.257	<.001	.207	<.001	.223
ALA-U	192	<.001	.112	<.002	.097	<.001	.085
Pb-U	192	.660	.001	.907	.001	.089	.018
Hct	192	.095	.031	.225	.007	.300	.004
ALA-D	192	.099	.012	.248	.010	.463	.002
Pb-B	192	.245	.005	.437	.003	.368	.003
Hb	192	.457	.002	.498	.002	.160	.008

Contractor: University of Glasgow, Department of Medicine  
Contract No. : 166-77-ENV UK  
Project Leader: Professor A. Goldberg, Dr. M.R. Moore  
Title of Project: The evaluation of certain aspects of lead exposure in terms of biochemical effects and consideration of bio-analytical indices of exposure

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### Objectives of the research

1. Mental retardation and neurophysiological studies.  
These aim at continued examination of children in high lead exposure areas coupled with studies in animals of the effect of lead and delta-aminolaevulinic acid.
2. Hypertension and renal effects.  
Following initial studies which indicated that elevated blood lead might be associated with renal insufficiency, the current studies have examined in greater depth the effects of lead upon the kidney and in the development of hypertension.
3. Indicators of exposure.  
The use of haem biosynthetic parameters as indicators of exposure to lead are to be examined, in particular the influence of extraneous compounds on the activity of ALA dehydratase will be measured.
4. Lead kinetics.  
It is intended to examine the rate of gastrointestinal absorption of lead in normal humans under differing dietary conditions, in addition to which percutaneous absorption of lead chloride will be examined.

### Results

1. Mental retardation and neurophysiological studies (i) Human Studies.  
In three studies of mothers and children in Glasgow we have examined the various factors which contribute to increased concentrations of blood lead. In the first of these studies, 236 pregnant women were studied and samples obtained of maternal blood, cord blood and breast milk together with samples of domestic first flush and running water. This study established that around 5% of blood lead values were in excess of 2.0 uM. Highly significant regressions were observed of the maternal blood lead and cord blood lead on the cube root of domestic water lead concentrations. Blood lead values were found to be higher in mother and foetus during the winter and a significant positive regression was found between maternal blood lead concentrations and maternal breast milk lead concentrations. On multiple regression analysis, the logs of the maternal blood lead concentrations and cord blood lead concentrations showed a significant negative correlation with the child's gestational age.



In the second study, of 119 mothers and their six week old children, 5 children were found to have blood lead concentration in excess of 1.75  $\mu\text{M}$ . Multiple regression analysis showed that the cube root of water lead had a significant regression on the log of the blood lead concentrations both for the mothers and for the children. These two studies established the distribution of blood lead in Glasgow which was confirmed by the general distribution in Glasgow (Lawther Report). This excess of high blood lead concentrations in Glasgow is attributed to increased uptake of lead from water supplies and especially through secondary uptake by foodstuffs during cooking (14, 15, 16, 17, 18).

In the last and currently ongoing study, we have identified a population of 885 pregnant mothers as part of the Duplicate Diet Study. From these three matched groups of pregnant women were identified, 1) high (greater than 1.5  $\mu\text{M}$ ), 2) medium (between 0.7-1.3  $\mu\text{M}$ ), and 3) low (less than 0.5  $\mu\text{M}$ ) lead exposure. The children from each of these groups are now being tested annually with the intention of maintaining a longitudinal testing for the next 7 years to cover both pre-school and early scholastic performance. Children are being tested for blood lead concentration, ALA dehydratase activity, blood protoporphyrin and when available deciduous tooth lead concentration will be measured. All will be examined for developmental milestones, head circumference, height and weight. Each child is being individually tested using standard psychometric procedures and questionnaires. At the present time, the first year of testing is nearing completion.

(i) In Vitro Studies and Animal Studies.

The association between the neurological effects of increased lead exposure and overproduction of delta-aminolaevulinic acid have been investigated further. ALA was found to inhibit spontaneous contraction and contractions induced by acetylcholine, barium chloride or ouabain in rabbit duodenum preparations. Muscle tone was decreased in proportion to the log of the ALA concentration between 25-1,000 mg/ml. These observed pharmacological effects of ALA are occurring by actions additional to sodium pump inhibition (5). In the frog sciatic gastrocnemius preparation, levels of ALA that did not interfere with nerve conduction inhibited the muscle response to nerve stimulation for 50-120 minutes. Ethological analysis has been used to study the behavioural effects following injections of ALA in mice. At a dose level of 1.6 mmol ALA/kg, male and female mice showed changed behaviour (4). These results continue to suggest that ALA may play some role in the development of neurological symptoms of lead poisoning (3).

Neurochemical correlates of lead exposure in rats are being examined by measurement of steady state levels of catecholamines, their metabolites and bio-synthesising enzymes, in rats treated acutely and chronically with lead. In the anterior and posterior hypothalamus increases in Noradrenaline levels in the lead-treated animals were associated with decreases in Tyrosine Hydroxylase activity. Phenyl N.-Methyl Ethanolamine Transferase activity was unaffected by lead treatment whilst Adrenaline levels were significantly elevated in the highest group. In other regions there were no significant changes in the levels of catecholamines and the activity of catecholamine

synthesising enzymes. These results demonstrate that lead exposure results in marked changes in central noradrenergic neuronal activity (12).

## 2. Hypertension and renal effects.

Following studies which linked hypertension and environmental lead exposure in man. We have examined the effects of chronic occupational lead exposure on renin release in man. We examined 11 male subjects with moderate to severe industrial lead exposure comparing the results with a group of normal control subjects. Plasma renin concentrations measured following intravenous administration of frusemide showed no significant difference in response. It would thus appear that industrial lead poisoning does not appear to affect renin release which may be related to the additional exposure to alcohol present in the previous studies (2). We repeated these studies in Birmingham where no relationship was found between blood lead and prevalence of hypertension. These results support our initial findings (1). Although lead has been implicated in renal neoplasia in rats, no evidence could be found of carcinogenic potential of lead in man (19).

In a study of 18 patients on haemodialysis, erythrocyte ALA dehydratase (ALAD) activity, serum aluminium, blood zinc and blood lead concentrations were significantly increased following haemodialysis. The increase in erythrocyte ALAD activity was found to be significantly linearly correlated with the increase in blood zinc concentrations. In comparison with control subjects, the patients had significantly lower activities of ALAD and significantly increased serum aluminium concentrations. When zinc and lead concentrations were corrected for packed cell volume they were significantly higher in the patient group when compared to the controls. It is not possible on the basis of the present study to attribute the depression in activity in the patient group to the changes in the concentrations of aluminium, zinc and lead. However, if the activity of erythrocyte ALAD reflects the activity of this enzyme at the major sites of haem biosynthesis, the depression in activity may account, to some extent, for the decline in haemoglobin concentration associated with haemodialysis (6).

## 3. Indicators of exposure.

In studies of the effects of lead on haem biosynthesis and biodegradation, we have shown that lead pre-treatment increased hepatic delta-aminolaevulinate synthase activity and increased microsomal haem oxygenase activity with concurrent decreases in total haem concentration. These results provide further evidence for the action of haem in the control of haem biosynthesis (8).

The effects of zinc, carbon monoxide and aluminium and diurnal rhythms have been examined on erythrocyte ALA dehydratase activity in man (6, 9, 10, 21). Regression analysis indicates that zinc, aluminium and carbon monoxide cause a small but significant change in the ALA dehydratase activity which does not negate the value of ALA dehydratase activity as a bioanalytical measure of environmental lead exposure. ALA dehydratase shows a diurnal rhythm throughout the day. The amount of this fluctuation lies between 5-10% of measured activity in normal subjects.

The use of haem biosynthetic parameters as indices of lead exposure and altered haem biosynthesis has been evaluated. Erythrocyte ALAD activity, blood protoporphyrin (PROTO) levels, blood and urinary ALA levels and urinary coproporphyrin (COPRO) levels were determined in control and lead exposed subjects. A predictive validity study showed that ALAD is a sensitive index of both environmental and industrial lead exposure whilst blood PROTO is a practical and fairly sensitive index of industrial exposure (7, 20). The relationships between erythrocyte ALAD activity and leucocyte ALA synthase activity, and of blood PROTO and leucocyte ALA synthase activity, suggest that blood PROTO more accurately reflects haem synthesis than does erythrocyte ALAD activity (11).

#### 4. Lead kinetics.

Studies of the gastrointestinal absorption of lead 203 chloride have been carried out in 11 normal subjects, 7 males and 4 females. Whole body counting of these subjects after administration of 74 KBq (2 uCi) of cyclotron produced lead 203 chloride showed a mean absorption of 21.3% of the dose with a range from 10.4-47.7% (male 18.1%; female 26.9%). Although this difference did not reach statistical significance it is postulated that these differences in absorption may relate to differences in iron saturation of the subjects (22). To study this, oral lead and iron absorption were measured in 10 fasting subjects, five of whom had low iron stores as shown by low serum ferritin concentration. The absorption of iron and of lead was abnormally high in these 5 subjects and in 1 subject with normal serum ferritin. The statistically significant correlation between lead and iron absorption would suggest that a certain segment of the population who are over absorbing iron may absorb 2-3 times more lead than iron replete subjects. These studies are being continued in a group of normal volunteers fed a standardized meal (25). The whole body retention of intravenous Pb 203 lead was found to have a half life of between 60 and 85 days in six volunteers. Twenty-four hours after administration, 8% of the administered dose had been excreted in the urine and 40 to 50% of the residual dose was in the blood. Urinary excretion in each subject was found to be much greater than the faecal lead excretion. The percutaneous absorption of lead from two hair darkening cosmetic preparations containing lead acetate has been measured by radioisotopic tracer techniques, using lead 203 acetate, in 8 normal male subjects. There was little absorption of lead through the skin, ranging between 0 and 0.3% of the dose applied. Greater absorption was found when the skin was broken. The potential hazard of the use of such cosmetic preparations is therefore considered to be negligible (23).

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Contractor : University of Aston and City of Birmingham

Contract No: 167 -77 ENV-UK

Project Leader : Prof. J. A. Blair, Dr. M. E. Hilburn

Title of Project : The intestinal absorption of lead and its  
subsequent neurotoxic effects

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#### OBJECTIVES OF THE RESEARCH

The major objective of the work carried out was to define a model which would describe the transport of inorganic lead across the mammalian small intestine. The details of the mechanism of lead transport could then be used to predict and to explain how certain dietary intakes or deficiencies, metabolic or physiological phenomena or disease states will alter the amount of lead entering the body cavity.

Another objective of the programme was to define the conditions and elucidate a possible mechanism by which lead interferes with the synthesis of neurotransmitters. This was achieved by studying the effect of inorganic lead on tetrahydrobiopterin synthesis and the effect of lead on the salvage pathway of biopterin, in the rat.

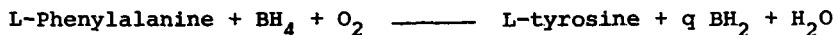
#### MATERIALS AND METHODS - A. LEAD TRANSPORT STUDIES

The transport of the lead cation across the rat small intestine was investigated using the everted sac technique and measuring the characteristic gamma emission from  $^{203}\text{Pb}$ . Details of the experimental procedures and the assessment of preparation viability have been fully described in publications related to this work. (1-8)

Lead transport across human intestinal tissue was assessed, after volunteers had ingested  $^{203}\text{Pb}$ , by monitoring the excretion of  $^{203}\text{Pb}$  in urine and faeces over a six day period and measuring retention in the body in a whole-body counter. Human biopsy samples and material obtained from surgical operations were also used to assess the characteristics of lead transport, by mounting the tissue in Ussing Chambers and exposing it to  $^{203}\text{Pb}$ .

## B. BIOPTERIN STUDIES

The level of tetrahydrobiopterin ( $BH_4$ ) is rate limiting in the synthesis of the CNS neurotransmitters catecholamines and serotonin.



The cellular concentration of  $BH_4$  is maintained by both a de-novo synthesis and a salvage pathway in which quinonoid dihydrobiopterin ( $qBH_2$ ) is reduced to  $BH_4$  by dihydropteridine reductase (DHPR) and NADH.

Rat whole brain was homogenised and biopterin determined using the Crithidia fasciculata assay. (Leeming R. J. & Blair J.A. Biochem. Med. 11 122-128 1974). DHPR activity was measured according to the method of Craine J. Hall E. Kaufman S. J. Biol. Chem. 247 6082-6091 1972.

## RESULTS

Unchelated lead cations cross the rat epithelial barrier extremely slowly and no evidence for the saturation of transport to the serosal space was observed when the luminal lead concentration was between  $10^{-7}M$  and  $10^{-2}M$ . (1-3) The rate of lead transport was similar at all sites along the small intestine and little affected by anoxia, lowering the incubation temperature, depletion of glucose or the presence of the metabolic inhibitors sodium azide, iodoacetate or dinitrophenol. The presence of other cations (Zn, Fe, Al, Be, Cd, Sr, Ce, La) when present individually in a thousand-fold excess concentration, inhibits lead transport by competing for access to extracellular pathways. In contrast, increased hydrogen ion concentration reduces the anionic charge within the tight junction channels (the area of contact between adjacent epithelial cells) and permits relatively more lead movement. (8)

The experimental data (1-8) and other published information on the regulation of ion transport across the small intestine suggests that free lead cations are passively transported between epithelial cells and that any change in the charge and size of the extracellular channels could promote lead transport.

Chelation of lead with dietary (citrate, ascorbate) or synthetic (EDTA, DTPA, NTA) ligands enhances transport to the serosal space. The increased lipid solubility and size of the created complex means that they are more likely to cross the epithelium by an intracellular route, through the epithelial cells, and not as the free cation between the cells. (11)

Concurrently with the slow passive transepithelial movement of lead ions there is a more rapid and much larger uptake of lead onto the epithelial cell surface. (1-6) The surface adsorption phenomenon is thought to be due to the hydrolysis of ATP within the glycocalyx, which is responsible for both the presence of an acid microclimate at the surface of the small intestine and the presence of phosphate ions which form lead phosphate. The amount of lead adhering to the cell surface is proportional to the mucosal lead concentration, but can be decreased under very acidic conditions or in the absence of glucose. Under conditions when the surface microclimate becomes less acidic (for example, in the presence of  $10^{-4}$  M sodium deoxycholate) the proportion of  $\text{HPO}_4^{2-}$  to  $\text{HPO}_4^-$  groups increases, and thereby creates the facility for greater lead binding to the cell surfaces. (1-3)

It is clear that the surface binding mechanism plays an essential role in excluding ingested lead from the body, as lead bound to the gut wall surface is not available for transport, but is lost as lead phosphate in the faeces. Dietary components on the other hand which act as an alternative binding site for lead, can either increase lead absorption if the chelated product is sufficiently lipid soluble or decrease lead absorption if the product is insoluble. The large uptake of lead onto the tissue (estimated to be 90% of the ingested lead in the absence of other dietary products) explains why only about 10% of an oral dose of lead is absorbed by a normal adult. Clearly conditions which reduce the lead-tissue interaction will result in an increase in the percentage of lead absorbed.

Studies using human tissue broadly confirm the conclusions drawn from experimentation with rats. However, the human intestine appears to be less permeable to lead ions than the

rat intestine. Other differences from the rat studies were that more lead was taken up by the human ileum than the jejunum, and at both regions the lead was less tenaciously bound and appeared to be associated with mucus secretions.

#### B. BIOPTERIN STUDIES

Inorganic lead compounds significantly inhibited tetrahydrobiopterin biosynthesis in rat brain extracts at a lead concentration of  $10^{-8}$  M. Concentrations of  $10^{-5}$  M lead and above, significantly inhibited the salvage of  $BH_2$  to  $BH_4$ . Dialysis of lead-inhibited DHPR resulted in no recovery of enzyme activity. (9)

Inhibition of the synthesis of  $BH_4$  and/or interference with the salvage pathway reduces the conversion of phenylalanine to tyrosine, and reduces the rate of neurotransmitter formation. Deviation from normal neuro-physiological development has been observed in patients with a deficiency of DHPR.

Mean serum biopterin derivative levels in adult males and females were 1.75  $\mu$ g/L and 1.53  $\mu$ g/L respectively. Serum levels were significantly decreased in lead poisoning. (12)

#### CONCLUSIONS

##### A. LEAD TRANSPORT STUDIES

The experimental observations suggest a model which (a) describes the mechanism of lead transport (b) demonstrates that the intestinal tissue protects an animal against exposure to lead and (c) predicts other conditions in which lead absorption may be markedly affected.

The rate of transport of the lead cation across the mammalian epithelium into the body cavity is dependent upon the initial uptake of lead by the surface of the intestinal tissue. The best model for lead transport suggests that under normal conditions uncomplexed cations, not bound to the tissue, passively diffuse across the intestinal epithelium via tight junctions.

Increased body burdens of lead will be found either when there is a decrease in tissue uptake of lead or under conditions in which the tight junctions become larger. The former situation will occur when the glycocalyx is either eroded by large



secretions of bile acids or is diminished as in Coeliac or Crohn's disease. Conditions of fasting, glucose deficiency or anaemia may result in an increased body burden of lead due to the decreased level of ATP associated with the glycocalyx.

Neonates, Vitamin D deficiency states and other conditions that promote calcium deficiency are associated with increases in tight junction dimensions. As a result lead absorption may be increased.

#### B. BIOPTERIN STUDIES

The concentration of the coenzyme tetrahydrobiopterin in the neurone is rate controlling for the synthesis of dopamine and noradrenaline. As  $BH_4$  does not cross the cell membrane, an inhibition of the synthesis of  $BH_4$  by low concentrations of lead cannot be compensated for by increased dietary intakes. Changes in  $BH_4$  metabolism due to the presence of lead could have serious neurological consequences for children and adults similar to the profound dementia found in malignant hyperphenylalaninaemia and the senile dementia of the Alzheimer type.

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Contractor: University of Aarhus, Denmark  
Contract n<sup>o</sup>: 171-77 ENV-DK  
Project leaders: J.C.Hansen and G.J.Bonde  
Title of project: Comparative Toxicology of Heavy Metals:  
Cellular Distribution and Interaction  
of Mercury and Cadmium in Relation to  
Selenium Intake

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Original objectives: To clarify the antagonistic effects of  
Se with regard to Hg and Cd in target  
organs

The original design was based upon experiments using rats with chemical determination of metals, but as equipment for isotope studies was made available in the beginning of the contract period, it was decided to replace chemical analysis by isotope technique since this would expand the analytical capacity considerably. It was furthermore necessary to use mice instead of rats to perform wholebody countings, as a wholebody scanner suited for counting of rats would be extremely expensive. Rats were only used in two initial non-isotopic technique studies.

The study of cadmium mentioned in the objectives has not been carried out as it was considered necessary to perform an extensive study of selenium kinetics before the metal interaction studies in order to create sufficient basic information. Only limited information on this topic was found in the literature.

Except for the delayed start of the project (July 1, 1977), and the changes in the objectives mentioned, progress has been made according to schedule.

### Materials and methods

The studies were carried out on female mice to which  $^{75}\text{Se}$  labelled Selenite ( $\text{SeO}_3^{--}$ ) and  $^{203}\text{Hg}$  labelled inorganic mercury ( $\text{Hg Cl}_2$ ) were administered in various molar ratios ( $< 1$ ,  $= 1$ ,  $> 1$ ), dosage levels (tracerdoses and subtoxic), and routes of administration (I/P, oral, continuous through drinking water). Hg vapours were administered in special exposition chambers in doses near the occupational TLV of  $50 \mu\text{g}/\text{m}^3$ . An initial study was carried out on rats exposed to Hg vapours and selenium in drinking water using non-isotopic technique. A supplementary study was carried out on pigs injected I/P with  $^{203}\text{Hg Cl}$  and  $^{75}\text{SeO}_3$ .

Determination of selenium and mercury was carried out by means of a Searle-Nuclear Solid Scintillation Spectrometer (2 channels) connected with a 2" x 2" Na (TI)-crystal.

To separate simultaneous countings from the two isotopes a technique involving "channel ratio" was used. All countings were corrected for background and radioactive decay by reference to  $^{75}\text{Se}$  and  $^{203}\text{Hg}$  standards counted simultaneously.

A special programme has been developed for data treatment at 'The Regional EDP-Center at the University of Aarhus'.

### Results

#### 1. Interaction between selenium and the mercuric ion at sub-toxic dosage levels.

Experiments on mice dosed with mercurychloride at a subtoxic level (0.25-2.5  $\mu\text{g}/\text{g}$ ) and selenium in various molar ratios to mercury (0.1, 1, and 10) have shown that selenium induced higher wholebody retention of mercury compared to mice receiving only mercury. The highest retention is obtained when selenium is given in equimolar ratio or in excess. Gradually the wholebody content of the elements will be found in a ratio close to unity irrespective of the ratios in the injected doses. Concentration of mercury in liver and spleen was increased by selenium. Also kidney concentrations increased. Comparing our results with literature have justified the statement that with regard to kidney retention of mercury there is a species difference as selenium increases kidney mercury in mice while in rats a decreased retention is observed. In the

experiment with pigs decreased kidney retention was also observed. Except for the kidney distribution it is concluded that both in mice and pigs the formation of a biologically inactive complex containing the elements in an equimolar ratio seems to be an essential mechanism in the detoxifying effect of selenium on mercury toxicity. The complex is primarily stored in the liver. According to the increased retention of mercury selenium was found to decrease mercury excretion in both urine and feces. Pretreatment with zinc alters the initial organ distribution of mercury presumably through induction of thionein in kidneys, but zinc appears not to be in true interaction with mercury.

## 2. Interaction at a tracer dose level.

When mercury and selenium were injected to mice in a tracer dose level (0.04  $\mu\text{g/g}$  Hg and 0.016  $\mu\text{g/g}$  Se) no influence of selenium neither on wholebody retention nor on organ distribution was observed, only in case of administration of excess selenium an increased retention due to increased kidney concentration was observed. It has been concluded that in mice there exists a functional threshold for induction of the mercury-selenium interaction determined partly by the dose and partly by the abundance of reactive selenium.

## 3. Interaction after exposure to mercury vapours.

A study on rats and a study on mice have both shown that selenium influences the organ distribution of mercury after exposure to low doses of vapour in both species but is without influence on pulmonary absorption. Also wholebody retention was unaffected unless selenium was administered in excess, which is in accordance with the tracer dose study. The organ distribution varied fundamentally in mice and rats as in mice kidney and lung retention was increased while in rats kidney retention was unaffected, while liver, spleen, and blood showed increased retention after concomitant selenium administration, and lungs showed decreased mercury content. The experiments

have shown that in both species selenium influences the oxidation process  $\text{Hg}^0 \rightarrow \text{Hg}^{++}$  which is regulated by catalase. Enzymatic differences in mice and rats may explain the different distributional patterns.

### Conclusion

Selenium reacts with inorganic mercury under formation of a biologically inert complex which is stored in the liver and is excreted at an extremely low rate. Selenium reacts in the organ in competition with natural binding sites for this reason there is a functional threshold for the interaction processes.

Beside the formation of a complex with the mercuric ion selenium also exhibits influence on the oxidation of mercury vapour in the organism, this latter process obviously being without any threshold. Fundamental differences have been demonstrated between the reaction in mice and rats and therefore further research is highly needed of the influence of selenium on the oxidation of mercury vapours, including studies of effect on the peroxide metabolising enzymes catalase and glutathione peroxidase in various mammal species.

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**TOPICS 12 + 14 : ORGANIC MICROPOLLUTANTS & NEW CHEMICALS**  
**Ecology**

**Contractor: Gesellschaft für Strahlen- und Umweltforschung mbH  
München**

**Contract No.: 187-77-1 ENV D**

**Project leader: Prof. Dr. F. Korte**

**Title of project: Behaviour and Ecological Effects of 14-C-labelled  
Organochlorine Compounds and a Model Ten-  
side in a Small Pond**

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## 1. Objective of the Research

The project, started in 1976 with five xenobiotics applied into natural aquatic ecosystems, was continued. The investigations now cover three vegetation periods. The objective was to study potential effects and the long-term fate (dispersion, accumulation and metabolism) of hexachlorobenzene (HCB), pentachloronitrobenzene (PCNB), pentachlorophenol (PCP), 4-chloroaniline (4-CA) and dodecylbenzenesulfonate (DBS) in different parts of the biotopes. To study biological effects on population dynamics, a short-term experiment was started with 2,4,6-trichlorophenol (TCP) and pentachlorophenol in selected aquatic compartments.

## 2. Materials and Methods

A detailed report of the implementation and application of the five 14-C-traced chemicals was given in March 1979. Several sub-closes aiming at 50 ppb concentrations were applied over 3 - 6 weeks. Samples were continuously collected and analyzed for total radioactive residues.

In summer 1980 additional biotopes were layed out. One of these was divided into six compartments by tubes of 50 cm diameter for the application of TCP and PCP.

### 3. Results and Discussion

In the sediment of the PCNB treated biotope (Fig. 1) there is a build-up phase of residues over several weeks followed by a very slow declining phase. A similar decrease of residues can be observed in the fauna species. Comparable residual concentrations were measured for all analyzed samples in the HCB-biotop.

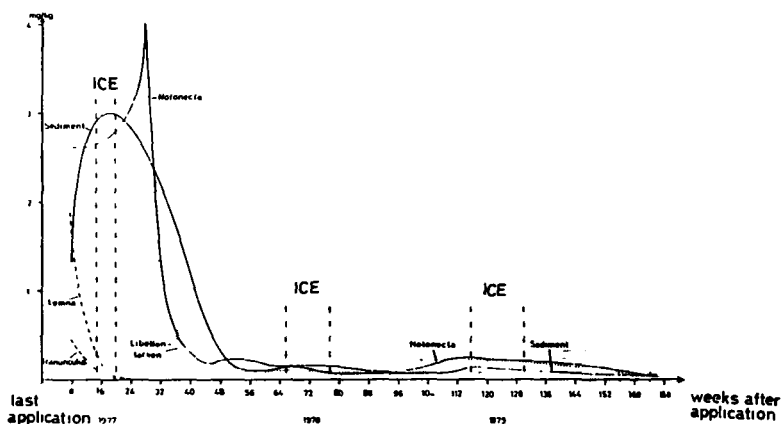


Fig. 1: Residues in sediment and selected fauna- and flora-species after PCNB-application.

The course of chemical concentration in the sediment in the PCP-treated biotop (Fig. 2) in 1979/80 is comparable with the initial course. A general rapid decrease of residues in water insects - except weak seasonal variations - can be observed in the 2nd investigation period. In contrast to the other biotopes, flora species (*Juncus spec.*) still contained PCP-derived residues, which were comparable to those of the fauna species.

The data obtained from the DBS biotope (Fig. 3) suggest that the persistence of this chemical is much higher in this natural system as expected from laboratory tests. After initial high values (2 - 8 ppm) the course of concentration in the sediment

shows a steady declining to about 500 ppb after 120 weeks, comparable to the data obtained from the HCB/PCNB biotopes. In the biotic parts, however, significantly higher DBS-derived residues can be found as compared to the other ponds. It should be mentioned, that the analytical technique used does not exclude that reassimilation of detergent derived CO<sub>2</sub> is included in these figures.

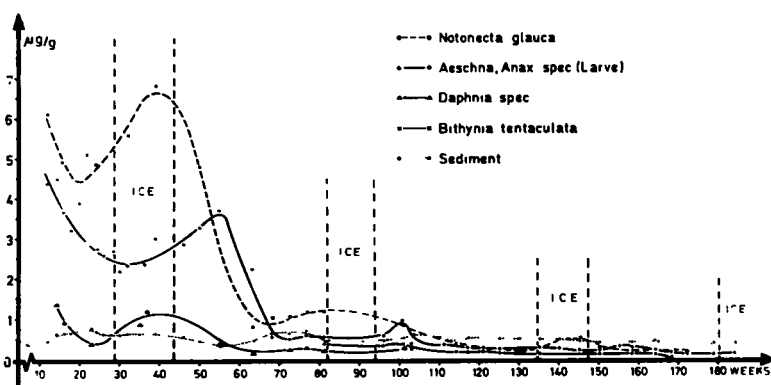


Fig. 2: Residues in sediment and selected fauna species after PCP application.

The residual behaviour of 4-chloroaniline (Fig. 4), although chemically quite different from the other model substances, follows a similar residual pattern resulting in relatively high concentrations in the biota and a slow decline in the sediment. Residue investigations of deeper soil layers (walls and ground) showed that about 95 % of the residues, located there, were present in the 0 - 10 cm soil layer.

In all experiments in which the chemical's concentration was kept at 50 ppb or lower in the water no unequivocal effects on fauna or flora populations or microbes were detected.

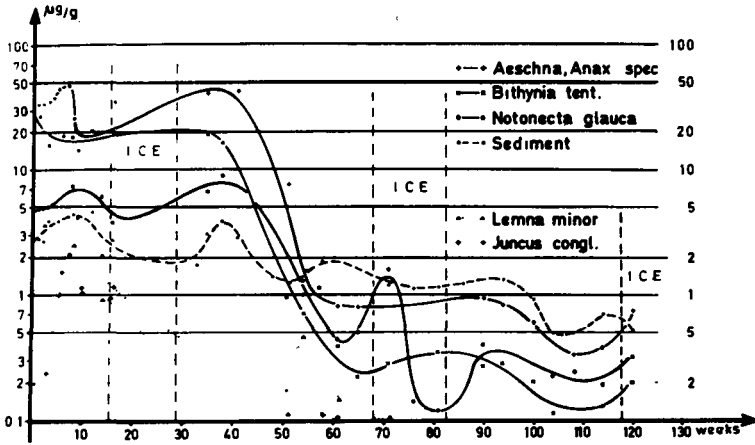


Fig. 3: Residues in sediment and selected fauna and flora species after DBS application.

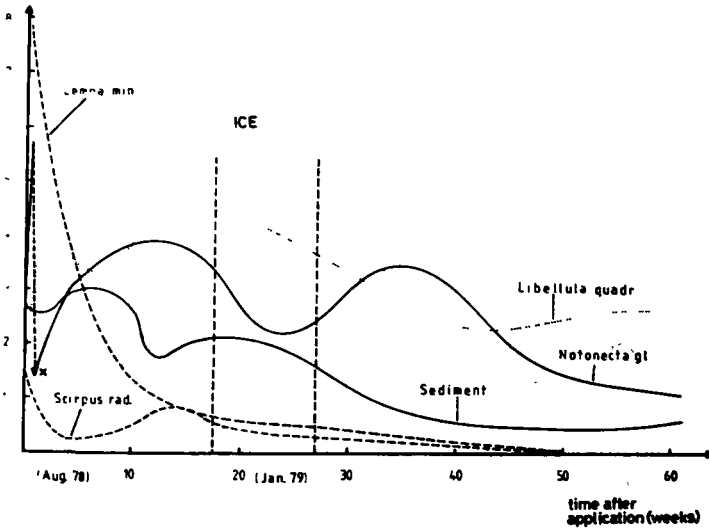


Fig. 4: Residues in sediment and selected fauna and flora species after 4-CA application.

The short-term experiments with compartmentalized ponds and using higher concentrations of TCP and PCP demonstrated a decrease in daphnia population, autotrophic phytoplankton and oxygen concentration, while flagellates and heterotrophic microorganisms increased.

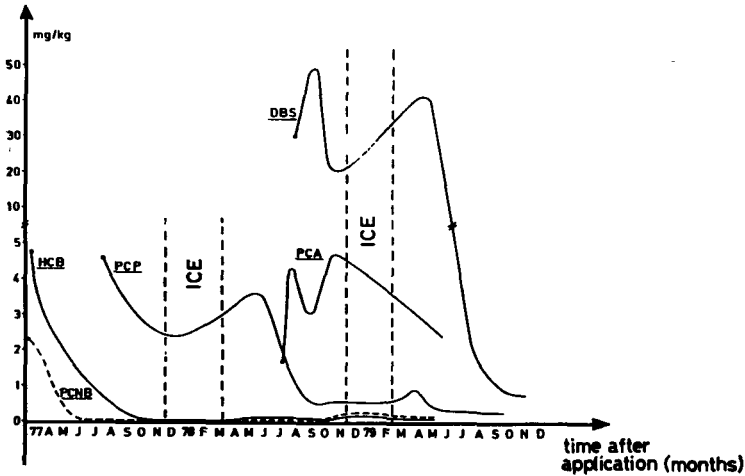


Fig. 5: Comparison of the residues of all chemicals in the selected fauna species libellula larvae.

#### 4. Conclusions

- Due to a strong initial accumulation and a slow disappearance of chemicals in the subsequent vegetation periods, residues can still be found in the sediment even after 3 seasons.
- The biodegradable substances DBS and 4-CA did not show the expected low persistence under the experimental conditions used. In spite of initial great differences in residual behaviour, caused by the physico-chemical properties of the chemicals and by the biological variety of the ecosystems, a principal similar decrease of residues was observed at the end of our investigations.

## 5. References

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- W. Klein, F. Korte, Case and Comparative Studies on Xenobiotics in Terrestrial and Aquatic Ecosystems in Agrochemical Residue-Biota Interaction in Soil and Aquatic Ecosystems, IAEA, Vienna 1980, STI/PUB/548.

Contractor : Gesellschaft für Strahlen- und Umweltforschung mbH München

Contract n<sup>o</sup> ENV - 371 - D Project 1

Project leader : Prof. Dr. F. Korte

Title of project : Photomineralization and Ozonolysis of Organochlorine  
Compounds

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#### Objective of the research

In the GSF test light-induced total oxidation of organic chemicals adsorbed on silica gel in an oxygen atmosphere is measured (photomineralization). In order to be able to extrapolate the test results to actual environmental processes, we are determining the effects of several important variables: the nature of the solid, its surface-to-volume ratio, the amount of adsorbed organic compound, the light source, etc. An important step toward finding a relationship between the test results and environmental processes, though it still does not allow calculation of environmental lifetimes, is comparison of the relative photomineralization (ranking) of compounds in the test system and under environmental conditions, which are approximated by using natural desert sands, very low surface coverage (use of C-14-labeled substances), and sunlight.

#### Materials and methods

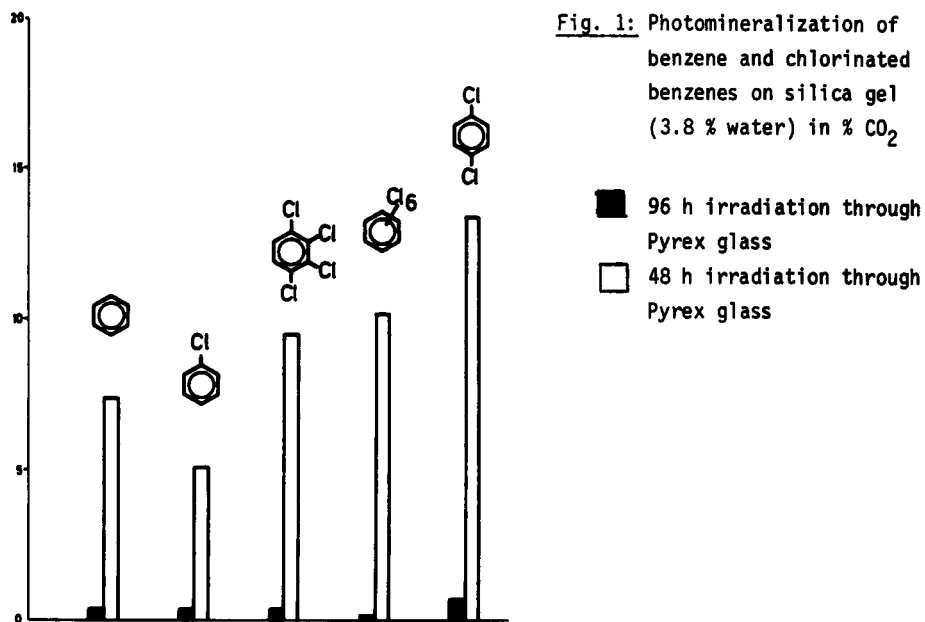
A 2-3 mm thick layer of silica gel with adsorbed organic test-substance is irradiated in the GSF microreactor with light from a HPK 125 W Philips lamp in a Pyrex cooling well<sup>1)</sup>. Use of a double-chambered reactor provides a "quasi-internal" standard. In other experiments a moving-bed reactor is used. Here the solid tumbles continuously, so that the irradiation is quite even<sup>2)</sup>; this is advantageous in experiments with natural solids (sands, soils), which are nearly opaque to UV light. The CO<sub>2</sub> formed is analyzed by various methods (wet chemical analysis, ir-gas-analysis, scintillation counting)<sup>1,2,3,4)</sup>.

#### Results

The photo-oxidative degradation of chlorinated alkanes (1,2-dichloropropane, 1,3-dichloropropane) and alkenes (1,1-dichloroethylene, trichloroethylene, tetrachloroethylene, 1,1-dichloro-1-propene, 2,3-dichloro-1-propene, hexachlorobutadiene, and hexachlorocyclopentadiene) depends strongly on the state of activation of the surface<sup>5,6,7)</sup>. After 4 days' irradiation ( $\lambda > 290$  nm)



on silica gel (3.8% surface water), 2-7% of the total carbon, and 3-14% of the total chlorine can be measured as  $\text{CO}_2$  and  $\text{Cl}^-$ , respectively. By contrast, use of silica gel dried for 24 h at  $150^\circ\text{C}$  (0.3% surface water) gives 50-100%  $\text{CO}_2$  and 30-70%  $\text{Cl}^-$ . The exception here is 1,1-dichloro-1-propene, which gives 37%  $\text{CO}_2$  and 73%  $\text{Cl}^-$  even on the undried surface. These chlorinated alkenes produce chlorine as well as chloride on the dried surfaces. The wavelength of the light plays a significant role in the photomineralization of benzene and chlorinated aromatics (Fig. 1).



While benzene, chlorobenzene, 1,2,3,4-tetrachlorobenzene, hexachlorobenzene, and 1,4-dichlorobenzene are only slightly degraded to  $\text{CO}_2$  (0.2-0.7%) and  $\text{Cl}^-$  (1.3-4.4%) on undried silica gel with Pyrex-filtered light, with quartz-filtered light the mineralization is much higher (5.1-13.4%  $\text{CO}_2$  and 22.7-71.9%  $\text{Cl}^-$ ). Similar behaviour is also observed in the cases of 1,3-dichloropropane and hexachlorocyclopentadiene. Four days' irradiation of

1,1-dichloro-1-propene ( $\lambda > 290$  nm) gives almost quantitative degradation to  $\text{CO}_2$ , and short-wave UV light (quartz filter) does not produce higher mineralization. Hexachlorobutadiene and the chlorinated ethylenes, on the other hand, are somewhat more effectively mineralized by quartz-filtered

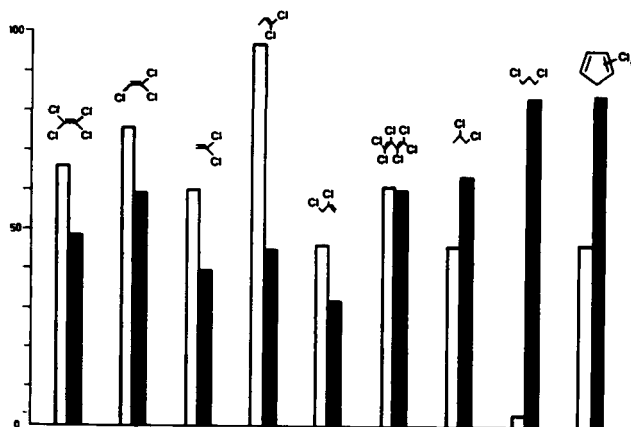


Fig. 2. Photomineralization of chlorinated alkanes and alkenes on dried silica gel (0.3% water) in %  $\text{CO}_2$

■ 96 h irradiation through Pyrex glass  
 □ 48 h " " quartz glass

light than with Pyrex-filtered (Fig. 2). These degradation rates are only valid for a relatively narrow range of concentrations (the concentration used in the experiments above was 8 mmol of substance on 400 g silica gel). Preliminary experiments show that the mineralization decreases with increasing concentration of the substance on silica gel (surface coverage). This decrease is less conspicuous in the case of hexachlorobenzene than for 4-nitrophenol in the range examined (0.02-1.25  $\mu\text{mol}$  per gram of silica gel, or  $3 \times 10^{13}$  -  $1.8 \times 10^{15}$  molecules per  $\text{m}^2$ ). We are now determining whether the ranking obtained in the mmol range is also valid for the lower concentrations.

### Conclusions

The chlorinated compounds all show decreasing photomineralization in the sequence alkenes > alkanes > aromatics. In the case of the chlorinated aromatics the mineralization is faster for substitution products which are likely intermediates in the degradation than for the parent compound (for example: hexachlorobenzene, 0.2% CO<sub>2</sub>, 1.6% Cl<sup>-</sup>; pentachlorophenol, 5.7% CO<sub>2</sub>, 19.7% Cl<sup>-</sup>). This suggests that the degradation rate is essentially determined by the primary reaction steps. The importance of using Pyrex-filtered, rather than quartz-filtered light for determining tropospheric degradation is revealed by the behaviour of the chlorinated aromatics, where not only the degradation rates, but also the ranking is different for the two light sources<sup>5,6,7</sup>.

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- 4) Gäb, S., Schmitzer, J., Turner, W. V., Korte, F.: Mineralization of CCl<sub>4</sub> and CCl<sub>2</sub>F<sub>2</sub> on Solid Surfaces. Z.Naturforsch. 35b, 946 (1980)
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- 6) Parlar, H., Gäb, S., Kotzias, D.: Eine neue Methode zur Bestimmung der Photostabilität von Umweltchemikalien adsorbiert an Oberflächen. Internationale Arbeitstagung über Prüfmethode und Bewertungsverfahren zur Bestimmung des photochemischen Abbauverhaltens von chemischen Substanzen, Umweltbundesamt und Fraunhofer-Institut, Berlin 2.-4.12.80
- 7) Gäb, S.: Abiotic Conversion and Degradation of Organic Chemicals. Seminar on Management of Pesticide Application in Agriculture, Cairo, 16.-19.2.81

- Contractor: Gesellschaft für Strahlen- und Umweltforschung  
Contract No.: ENV-371-D Project 2  
Project leader: Prof. Dr. F. Korte  
Title of project: Balance of mobility and conversion of environmental chemicals in terrestrial ecosystems.
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Objective of the Research:

Numerous publications are available on residues of pesticides and other chemicals in soil, plants, and vegetable food. However, the analytical methods used for the detection of the parent compounds are applicable to conversion products only to a limited extent, i.e., only to those conversion products whose chemical and physical properties and chromatographic behaviour are similar to those of the parent compound. In order to establish the complete balance of all residues derived from the chemical, including non-extractable (bound) residues, in all parts of terrestrial ecosystems, open-air experiments with  $^{14}\text{C}$ -labeled chemicals must be carried out, the experimental conditions being as close as possible to environmental conditions. This report describes model experiments on the uptake and conversion of chemicals by plants after application to soil. Previously, it had been shown that the results obtained with this model are largely identical to those of field experiments under quantitative respect <sup>1</sup>.

Materials and Methods

The experiments were carried out under outdoor conditions in water-resistant plywood boxes (60x60x60 cm) with a perforated base to drain the excess water, placed in a metal tray. The outside of the boxes was wrapped in aluminium foil to prevent temperature increases from direct sunlight. The bottoms of the boxes (2.5 cm) were layered with pebbles of nearly 2.5 cm diameter which in turn were covered with a layer of well-rotted turf. The boxes were filled with soil (160 kg) to 1 cm from the top and kept in a large pit with the upper surface of the soil level with the surrounding ground. Before treatment with chemicals, the soils were allowed to settle for nearly 4 weeks.  $^{14}\text{C}$ -labeled pesticides were applied to the soils as in agricultural practice. Other chemicals were dissolved in acetone, applied dropwise on

the soil surface and incorporated to a 10-cm depth. Crops were sown or planted immediately. During the vegetation period, the water drained from the box was collected and checked for radioactivity.

At harvest, roots, leaves, and edible parts were analysed separately. Soil samples (approx. 1 kg each) were taken at depths of 0-10, 10-20, 20-30 and 30-40 cm immediately after the harvest. Plant and soil samples were extracted continuously with methanol for 48 hours, except for substances which could undergo chemical reaction during such a procedure (pentachloronitrobenzene, metribuzin, monolinuron). These were extracted with cold methanol.

The radioactivity in extracts and in leaching water was determined by counting samples of 100, 200 and 500  $\mu$ l in a liquid scintillation counter (Tri-Carb Model 3380 or 3375, Packard). Unextracted radioactivity in soil and plants was determined by the combustion of 200 mg aliquots (Oxymat, Intertechnique) after extraction and drying in a vacuum desiccator at room temperature. For the determination of parent compounds and conversion products, the individual extracts were concentrated in a rotary evaporator. The extracts were subjected to TLC analysis on silica gel plates, zones of 1 cm were removed from the plates and the radioactivity of each zone was counted in a liquid scintillation counter. Isolation and identification of conversion products was carried out by appropriate chromatographic procedures.

### Results

Table 1 presents results obtained for the edible parts of the crops planted in soil containing various chemicals; further results are published elsewhere 1-11. For the edible parts, the observations made during these investigations may be assessed as follows:

1. For all compounds tested, residues of total radioactivity are present at measurable levels, even for those substances for which the residue of parent compound - (detectable by normal analytical methods) - is negligible. For non-persistent compounds, e.g. allyl alcohol, metribuzin, monolinuron, the total residues consist only of conversion products; for persistent

compounds, substances such as dieldrin, kepone, and PCB-isomers, the parent compound residue equals nearly the total residue.

Table 1: Residues of Environmental Chemicals and their Conversion Products in Vegetable Food (in ug/g fresh weight; one vegetation period after application to soil; outdoor experiments except allyl alcohol which was used in a green house according to agricultural practice)

Chemical	Application rate (kg/ha)	Food	Residue parent compound	Total residue (parent compound and conversion products)
Aldrin	2.9	Wheat grain	<0.01	0.01
Aldrin	2.9	Maize grain	<0.01	0.01
p-Chloroaniline	1.4	Barley grain	<0.01	0.01
3,4-Dichloroaniline	1.4	Barley grain	<0.01	0.02
Allyl alcohol	170	Lettuce leaves	n.d.	2.0
Pentachloronitrobenzene	36	Peeled onions	n.a.	<0.1
Dieldrin	0.8	Peeled kohlrabi	0.01	0.01
Aldrin	2.9	Peeled sugar beets	<0.01	0.06
Kelevan	1.8	Peeled potatoes	n.d.	<0.001
Kepona	1.2	Peeled potatoes	0.01	0.01
Hexachlorobutadiene	6.2	Peeled potatoes	n.a.	0.02
Trichloroethylene	5.4	Peeled potatoes	n.a.	0.03
Aldrin	2.9	Peeled potatoes	<0.01	0.06
2,4,6-Trichlorophenol	1.0	Peeled potatoes	n.a.	0.08
Metribuzin	1.4	Peeled potatoes	<0.01	0.14
Monolinuron	2.5	Peeled potatoes	n.d.	0.90
Pentachlorophenol	1.1	Carrots	<0.01	0.03
2,2'-Dichlorobiphenyl	1.1	Carrots	0.24	0.25
2,4,6,2',4'-Pentachlorobiphenyl	1.1	Carrots	0.41	0.50
2,5,4'-Trichlorobiphenyl	1.3	Carrots	0.71	0.86
Chloroalkylene-9	1.3	Carrots	0.63	0.95
Allyl alcohol	170	Carrots	n.d.	4.5

n.d. = not detected

n.a. = not analyzed

2. As expected, the residue level is higher for roots and tubers than for aerial plant parts (grain, leaves). This was also confirmed by comparing the residues of different parts of the same plant 1-11.
3. Species differences are evident, especially when comparing the residue levels in carrots and the other crops; for allyl alcohol it was found that the residues in carrot roots were 1.4 times higher than in lettuce roots, and in carrot tops 2.3 times higher than in lettuce tops 11. This fact is due to the relative high oil content of the carrot.
4. Surprisingly, no correlation was apparent between chemical structure (e.g. number of chlorine atoms) and uptake into plants for the series of polychlorinated biphenyls investigated (2,2'-dichlorobiphenyl, chloroalkylene-9 with 2 chlorine atoms, 2,5,4'-trichlorobiphenyl, 2,4,6,2',4'-pentachlorobiphenyl).

### Conclusions

It may be concluded that the determination of residues of parent chemicals in plants is not sufficient for the assessment of their content of unwanted foreign residues, at least not in the case of less persistent chemicals. In order to predict the total residue levels of a foreign compound in edible plant parts, persistence of chemical as well as plant species and plant part (root or aerial) should be taken into account.

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6. K. Sandrock, Doctoral Thesis, University of Bonn, Federal Republic of Germany (1974).
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9. P.N. Moza, I. Scheunert, and F. Korte, Arch. Environ. Contam. Toxicol. 8, 183-189 (1979).
10. P.N. Moza, I. Scheunert, W. Klein, and F. Korte, J. Agric. Food Chem. 27, 1120-1124 (1979).
11. I. Scheunert, unpublished results.



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Contract n° 188-77-1 ENV F

Project Leader : G. BORIES  
Laboratoire de Recherches sur les Additifs Alimentaires  
180, chemin de Tournefeuille - 31300 - Toulouse

Title of project : Study of the water contaminants paraffinic and naphthenic hydrocarbons in fish : metabolic pathways, enzymatic mechanisms of oxidation, attempt of explanation of the depressive effect on growth

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#### OBJECTIVE OF THE RESEARCH

The aquatic environment is a repository for numerous foreign organic chemicals that occur as environmental pollutants and among these, hydrocarbons are very important due to accidental or intentional spillage during shipping, to seepage of hydrocarbons from underwater oil, but also due to uncontrolled reject of fuel, cutting oils or mineral oils in freshwaters. Our interest in studying the fate and effects of hydrocarbons in aquatic species stems from not only our desire to understand what these substances might do to fish but also because such studies might forecast safety problems which might arise in man.

Saturated hydrocarbons were studied at first because :

- on the basis of the extended results in our laboratory, in rat and chicken, it was of major interest to develop comparative metabolic studies.

- these are the main components of petroleum : n-alkanes occur in most petroleum as the most abundant series of hydrocarbons, cyclo-alkanes are found in all crude oils in quantities ranging from 30 to 60 %, while iso-alkanes occur at a minor scale.

In a previous report concerning the work sponsored by E.E.C., it was indicated that dodecylcyclohexane and pristane chosen as model of cycloparaffins and branched paraffins, resulted in an important decrease in the growth rate of trouts, when distributed in their diet ; the first nutritional and biochemical investigations underlined the hypothesis of a metabolic inhibitory process in which lipid metabolism was to be involved (incorporation of unusual fatty acids in the functional lipids).

Thus it was of the main interest to conduct new experiments confirming the depressive effect on growth of such petroleum components. Furthermore, it was observed that every hydrocarbon tested was largely absorbed, and led to a cumulative process with a preferential storage in fatty deposits ; moreover the main part of the absorbed hydrocarbon was metabolized and thus investigations were to be directed on the metabolic behaviour, specially oxidation pathways, of the different classes of saturated hydrocarbons. Lastly, in view of recent reports describing the induction of fish mixed function oxidases (MFO) by exposure to petroleum hydrocarbons, we have investigated the effect of pristane and dodecylcyclohexane on the activities of two MFO reactions and on cytochrome P<sub>450</sub>

(believed to be the terminal oxidase in such transformations); in the trouts after the nine weeks period of hydrocarbon diet ingestion.

#### MATERIALS AND METHODS

The effect on the growth rate of Rainbow trouts was tested using diets in which hydrocarbons were incorporated at the 1 % level into the complete pelleted feed. There was a control group and two experimental groups receiving dodecylcyclohexane or pristane. Two more control groups were used, that received a quantity of control feed equivalent to that ingested the day before, by the pristane or dodecylcyclohexane groups (pair control feeding). Each group consisted in 200 trouts (initial weight 120 g.) and was in duplicate ; the experiment was stopped after nine weeks.

Hydroxylation of saturated hydrocarbons was studied in vitro using the liver microsomal fraction of trouts ; microsomes were prepared according to the method of Remmer using ultracentrifugation ; the microsomal pellet was resuspended in phosphate buffer. Hydroxylase activity was assayed in a mixture containing 10 mg microsomal protein, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP,  $MgCl_2$ , nicotamid and 0.1M phosphate buffer pH 7.4, in optimal proportions. Incubations were performed at 25°C for 30 min. with  $^{14}C$ -heptadecane,  $^3H$ -dodecylcyclohexane or  $^3H$ -pristane, then stopped with perchloric acid. Reaction products were extracted with ethyl acetate, then separated by T.L.C. or silica-gel. Identification of products was performed using GC-MS analysis.

Induction of the mixed function oxidases enzymes was assayed using liver microsomal suspensions. Aniline hydroxylase activity, aminopyrine demethylase activity and Cytochrome P450 content were measured according to the classical methods.

For the study of the metabolic fate of dodecylcyclohexane, trouts were held individually in aquarium (50 l.) supplied with continuously flowing well water, and they received a commercial diet until 48 h. prior to an experiment. Impregnation of pellets with a 50  $\mu Ci$   $^3H$ -dodecylcyclohexane dose obtained after soaking in hexane ; the pellets were distributed then, the water flow was cut off and the water was artificially aerated. After 30 min. period trouts were anaesthetized and a catheter was inserted in the urinary duct. Urine was collected during one week ; samplings of environmental water were made every day ; the whole feces and carcass were analyzed too. All the analytical methods available in the laboratory were used for identification and quantification of the metabolites in urines, feces, carcass and water : liquid scintillation counting, radio-thin layer chromatography, radio-gas-chromatography, gas-liquid chromatography, gas chromatography-mass spectrometry.

#### RESULTS

##### *1 - Effect of pristane and dodecylcyclohexane on trout growth rate*

On Fig. 1 are indicated the growth curves of the five groups of trouts. Incorporation of 1 % hydrocarbons in the diet slows down very dramatically the growth of trout. The presence of hydrocarbon in the diet

results in a reduction of food consumption ; moreover food efficiency is decreased as indicated by the growth curves of control groups receiving the same amount of food as hydrocarbon treated groups.

## 2 - Hydrocarbon hydroxylation and effect on the mixed function oxidase system

After incubation, the reaction products were extracted then separated by thin layer chromatography and analysed by radio gas-liquid chromatography. A scheme similar to the one describe for  $\omega$ -oxidation of fatty acids was established for any kind of paraffin : one methyl group is oxidized to yield a primary alcohol that is converted to the corresponding fatty acid, very likely via the intermediary aldehyde. Moreover, formation of a secondary alcohol as a result of subterminal oxidation, was also demonstrated for the three hydrocarbons.

The activity of the hydrocarbon hydroxylating system was determined by measuring the radioactivity in the reaction products. It is noteworthy that hydroxylation yield is higher for n-heptadecane than for pristane or dodecylcyclohexane ; in these cases it is conceivable that methyl groups or cyclohexane ring result in a steric hindrance.

Analysis of the mixed function oxidase system involved in the classical xenobiotic transformations did not reveal any significant difference between the control, pristane or dodecylcyclohexane groups. Aniline hydroxylase and aminopyrine N-demethylase activities were unmodified ; the Cyt. P450 content (per mg of microsomal protein) was the same in every group of trouts.

## 3 - Metabolism of dodecylcyclohexane

### Excretion routes

- *Fecal excretion* : It is via this route that the most important amount of unchanged dodecylcyclohexane is eliminated into the water. The absorption level (73,5 %) shows that this foreign compound is well transferred through the gut.

The radioactivity detected in the feces is due to unchanged dodecylcyclohexane, indicating that not attack at extended level occurs by intestinal flora.

- *Elimination across the gills* : It was generally accepted that fishes process lipid soluble organic substances by the rapid elimination of these substances into the external aqueous environment through the activity of the gills. Dodecylcyclohexane is highly soluble in fat solvents and actually excreted via this route : 8 % of the ingested hydrocarbon follow this way. Meanwhile, 30 % of radioactivity ingested passes into the water, and is due to tritiated water and at a minor scale to a metabolite, cyclohexylacetic acid, conjugated to taurine.

- *Urine* : No trace of hydrocarbon was detected in urine when 20 % of radioactivity were excreted by this route, indicating a metabolic transformation of dodecylcyclohexane.

### Urinary metabolites

Only 40 % of the urinary radioactivity could be extracted with ethyl acetate. In this extract, many metabolites have been identified, in various amounts according to samples ; they are mainly cyclohexyl acetic acid, and the corresponding hydroxylated products (OH in position 1, 3 or 4 on the cyclohexane ring). Few per cent of extracted radioactivity are regarding the glucuronide conjugates of phenylacetic acid and cyclohexyl acetic acid.

Unextracted radioactivity is due for half to tritiated water and the other part proceeds from conjugated metabolites. Two types of urinary conjugates were observed ; the first is a glucuronide conjugate which is the major way of detoxication in fish. The second is a taurine conjugate which have been described in some marine species by James. The conjugated compounds are principally cyclohexyl acetic acid and phenylacetic acid. The other metabolites are above all excreted as free acids.

### Unexcreted radioactivity

Its appears, in striking the metabolic balance of dodecylcyclohexane, that 30 % of the labeling remain in carcass a week after ingestion of this foreign compound. 70 % of this radioactivity is due to dodecylcyclohexane ; it is stored especially in adipose tissue and lipidic fraction of other tissues. Two intermediary metabolites have been isolated from carcass, namely cyclohexyldodecanoic acid and cyclohexyldodecan-1-ol. They are localized principally in liver. According to the identification of all these metabolites we can offer a scheme of biotransformation of dodecylcyclohexane by trout (Fig. 2).

### CONCLUSION AND COMMENTS

The main effect observed is the important growth slow down of trouts receiving hydrocarbons in their diet. The involvement of a metabolic inhibitory effect is supported by the results of the experiment with paired control groups, and by the compensatory growth observed in the previous study when hydrocarbons were withdrawn ; nevertheless, it remains to be explained.

Hydrocarbon hydroxylation study allowed us to give a general scheme for the first step of saturated hydrocarbon metabolism in trout ; the formation of the corresponding acid after  $\omega$ -oxidation, and its incorporation into the functional lipids may raise a problem of toxicological significance when unusual branched or naphthenic fatty acids are concerned. Furthermore, evidence was obtained of the subterminal oxidative of the carbon chain leading to  $\omega$ -1 alkanols ; these long chain alcohols are incorporated into the mammalian glycerophospholipids (plasmalogens), and this point is to be investigated with fishes.

Experiments concerning the fate of dodecylcyclohexane gave evidence that intense metabolization occurred leading to metabolites excreted via the gills and the urines. Identification of the biotransformation products proved that rainbow trout possesses many of classical enzymes involved in the elimination of lipophilic environmental contaminants. Thus, it would be of interest to develop automatizable tests for the measurement of various enzymatic activities which could be used for the assessment of contamination of the aquatic environment.

PUBLICATIONS AND COMMUNICATIONS

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- 2 - Effects and storage of different types of saturated hydrocarbons in the growing trout.  
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- 3 - Incidences, sur le poisson, d'une contamination alimentaire par un hydrocarbure naphthénique. Métabolisme du dodécylcyclohexane chez la Truite arc-en-ciel (*Salmo Gairdneri*).  
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- 4 - Metabolic pathways of a naphthenic hydrocarbon (dodecylcyclohexane) in rainbow trout.  
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- 5 - Tentative approach of the effects of branched and naphthenic hydrocarbons in trout : mechanism of oxidation and interaction with liver mixed function oxidases.  
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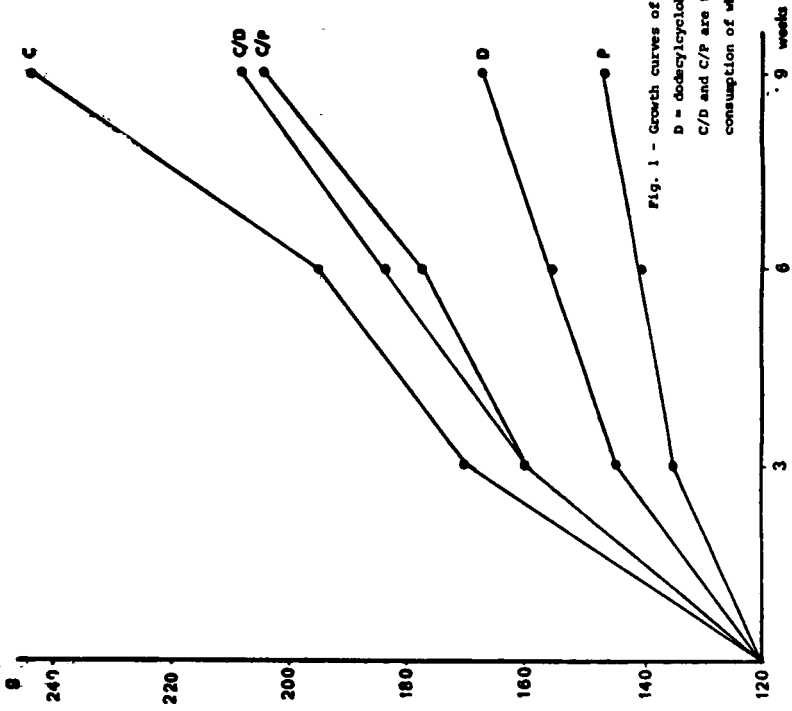
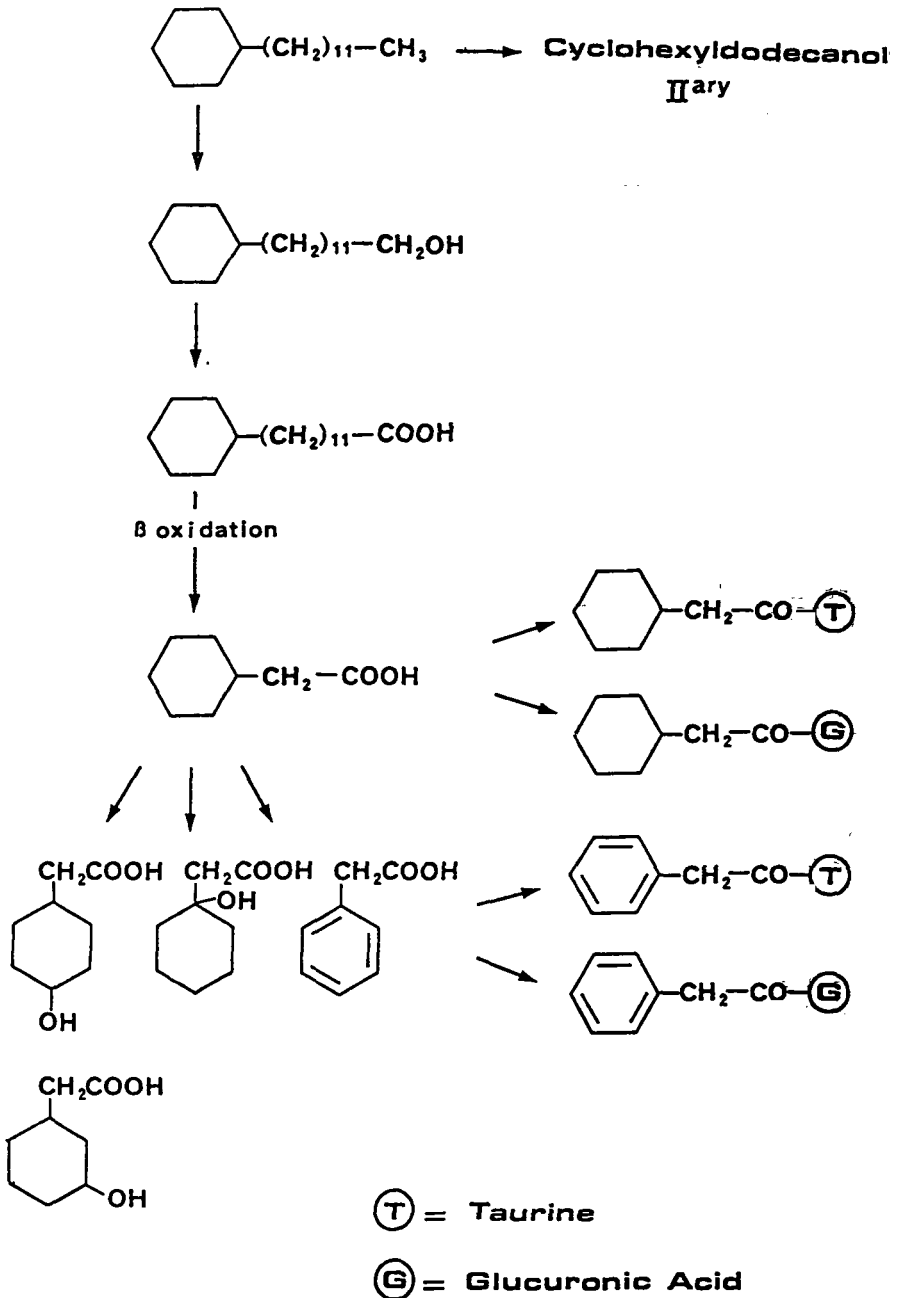


Fig. 1 - Growth curves of trout. - C = control group  
D = dodecylcyclohexane group ; P = pristane group  
C/D and C/P are the pair fed control group the food  
consumption of which are adjusted on groups D and P

Fig. 2 - Metabolic pathways of dodecylcyclohexane in rainbow trout.



Contractor : Donegani Research Institute, Novara

Contract : n° 284-77-1-ENV I

Project leader: dr. G. Borgonovi

Title of the project: Biodegradability of organic substances

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### Objectives of the research

- Development of a simple method, based on unspecific analysis, to determine aerobic biodegradability of insoluble organic compounds.
- Methods for determining anaerobic biodegradability of soluble and insoluble compounds.

### A) Aerobic biodegradation

#### Materials and methods

Products: Diethylene Glycols (DEG), Polyethylene Glycols 1000 (PEG), dodecane, pentadecane, tetradecane-1 ol (myristic alcohol), cellulose ( $\alpha$  cellulose  $>99\%$ ), glucose and inorganics have been used.

Experimental apparatus and procedure: A closed type system was used which consists of a glass air-tight stirred reactor connected with a CO<sub>2</sub> trap containing a KOH solution. Forced air circulation is maintained by a pump into the system and oxygen is provided to ensure aerobic conditions. After addition to the reactor of mineral solution and test compound, the system is freed of all CO<sub>2</sub>-C by circulating air through the trap and back to the reactor. After equilibria have been reached, inoculation is carried out in both the test and blank unit. Samples for CO<sub>2</sub>-C determinations by Total Carbon Analyzer are directly extracted from the trap with a micro-syringe. Inoculum adaptation was obtained in different ways and acclimation of sludges was checked, for insoluble compounds, by oxygen uptake rate measurements.

#### Results

The apparatus was formerly tested for biodegradation of PEG, DEG and glucose by contemporary measurements of evolved CO<sub>2</sub> and D.O.C. in order to generally establish when the biodegradation could be considered accomplished, notwithstanding the continuous CO<sub>2</sub> evolution due to endogenous metabolism. The results on tested compounds showed that to a curve representing CO<sub>2</sub> evolution for substrate consumption follows a stright



line representing endogenous respiration and that the onset of the linear production corresponds to an advanced substrate exhaustion, by D.O.C. measurements. Such results were extrapolated to insoluble compounds, where D.O.C. measurements are not significant.

The trials on liquid and insoluble compounds showed that reliable results could be obtained only if adapted sludges, under certain conditions, are used for inoculation. Biodegradation tests gave following results:

- n-alkanes ( $C_{12}$  and  $C_{15}$ ): at least 50% of C evolved as  $CO_2$  was obtained;
- myristic alcohol and cellulose: 43% and 58% respectively of the theoretical  $CO_2$  was attained.

Some difficulties during the adaptation have been met in attaining the real endogenous state, because of the presence of slowly biodegradable materials not separable by simple washing.

#### Conclusions

Main conclusions and point of possible development are:

- within the tested compounds the proposed apparatus has given satisfactory results, and it seems very simple both in assembling and in handling.
- Ring tests may be needed for a wider evaluation.
- In the case of solid compounds a careful inoculum management has to be performed during adaptation, to avoid residual substrate content at test starting.
- Great importance has to be attributed to inoculum and its adaptation; the right procedure must be experimentally chosen, and an appropriate method should be used to follow this process: in the case of insoluble substances oxygen uptake rate measurements could be suggested.

#### B) Anaerobic biodegradation

##### Materials and methods

Products: Polyethylene Glicol 4000, cellulose, glucose meat extract and inorganics were used.

Experimental apparatus and procedure: The approach to anaerobic biodegradability evaluation of soluble and insoluble substances is based on three different types of testing:

Continuous test simulating an anaerobic digester in bench scale: the apparatus substantially consists of a heated and stirred glass digester equipped with sampling, feeding and gas measuring devices. The reactor is filled with anaerobic sludges from a full-scale municipal digester suspended in test medium, under pure nitrogen fluxing. Tested compounds and municipal sludges, in the

mineral medium; in different concentrations are added after having discharged an equal volume of mixed liquor. Decreasing doses of municipal sludges and increasing doses of tested compound, have been initially fed, till 100% of the latter. D.O.C., suspended solids, pH, redox potential and gas evolution are recorded daily.

Discontinuous test: The apparatus consists of a spherical flask with a rubber stoppered outlet, connected to a gasometric burette for gas measurement and pressure adjustment in the apparatus. The substance and the medium are introduced, after careful homogeneization, under pure nitrogen fluxing. Then, the digester content is heated to the test temperature and the anaerobic sludges are injected. Gas evolution and, on liquid samples, D.O.C. and pH are recorded.

Closed reactor test: The apparatus consists of a reactor flask, stirred at the desired temperature, provided with a rubber stoppered outlet. Under nitrogen fluxing, an appropriate volume of medium and inoculum are injected into the flask. Gas samples are withdrawn and directly analyzed for total carbon.

#### Results

Using the anaerobic bench scale digester data on PEG 4000 showed that 59% of carbon has been transformed to gas, 32.0% to biomass, 8,7% to soluble carbon; corresponding values for cellulose are 39%, 30.2% and 21.3%. In this latter case the carbon balance is affected by an error due to the presence of some undegraded cellulose in the sample. Tests has been carried out respectively at 35°C with a mean hydraulic retention time of 15 days and at 25°C of 30 days; redox potential was in the range from -150 to -250 mV in both cases. Experimental time has been 50 days for PEG and about 100 days for cellulose, in order to obtain a steady state and reliable data.

Discontinuous tests have been performed on cellulose and PEG 4000 at 35°C using 1 g of both substance and sludge, and cellulose at 25°C using 40 mg of substance, as carbon and 80 mg, as dry matter, of inocula. Only tests performed with acclimated inoculum yielded positive results, but those carried out, on cellulose, with a lower level of inoculum and substrate showed unreliable results.

Closed reactor tests, performed using 10 mg of substance (as carbon) and 20 mg of unadapted inoculum, did not yield any positive results by feeding cellulose, PEG and glucose. A pH lowering was observed at the end of 40 days test and about all the starting D.O.C. was again found.

### Conclusions

The results may be considered as a preliminary contribution to the development of anaerobic biodegradation methods. Although many parameters are still to be investigated, the following features can be suggested:

- bench digester test is simple in handling and give positive and accurate results, but more sperimentation is needed to check the operational conditions.
- Discontinuous test is very simple in handling, but adapted sludges are required to obtain reliable results. Inoculum and substance concentration and their relative ratio, seem to be limiting factors; this could explain failure of tests performed with low levels of inoculum and substance and they show that to assure proper anaerobic condition not only an oxygen free atmosphere, but also high concentration of both substances and sludges could be secured.
- Closed reactor test: unsatisfactory results have been obtained, it may be for pH decrease and low substrate and inoculum concentrations.

A simple and more economical method for anaerobic sludge adaptation than the use of anaerobic bench digester is needed, like for example, culturing inoculum in test substrate enriched media.

Results of the first part of this research have been presented at "Freshwater Contact Group" on October 16<sup>th</sup> and 17<sup>th</sup> in Dublin, and a publication about this research is under way.



**TOPICS 12 + 14 : ORGANIC MICROPOLLUTANTS & NEW CHEMICALS**  
**Health effects**

Contractor: Université Pierre et Marie Curie  
(Faculté de Médecine Pitié-Salpêtrière) Paris, FRANCE

Contract n° ENV/374 F

Project leader: Pr Ag. Yvan Touitou

Title of project: Effects of op'-DDD on adrenal steroid synthesis in man

**OBJECTIVE OF THE RESEARCH:** DDD (rothane) is an insecticide which presents a high toxicity towards adrenocortical glands. This toxicity has been attributed to one of its major isomers, op'-DDD. The metabolism and mechanism of action of op'-DDD are poorly known although this compound has been used in the treatment of Cushing's syndrome and adrenal cortical carcinoma to reduce cortisol hypersecretion.

The aims of the present study were: 1) to put in evidence and document tissular levels of op'-DDD and one of its unsaturated metabolite (op'-DDE) in various biological materials obtained from patients treated for an adrenocortical carcinoma. 2) to study the *in vitro* biosynthesis of steroids in cortical adrenal carcinoma obtained from op'-DDD-treated or untreated patients.

**MATERIALS AND METHODS:** The patients' main features are presented in Table I. op'-DDD and op'-DDE determinations. Two 400 mg samples of each analyzed tissue were homogenized in acetone with a Potter homogenizer in presence of 50 µg of pure pp'-DDD as internal standard and to allow correction for losses. Assays were performed by Dr. Moolenaar (Leyden University, The Netherlands) by gas-liquid chromatography (electron capture detection) according to a previously published method (Clin.Chim.Acta, 1977, 76, 213). In vitro incubations. Adrenocortical carcinoma tissues were homogenized in Earle's medium and were incubated in presence of tritium labelled steroid precursors and of an NADPH-generating system. Enzymatic reactions were stopped by precipitating the proteins with acetone. Trace amounts of carbon-14 labelled authentic steroids were added to incubation media prior to extraction in order to allow correction for losses. Results are expressed as a percentage of the incubated precursor. Details on incubation procedure, steroids separation and characterization have been already published (1.2.).

**RESULTS:** op'-DDD has been identified in any analyzed tissue, adrenocortical carcinomas as well as metastasis or *in situ* recurrence of cancer (Table II). Concentrations were often quite different from one sample to another (mean  $\pm$  SEM =  $61.5 \pm 28.9$  µg/400 mg tissue). op'-DDE was identified in 7 out of 10 analyzed tissues (Table II). Large variations of concentration could also be seen (mean  $\pm$  SEM  $9.2 \pm 4.6$  µg/400 mg tissue). Due to the well known affinity of these molecules for lipids the total lipid content was determined in each sample and was also found very variable (Table II). Therefore the op'-DDD and the op'-DDE to lipid ratio were computed and were found much more reproducible (respectively  $2.0 \pm 0.2$  and  $0.38 \pm 0.05$ , mean  $\pm$  SEM).

Table III displays the results obtained with adrenocortical carcinomas removed from patients treated or not with op'-DDD and incubated in the presence of progesterone or 17-hydroxy progesterone as precursors. The large variability of synthetic activity encountered was probably in relation with differences in the *in vivo* secretory ability of the studied tissues and possibly with their histological heterogeneity. However, as a mean, op'-DDD seemed to be more active on 17 $\alpha$ -hydroxylase than on 21-hydroxylase whereas 11  $\beta$ -hydroxylation was most often diminished.

This study leads to the following conclusions:

- op'-DDD was identified in all analyzed biological materials
- op'-DDE, an unsaturated metabolite was found in 7 out of 10 studied tissues. Its concentration was about 10% that of op'-DDD.
- op'-DDD and op'-DDE tissular levels increased parallely with that of total lipids content.
- There was a very large variation of the lipid content from one tissue to another and within a tissue from one sample to another.
- Therefore, determination of op'-DDD (or op'-DDE) concentration in a tissue has no signification if it is not related to the lipid content of the sample as only the op'-DDD/lipid ratio seems able to provide a reproducible index of the tissular impregnation.
- op'-DDD apparently inhibited 17-hydroxylase activity in adrenocortical tissue but did no affect that of 21-hydroxylase. This comes in addition to our former reports on 11  $\beta$ -hydroxylase and 18 hydroxylase inhibition by op'-DDD.
- op'-DDD is an insecticide isomer which concentration along the food chain may interfere with adrenocortical steroid synthesis (among others) in man or animal. Its accumulation is certain in any tissue with a high lipid content. The storage of this agent in man or animal will increase as a function of the adipose mass.
- The target organs, among which the adrenals, would be thus affected not only by the direct exposure to the toxic compound but also to the release of op'-DDD from his storage organ, i.e. the adipose tissue.

These elements should be kept in mind in the appreciation of the toxicological effects of DDD, DDT and their analogs.

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Table I : Main features of patients

	Age	Sex	tumour localization	tumour weight	Major <u>in vivo</u> steroid secretion
treated patients					
Ma.	26	F	epiloon metastase	3390	cortisol
Lec.	73	F	R. adrenal	336	androgens + cortisol
Ch.	49	F	R. adrenal	106	androgens + cortisol
Vr.	62	F	L. adrenal	1044	androgens
untreated patients					
An.	42	F	R. adrenal	-	deoxycorticosterone
Car.	16	F	R. adrenal	-	androgens
Tro.	33	F	L. adrenal	-	androgens + cortisol
Vos.	44	M	L. adrenal	-	non secreting tumour



Table II op'DDD, op'DDE and lipids levels in tumours (two 400 mg samples by tissue)

Patients	analyzed tissue	op'DDD µg	op'DDE µg	lipids mg	op'DDD lipids µg/mg	op'DDE lipids µg/mg	op'DDD op'DDE µg/mg	op'DDD total dosage g
Ma	epiloon metastase	13-15	2.4-2.5	6.6-5.1	2.0-2.9	0.36-0.49	5.4-6.0	3390
Lec	adrenal	12-19	1.1-1.5	9.9-11.6	1.2-1.6	0.11-0.13	10.9-12.7	336
Ch	adrenal	20-11	-	42.2-25.1	0.47-0.44	-	-	106
Vr	adrenal	192-145	9.4-23.5	37.6-134.0	5.1-4.1	0.25-0.18	20.4-23.2	1044
Vr	local recurrence	6-7	1.1-1.5	3.9-3.9	1.5-1.8	0.28-0.38	5.5-4.7	1832
Vr	liver metastase	21-32	5.3-6.0	12.3-10.1	1.7-3.2	0.43-0.59	4.0-5.3	
Vr	adrenal loggia metastase	16-234	4.2-65.4	6.4-99.0	2.5-2.4	0.66-0.66	3.8-3.7	
Mo	lymphatic ganglion	12-17	-	9.4-7.4	1.3-2.3	-	-	864
Di	local recurrence	9-24	-	7.2-17.0	1.2-1.4	-	-	450

Table III Steroid synthesis by tumour tissues in vitro

DT	Precursor steroid	P	17 OH P	S	F
ND	Synthesized steroid	DOC			
Ma.	Ma.	66	0.4	15	4
Lec.	Lec.	20	0.8	67	2
Ch.	Ch.	2	20	13	31
Vr.	Vr.	12	14	6	0.4
Am.	Am.	35	41	61	5
Car.	Car.	1	21	16	3
Tro.	untreated patients	0.5	0.6	0.4	65
Vos	Vos	-		1.5	3

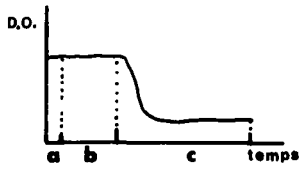


fig. 1

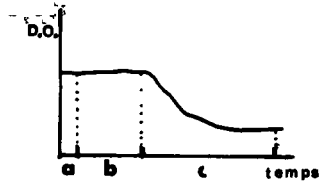


fig. 2

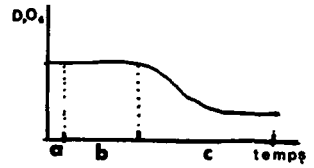


fig. 3

Fig. 1,2, 3. Development of optical density during flocculation

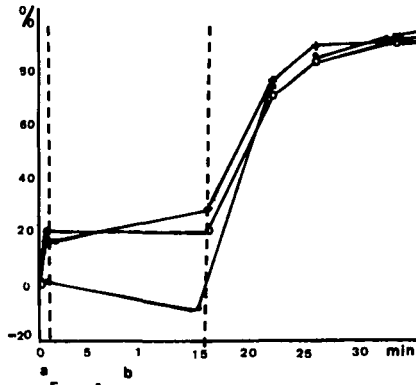


Fig. 4

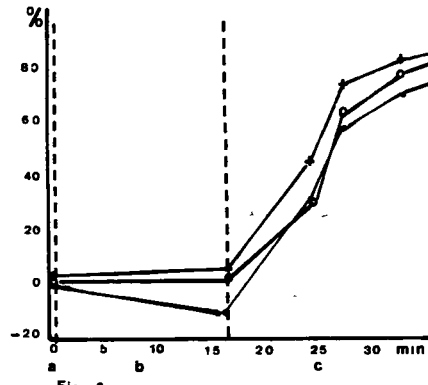


Fig. 5

Fig. 4,5, 6. Percentage for the elimination of the parameters controlled, depending on period

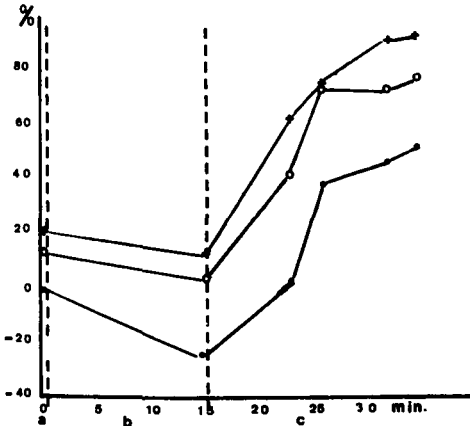


Fig. 6

- + E. coli
- o S. faecium
- o D.O.
- a. rapid mixing time
- b. slow mixing time
- c. setting time

Contractor : Tumour Center and Institute of Oncology, Bologna,  
Italy

Contract No. 323-79-4-ENV-I

Project leader : Prof. Dr. Cesare Maltoni

Title of project: Assessment of a Shorter Model of Carcinogenicity Bioassays for Organic Chemicals.

---

I) Objective of the research.

At present, long-term carcinogenicity bioassays, performed on laboratory rodents (usually mice and rats), are of long duration.

The treatment period varies from 78 or 104 weeks to life span, for mice and rats respectively. Often, even when the treatment is stopped at 78 or 104 weeks, the animals are kept alive until spontaneous death.

The objective of this research is to assess the feasibility and the adequacy of medium-term bioassays for carcinogenicity.

The availability of such an experimental model would accelerate the production of scientific information, lower costs, and enable the few existing laboratories to produce basic data on a larger number of compounds.

For such research the following points were borne in mind:

- 1) embryos and newborns are usually more responsive than adults to oncogenic agents;
- 2) many chemicals and their active metabolites are concentrated in and secreted with milk;
- 3) the higher the dose of a carcinogen the greater the tumour response is.

Based upon these considerations, a series of compounds, whose carcinogenic effects are largely or partially known (table 1), have been tested with an experimental protocol, aimed at providing data on the shorter, although effective, treatment and biophase period (table 2).

Table 1. Available data on the carcinogenicity of the tested compounds.

Compound	Carcinogenicity		
	Rat	Mice	Man
Vinyl chloride (VC)	+	+	+
Vinylidene chloride (VDC)	-	+	
Acrylonitrile (AC)	+		?
Benzene (Be)	+		+
Methylene chloride (CMT)	?		

Table 2. Experimental protocol

Animals	Treatment (weeks)	Biophase	
		52 weeks sacrifice	104 weeks sacrifice
Breeders	52		All the survivors
Offsprings	15	10 M and 10 F	All the survivors
	52	10 M and 10 F	All the survivors

## II) Materials and methods.

All the five compounds used (VC, VDC, ACN, BE, CMT) are chemically defined.

Sprague-Dawley rats are employed.

The plan of the experiments is presented in tables 3-8.

The animals are exposed, by inhalation, in the inhalatory chambers of our laboratory. The level of exposure during the treatment is monitored by gas chromatograph.

The animals are controlled and weighed respectively: during the first three months of the experiments weekly and every two weeks, up to one year every two weeks and every four weeks, and after one year every two weeks and every eight weeks.

The sacrifice is performed by ether inhalation.

A complete autopsy is made of every animal. Histological specimens include the brain, Zymbal glands, interscapular brown fat, salivary glands, tongue, lungs, liver, spleen, kidneys, stomach, different segments of intestine, bladder, lymphnodes, and any other organs with pathological lesions.

The experiments were started on June 10, 1980.

## III) Results (preliminary).

The results, up to the 36th week, are shown in tables 3-8.

In the last 10 weeks (from 36 to 46) several VC treated offspring, mainly from the group for which 52 weeks of treatment is planned, died, and nearly all with tumours known to be specifically correlated with the compound.

## IV) Additional comments.

In June, one year after the beginning of the experiments, 10 males and 10 females from each of the two subgroups of offspring, for each tested compounds, will be sacrificed, as planned in the protocol.

The autopsy and the histological examinations of these animals will provide early indications on the efficacy of the proposed experimental model.

The experimental biophase will end in June 1982, and the final report will be ready by the end of that year.

TABLE 3  
 Experiments WF4001-WF4006: Exposure by inhalation to Vinyl chloride (VC), Vinylidene chloride (VDC), Acrylonitrile (AN), Benzene (BZ) and Methylene chloride (MC), in air. Results after 36 week

EXPERIMENT	GROUPS	COMPOUND	ROUTE	TREATMENTS	ANIMALS (Sprague-Dawley rats)			TUMORS																
					Sex	Age at start	No. at start	No. autopsied	No. %	Primary tumors	No. %	Secondary gland tumors	No. %	Thyroid gland	No. %	Liver	No. %	Lymphatic nodes	No. %	Other	Type			
WF4001	I	VC	Inhalation	4h/d 50/w 7* and then 7h/d 50/w 8* at 2,500 ppm	M	12 weeks old (breeders)	54 (19)	53	98.1	2	3.8	0	-	0	0	-	0	0	-	1	1.9	1	Symbl gland adenoma	
					F	Embryos at the 12th day of pregnancy	60	56	93.3	0	-	1	1.8	1	1.8	0	-	1	1.8	0	-	1	1.8	1
		M	Embryos at the 12th day of pregnancy	60	60	100.0	0	-	1	1.7	0	-	0	-	0	-	-	-	-	-	-			
		M and F	Embryos at the 12th day of pregnancy	120	116	96.7	0	-	2	1.7	1	0.9	0	-	1	0.9	0	-	1	0.9	1	liver angiosarcoma		
TOTAL					M	Embryos at the 12th day of pregnancy	63	63	100.0	0	-	0	-	0	-	0	-	0	-	0	-	-		
					F	Embryos at the 12th day of pregnancy	65	61	93.8	0	-	0	-	1	1.6	1	1.6	1	1.6	1	1.6	0	-	-
					M and F	Embryos at the 12th day of pregnancy	128	124	96.9	0	-	0	-	0	-	1	0.8	1	0.8	0	-	-		
					M		123	119	96.7															
					F		179	174	97.2															
					M and F		302	293	97.0															

\* 1) between brackets number of pregnancies.

TABLE 4

Experiments W74001-W74006: Exposure by inhalation to Vinyl chloride (VC), Vinylidene chloride (VDC), Acrylonitrile (AN), Benzene (B) and Methylene chloride (MC), in air, 8 hours a day, 5 days a week, 10 weeks after: 36 weeks

EXPERIMENT NO.	GROUPS COMPOUND	ROUTE	TREATMENTS	ARTICLES (Sprague-Dawley rats)			TUMORS																			
				Sex	Age at start (Breeders)	No. at start (23)	No. with tumors	%	Mean # of tumors	4 mg/liters	No.	%	Mean # of tumors	Spleen gland carcinomas	No.	%	Liver and adrenals carcinomas	No.	%	Probable others	No.	%	Others	No.	%	Type
W74002	I	Inhalation	4N/4 50/m 7m and then 7N/4 50/m 7m at 100 ppm	M	12 weeks old (Breeders)	54	1	1.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	
				F	60	59	98.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	
				M and F	120	118	98.3	1	0.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-
	II	Inhalation	4N/4 50/m 7m and then 7N/4 50/m 7m at 100 ppm	M	Embryos at the 12th day of pregnancy	60	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	
				F	Embryos at the 12th day of pregnancy	60	1	1.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-
				M and F	Embryos at the 12th day of pregnancy	120	118	98.3	1	0.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-
TOTAL			4N/4 50/m 7m and then 7N/4 50/m 45m at 100 ppm	M	Embryos at the 12th day of pregnancy	62	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	
				F	Embryos at the 12th day of pregnancy	61	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-
				M and F	Embryos at the 12th day of pregnancy	123	122	99.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-
TOTAL				M		176	174	98.9																		
TOTAL				F		121	120	99.2																		
TOTAL				M and F		297	294	99.0																		

1) between brackets number of pregnancies.



TABLE 5

Experiments WF4001-WF4006: Exposure by inhalation to vinyl chloride (VC), vinylidene chloride (VDC), acrylonitrile (ACN), Benzene (BZ) and Methylene chloride (MTC), in air. Results after: 35 weeks

EXPERI- MENT	GROUPS COMPOUND	ROUTE	TREATMENTS	ANIMALS (Sprague-Dawley rats)			SUBROUS																		
				Sex	Age at start	No. at start (1)	No. at start vivors (2)	No. of vivors	No. of tumors	Spleen gland carcinomas	Liver meta- sarcomas	Neurofibro- sarcomas	Neurofibro- sarcomas	Others											
WF4003	I	Inhalation	4N/4 50/w 7w and then 7w/4 50/w at 60 ppm	F	12 weeks old (breeders)	54 (22)	100.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
					M	Embryos at the 13th day of pregnancy	60	96.7	0	1	1.7	0	0	0	0	0	0	0	0	0	0	0	0	0	
						Embryos at the 14th day of pregnancy	60	98.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
						Embryos at the 12th day of pregnancy	120	97.5	0	1	0.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
II	ACN	Inhalation	4N/4 50/w 7w and then 7w/4 50/w 4w at 60 ppm	F	Embryos at the 12th day of pregnancy	67	100.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
					M	Embryos at the 12th day of pregnancy	54	100.0	1	1.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
						Embryos at the 13th day of pregnancy	121	100.0	1	0.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
						Embryos at the 12th day of pregnancy	181	99.9	1	0.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TOTAL				M		181	179	99.4																	
				F		114	113	99.1																	
				M and F		295	292	99.0																	

1) between brackets number of pregnancies.

TABLE 6

Experiments PF-001-PF-006: **EXPOSURE** by inhalation to Vinyl chloride (VC), Vinylidene chloride (VDC), Acrylonitrile (AN), Benzene (BZ) and Methylene chloride (MC), in air. **RESULTS** after 35 weeks

EXPERIMENT	GROUPS COMPOUND	ROUTE	TREATMENTS	ARTHRALIS (Sprague-Dawley rats)			SYMPOMS											
				Sex	Age at start	No. at start	No. of survivors	Mammary tumours		Spleen gland sarcinoma		Liver aneis		Hepatobiliary aneis		Others		
								No.	%	No.	%	No.	%	No.	%	No.	%	No.
PF-004	I	Inhalation	4b/4 5d/4 7c/4 5d/4 12c/4 at 200 ppm and then 7b/6 5d/4 at 300 ppm	♀	12 weeks old (breeders)	24 (22)	100.0	0	0	0	0	0	0	0	0	0	0	
				♂	Embryos at the 12th day of pregnancy	70	100.0	0	0	0	0	0	0	0	0	0	0	
	II	Inhalation	4b/4 5d/4 7c/4 5d/4 12c/4 at 200 ppm	♀	Embryos at the 12th day of pregnancy	59	100.0	0	0	0	0	0	0	0	0	0	0	
				♂ and ♀	Embryos at the 12th day of pregnancy	129	100.0	0	0	0	0	0	0	0	0	0	0	
TOTAL				♀	Embryos at the 12th day of pregnancy	75	100.0	0	0	0	0	0	0	0	0	0	0	
				♂	Embryos at the 12th day of pregnancy	65	98.5	0	0	0	0	0	0	0	0	0		
				♂ and ♀	Embryos at the 12th day of pregnancy	140	99.3	0	0	0	0	0	0	0	0	0		
				♀		199	100.0											
				♂		124	123											
				♂ and ♀		323	322											

<sup>1)</sup> between brackets number of pregnancies.

TABLE 7

Experiments 224001-224006: Exposure by inhalation to Vinyl chloride (V), Vinylidene chloride (VDC), Acrylonitrile (AN), Benzene (Bz) and Methylene chloride (MC), in air. Results after 36 weeks

EXPERIMENT	ROUTE	COMPOUND	ROUTE	DURATION	MATERIALS (Sprague-Dawley rats)			TUMORS											
					Sex	Age at start of pregnancy (weeks)	No. at start of pregnancy	No. at 100 ppm	Mammary tumors		Fungal gland carcinoma		Liver carcinoma		Methrobl-astoma		Others		
									No.	%	No.	%	No.	%	No.	%	No.	%	No.
224005	I	OET	Inhalation	4h/4 5d/w 7e and then 7h/4 4d/w at 100 ppm	F	12 weeks old (heifers)	34	53	59.1	0	0	0	0	0	0	0	0	0	
							60	100.0	0	0	0	0	0	0	0	0	0		
							70	100.0	0	0	0	0	0	0	0	0	0		
224005	II	OET	Inhalation	4h/4 5d/w 7e and then 7h/4 5d/w at 100 ppm	M and F	Embryos at the 12th day of pregnancy	130	100.0	0	0	0	0	0	0	0	0	0	0	
							60	59.3	0	0	0	0	0	0	0	0	0		
							69	100.0	0	0	0	0	0	0	0	0	0		
224006	I	OET	Inhalation	4h/4 5d/w 7e and then 7h/4 5d/w at 100 ppm	M and F	Embryos at the 12th day of pregnancy	129	188	99.2	0	0	0	0	0	0	0	0		
							174	178	98.8										
							139	138	100.0										
TOTAL					M and F	313	311	99.4											

1) Between brackets number of pregnancies.

TABLE 8

Experiments RE4001-RE4006: **EFFICACY** by inhalation to Vinyl chloride (VCl), Vinylidene chloride (VDC), Acrylonitrile (AN), Benzene (Bz) and Methylene chloride (CH<sub>2</sub>) in air. **RESULTS** after 36 weeks

EXPERIMENT	GROUP COMPOUND	NOTE	TREATMENTS	ARTICLES (Sprague-Dawley rats)				TUMORS														
				Sex	Age at start (1)	No. at start	No. excised livers	Hemangio-sarcoma		Hepatocellular carcinoma		Hepatoblastoma		Adenoma		Others						
								No.	%	No.	%	No.	%	No.	%	No.	%					
RE4006 (1)	I None (Control)	Inhalation	6 1/4 56/m <sup>3</sup> 7 <sup>m</sup> and then 7 1/4 56/m <sup>3</sup>	7	12 weeks old (Meaders)	60 (24)	60 100.0	0	0	0	0	0	0	0	0	0	0	0	0	0		
				8	Embryos at the 12th day of pregnancy	80	79 98.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
				7	Embryos at the 12th day of pregnancy	70	70 100.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	II None (Control)	Inhalation	6 1/4 56/m <sup>3</sup> 7 <sup>m</sup> and then 7 1/4 56/m <sup>3</sup> 45 <sup>m</sup>	8 and 7	Embryos at the 12th day of pregnancy	150	149 99.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
				8	Embryos at the 12th day of pregnancy	76	77 98.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
				7	Embryos at the 12th day of pregnancy	79	76 95.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
				8 and 7	Embryos at the 12th day of pregnancy	157	155 98.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
<b>TOTAL</b>				8		218	216 99.0															
				7		149	148 99.3															
				8 and 7		367	364 99.2															

(1) These untreated rats serve as common control for the experiments RE4001, RE4002, RE4003, RE4004 and RE4005  
 1) between brackets number of pregnant.

CONTRACTOR: ISTITUTO SUPERIORE DI SANITA' , Roma

CONTRACT n. ENV/366 I

PROJECT LEADER: V.G. LONGO

TITLE OF PROJECT: Electroencephalographic investigation on the effects of organophosphorous pesticides and their metabolites on the central nervous system

#### Objective of the research

Aim of this work was to measure quantitatively effects induced by long-acting anticholinesterase (organophosphorous) compounds and their metabolites versus short acting anticholinesterase (physostigmine) agents on the electroencephalogram of laboratory animals.

Electroencephalographic investigations through computerized analysis (CEEG) have been then carried out to study also mechanisms of action of anticholinesterasic agents on mammals brain at low doses, which do not induce behavioral and/or biochemical alterations (Zapponi et al 1978; Longo et al, 1980). Paraoxon, methyl-parathion and physostigmine show in mice analogous CEEG patterns during the first hour after treatment: at high doses (about 10% of LD50 and up) all these drugs induce diminution in power of some CEEG frequency bands of spectrum. Following low doses (about 1 to 5% of LD50) however, a partially different effect may be noted: i.e. methyl-parathion and paraoxon induce a significant increment in power of 7.5 to 12 Hz frequency band, while physostigmine induce an unconsistent (not significant) increment of the same band power.

#### Materials and methods

Twenty DBA/2 male mice weighing 22 to 24 grams were chronically implanted with four cortical electrodes (anterior and posterior sensorimotor cortex). EEGs were recorded on paper and on magnetic tape according following schedules: control EEG was recorded for 2 consecutive hours; then mice were challenged with saline, 0,2 ml i.p.; EEG was again recorded for 2 minutes 5, 15, and there after every 15 minutes after treatment for at least 2 hours. On following day a recording session was performed with the same animal, which was treated with paraoxon (four animals per dose: 0.025; 0.05; 0.1; 0.2; 0.4 mg/kg). The EEG signal was then sampled, power spectrum analysis was performed, and power spectra, ranging from 0 to 63.5 Hz with 0.5 Hz discrimination, were stored on digital tape. On the basis of a preliminary correlation analysis (for methods see Loizzo et al 1979; Zapponi et al 1979) between frequencies, 6 frequency bands were chosen. Each EEG period was represented by a 8 components vector, i.e. 6 frequency bands: 0.5-3.5 Hz, 4-7 Hz, 7.5-12 Hz, 12.5-20 Hz, 20.5-40 Hz), plus total power and the mean frequency of the spectrum.

In order to reduce interanimal variability normalization of power values was performed dividing each absolute frequency value relative to post-drug interval by mean value of total power relative to predrug intervals in each animal.

### Results

After low doses of paraoxon no gross behavioral alterations were noted; while higher doses (0.8-0.4 and sometimes 0.2 mg/kg), induced transitory hypomobility and horripilation.

On visual inspection EEG records relative to control and treated animals with 0.025 to 0.1 mg/kg appeared normal. In two animals out of four treated with 0.2 mg/kg and in all animals treated with 0.4 mg/kg visual inspection of EEG records showed transitory slowing in frequency and diminution in amplitude, although morphology of EEG waves was not affected.

No clear signs of seizures were ever noted.

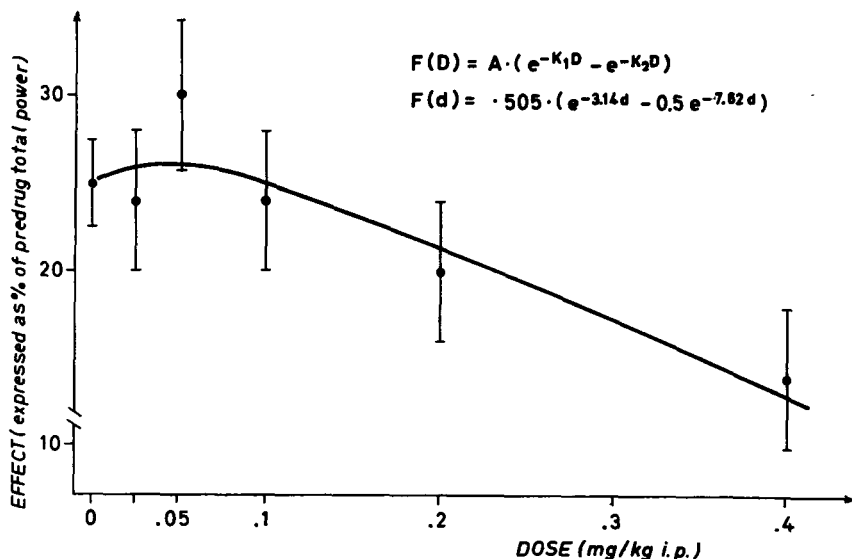
Computer analysis showed that the lowest dose of paraoxon did not induce significant CEEG modifications. However after 0.05 mg/kg a significant enhancement of power in intermediate bands of spectrum was evidenced (see table 1).

T A B L E I

Dose (mg/kg i.p.)	.4	.2	.1	.025	H2O	0.5
effect (in percent of predrug total power)	14	19	23	24	25	30
	<hr/>				<hr/>	
	* 5%				* 5%	
	<hr/>					
	* 5%					

Effect induced by low doses of Paraoxon on intermediate frequency band (7.5-12 Hz) of EEG power spectrum in mice. Asterisks indicate estimated levels of significance (Duncan test) between contiguous values. Wider intervals indicate higher significance levels.

After higher doses (0.1 mg/kg and up) a significant depression in the first hour after administration was seen in the same CEEG frequency bands. It was also possible to evaluate a function model for power values relative to 7-12.5 Hz frequency band of the spectrum (Fig. 1)



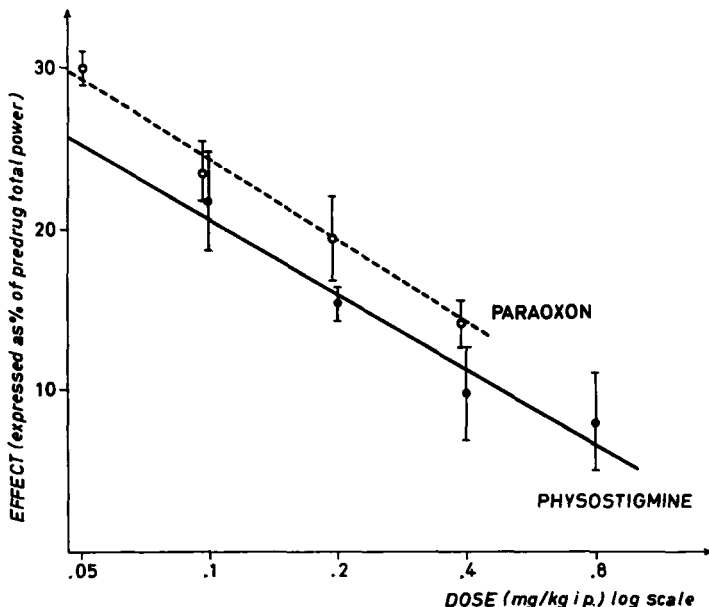
Exponential function interpolated from 7.5-12 Hz frequency band of spectrum power values in the first hour after i.p. administration of paraoxon.

Each point represent mean values  $\pm$  2 standard error, 4 animals per dose

#### Conclusions and comments

Present report can give confirmation of the strict concomitance of effects induced by higher doses of physostigmine and paraoxon.

Fig. (2) shows pattern distribution of power values relative to physostigmine and Paraoxon at equivalent doses (LD50 of Physostigmine salicylate is about 1 mg/kg i.p.; LD50 of paraoxon is about 1.8 mg/kg i.p.).



Titration curve relative to effects induced by paraoxon versus Physostigmine on CEEG in mice.

Normalized power value of 7.5 to 12 Hz band during the first hour after i.p. administration (Four animals per dose)

Abscissa: log of dose

Ordinate: Effect (expressed as % of predrug total power)

No significant difference between dose/effect relationships obtained from linear correlation curves of power values relative to the two drugs was found (titration value: 1.03) thus showing that biological (CEEG) activity trend of the two drug was analogous. These EEG effects have not been evidenced only with high doses of physostigmine (corresponding about to 10 to 80% of LD50) and paraoxon (corresponding to about 3 to 20% of LD50) therefore it may appear unreliable to interpret it as unspecific, toxic effect. However from these results following conclusions can be tentatively drawn:

- 1) Route of administration of the drugs should be taken into better consideration, as well as bioavailability of the drugs when biological (CEEG) effects are studied, although our methods seem to be very suitable to measure central effects induced by cholinesterase inhibitors on animals brain.



- 2) CEEG effects should be compared with other (behavioral and/or biochemical) effects in confrontable time lags after treatment.
- 3) From EEGrafical effects it is possible to hypothesize that anticholinesterasic reversible and irreversible drugs have a common site of action, while they seem to have different modulating effects on cerebral structures which are responsible for "production" of high frequency (7.5 to 12 Hz band) in mice dorsal hippocampus.
- 4) Hypothesis considered in previous final report (Longo, et al., 1980) requires probably more precise definition, since a parallel discussion between CEEG effects and biochemical effects induced by cholinesterase inhibitors (as reported in the literature) deserves further reconsideration. In this report only discussion of qualitative and quantitative EEG effects will be considered:
  - a) High doses of physostigmine, paraoxon and methyl-parathion induce a surprising similar CEEG effects (as it was seen from 7.5 to 12 Hz band values analysis).
  - b) A low dose of paraoxon (0.05 mg/kg i.p.) induce a significant transitory increment of the same frequency band. This effect was analogous to the effect induced by low doses (0.25 and 0.5 mg/kg p.o.) of methyl-parathion. A low doses of physostigmine (0.025 mg/kg i.p.) induced an increment of values in the same frequency band which however did not reach statistical significance.
  - c) All three drugs induce most important effects on 7.5 to 12 Hz frequency band, therefore a correlation between biochemical and CEEG effects induced by the three drugs, can be taken into caute consideration. As for as we know, it is the first time that a similar hypothesis has been, at least in part, supported by statistical analysis.

This report has been made with the participation of the following people:  
T. Battisti, E. Deodati G. Gallozzi, A. Loizzo, E. Ortolani, S. Palazzesi,  
Pezzola A., Zapponi G.A.

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- A. Loizzo, P. Ceccarelli, E. Ortolani, S. Palazzesi, G.A. Zapponi.  
L'analisi elettroencefalografica in fisiologia e farmacologia sperimentale. Rivista Italiana di EEG e Neurofisiologia, 1979 Vol. II Fase II pag.378-391.
- G.A. Zapponi, P. Ceccarelli, E. Ortolani.  
Alcuni metodi per la semplificazione della interpretazione dell'EEG. Rivista Italiana di EEG e Neurofisiologia, 1979 Vol. II Fase II pag. 394-400

Contractor: Istituto Superiore di Sanità, Rome, Italy

Contract n° ENV/367/I

Project Leader: Professor Gian Luigi Gatti

Title of project: Analysis of the mechanisms of the neurotoxicity of organophosphorus pesticides in relation to the inhibition of the isoenzymatic profiles of acetylcholinesterase

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PART 1. COMPARATIVE STUDIES ON WHOLE BRAIN SOLUBLE ACETYLCHOLINESTERASE AND ITS MOLECULAR FORMS DURING ACUTE INTOXICATION BY ISOFLUROPHATE AND PARAOXON IN RATS. REGIONAL DIFFERENCES IN RAT BRAIN SOLUBLE AChE AND ITS MOLECULAR FORMS AFTER ACUTE POISONING BY ISOFLUROPHATE.

PART 2. MOLECULAR FORMS OF THE RAT AND HUMAN ERYTHROCYTE ACETYLCHOLINESTERASE.

PART 1

OBJECTIVE OF THE RESEARCH: Previous experiments performed under contract (268-77-1 ENV I) on the fine mechanisms of neurotoxicity during acute intoxication by Isoflurophate (DFP) showed a differential inhibition and recovery of three main molecular forms of acetylcholinesterase (AChE) in the whole brain of rats. The recovery appeared particularly rapid for medium-molecular-weight forms and more pronounced in the soluble portion than in membrane-bound enzymatic activity. (1,4) The main objective of the present investigation was to extend the experiments to Paraoxon, the active metabolite of Parathion -- one of the most commonly used organophosphorus pesticides (over 2600 tons consumed in Italy in 1978), frequently involved in human poisonings. Moreover, in order to evaluate the dynamics of the phenomenon in a more detailed fashion the study on inhibition and recovery of multiple molecular forms during acute intoxication by DFP has been extended to some brain regions, such as cerebral cortex, hippocampus and striatum. These regions have quite different levels of AChE, presumably with different physiopharmacological significance and in each of them there is a good correlation between AChE and other important markers of cholinergic activity such as cholineacetyltransferase, high-affinity choline uptake and density of muscarinic receptors (for references see 10) which indicate the importance of their cholinergic systems.

MATERIALS AND METHODS. Wistar male albino rats (160-220 g) were treated subcutaneously with DFP (1.1 mg/kg in arachis oil) or Paraoxon (0.25 mg/kg in water). Separate groups of six rats (in experiments on whole brain) or eight rats (in experiments on brain regions) were sacrificed at various intervals from 90 min up to 12 days after treatment. Appropriate groups treated with the vehicle were run in parallel. Whole brain including cerebellum was removed (in experiments on brain regions dissected according to Glowinski and Iversen - J. Neurochem. 13, 655, 1966), homogenized using Tris-HCl buffer, 0.038M pH 8.5 and centrifuged at 100,000 g for 1 hr in a Beckman L2 65B ultracentrifuge. The enzymatic activity in supernatants was determined according to Ellman and coworkers (Biochem. Pharmacol., 7, 88, 1961) and corresponded to about 20% of the activity present in crude homogenate in controls. The separation of molecular forms was carried out by disc polyacrylamide gel electrophoresis, followed by enzymatic reaction with acetylthiocholine (AcThCh) and staining, as

described for the previous research on this contract (1, 4). The gels were subsequently scanned at 600 nm in a Gilford 2400 spectrophotometer and peak areas integrated using a Hewlett-Packard (9804) digitizer. The data were elaborated statistically by analysis of variance (ANOVA).

**RESULTS:**

Comparative studies on whole brain soluble acetylcholinesterase and its molecular forms during acute intoxication by Isoflurophate and Paraoxon in rats (publication 6).

Fig. 1 shows some differences in the rate of inhibition and recovery of soluble AChE following treatment by DFP and Paraoxon. Specifically, initial depression of enzyme activity was slightly more pronounced after Paraoxon than after DFP (e.g., to 17% versus 24% of control level at 90 min), while recovery was somewhat slower after the latter compound. In fact, a 2x 8 ANOVA yielded a significant interaction between type of organophosphate and interval ( $p < 0.02$ ).

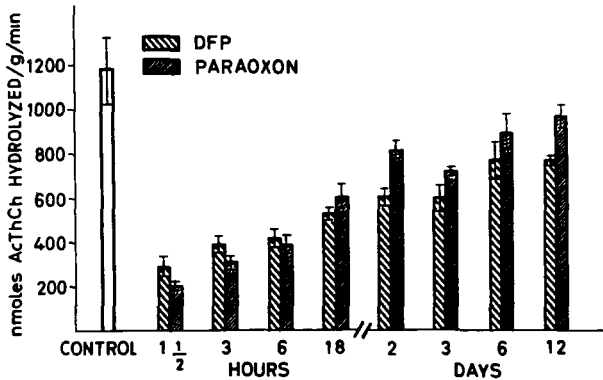


Fig.1. Recovery of rat brain soluble AChE following a single subcutaneous dose of DFP (1.1mg/kg) or Paraoxon (0.25 mg/kg)

The data of Fig. 2 indicate that during the initial phase of intoxication (90 min) the relative contribution to the enzymatic activity of individual molecular forms does not differ from that found in the controls in the case of DFP (A) but changes markedly in the case of Paraoxon (B): the low-molecular-weight forms, characteristic of cytosol, appear to be more depressed than high-molecular-weight-forms, probably in relation to the greater hydrosolubility of this compound. As concerns the subsequent recovery phase, the DFP data show a selective increase of medium-forms, reaching a peak at 72 hr, and a less marked increase of low-forms. These modifications of the pattern are partially reversed by the 6th day and disappear by the 12th day. In the case of Paraoxon the low-molecular-weight-forms, most depressed initially, show a very fast recovery, while the increase of medium-forms appears negligible and normalization of the pattern occurs by the 3rd day. These differences in trends were confirmed by 2x8 ANOVA's carried out separately for each molecular form showing significant effects of treatment (T) and intervals (I), with no interaction between the two, both in the case of high-weight-forms (T: $p < 0.001$ ; I: $p < 0.001$ ) and in the case of the low-weight-forms (T: $p < 0.02$ ; I: $p < 0.002$ ), while a significant T x I interaction appeared in the case of the medium-weight-forms ( $p < 0.05$ ).

Additional experiments in vitro using soluble AChE from animals sacrificed 90 min after treatment indicated a negligible spontaneous reactivation at 30°C of enzyme inhibited by DFP and its slow reactivation by Pralidoxime. The spontaneous and Pralidoxime-induced reactivation of AChE inhibited by Paraoxon was markedly faster. However, none of the individual molecular forms appeared to be reactivated preferentially in vitro whether after DFP or Paraoxon treatment.

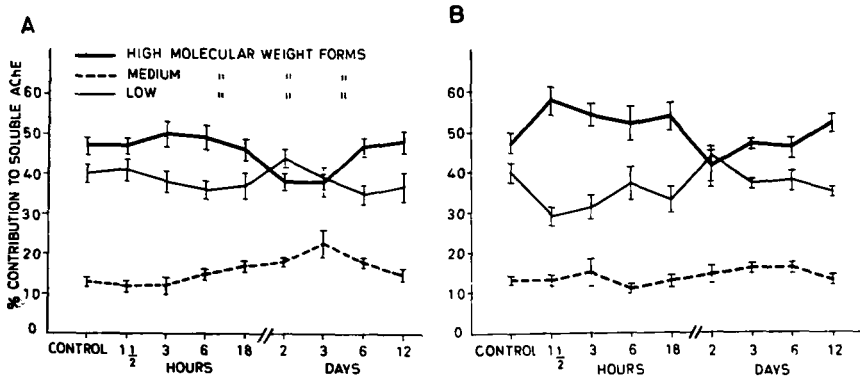


Fig. 2. Percentage contribution of individual molecular forms to soluble AChE during the intoxication by DFP (A) and Paraoxon (B).

Regional differences in rat brain soluble acetylcholinesterase and its molecular forms after acute poisoning by Isoflurophate (publications 5 and 9).

The activity of soluble AChE in the cerebral cortex, hippocampus and striatum was respectively of 766 - 39, 526 - 44 and 3645 - 356 nmoles of AcThCh hydrolyzed/min/g of tissue. Fig. 1 shows that at 3 hr maximum inhibition of the enzymatic activity occurs in the striatum while the effect appears the least pronounced in hippocampus. This means that there is an inverse relationship between the absolute level of soluble AChE in a given region and the percentage of inhibition. The subsequent rate of recovery appears to be directly related to the extent of the initial inhibition. For example, a recovery consisting in a return to 60% of control activity is observed in hippocampus and cerebral cortex already after 48hr and in striatum only after 12 days.

Fig. 2 shows that in spite of the considerable differences in absolute levels of enzymatic activity, the percentage contribution of individual molecular forms appears to be similar in the three regions of control rats. This contribution changes markedly already 3 hr after treatment (with a relative decrease of high-molecular-weight-forms and a relative increase of medium- and low-molecular-weight-forms. These alterations appear most pronounced in the striatum where the contribution of the former forms falls to one half of the controls. As concerns the subsequent recovery, in all regions there is a selective surge of medium forms reaching a maximum at 48 hr for cerebral cortex and hippocampus (similarly to whole brain) and at 18 hr in the striatum where there is an almost total inversion of the ratio high/medium forms (0.45 respect to 4.5 of controls). These modifications of the pattern are completely reversed in the striatum and partially reversed in the other

regions by the 6th day, followed by a trend towards normalization within 12 days.

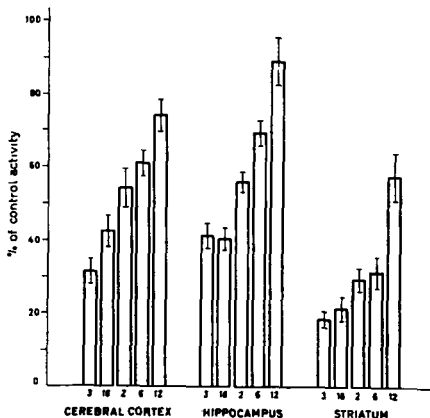


Fig. 3. Recovery of soluble AChE following a single subcutaneous dose of DFP (1.1 mg/kg) in various brain regions. The numbers under the columns indicate hours (3,18) and days (2,6,12) after treatment.

#### CONCLUSIONS:

As concerns the study on whole brain, the results indicate that the recovery of enzymatic activity after an organophosphorus exposure is accompanied by a transient increase in the percentage contribution of medium-and/or low-molecular-forms which appears to be the initial step in biosynthesis of new molecules in the soluble portion of AChE. The fine dynamics of the recovery following DFP and Paraoxon treatments appear, however, somewhat different. The molecular mechanisms of the recovery in the case of DFP and the importance of the soluble portion of AChE in this process have been fully discussed in the previous report of the research on this contract (4). In the case of Paraoxon, the selective inhibition of low-molecular-weight-forms (characteristic of cytosol), due to a marked hydrosolubility of the compound is followed by very fast recovery which depends also on a partial hydrolysis of phosphorylated molecules as suggested by experiments on *in vitro* reactivation.

As concerns the regional study following DFP treatment, the striatum appears to be particularly vulnerable to the toxic effects, showing greater initial inhibition and slower recovery relative to other regions. Moreover, in the striatum the sensitivity of high-molecular-weight-forms to DFP is maximal and subsequent selective recovery of medium-forms appears to be more pronounced than elsewhere.

It must be reminded that the cholinergic systems located in striatum are considered to have a critical role in motor control. Furthermore, severe disturbances appearing in the early phase of the intoxication by DFP show a fair correlation with the degree of inhibition of enzymatic activity. Therefore, it may be hypothesized that fast recovery of medium-forms play some role in short term behavioural compensation taking place before overall recovery of enzyme activity.

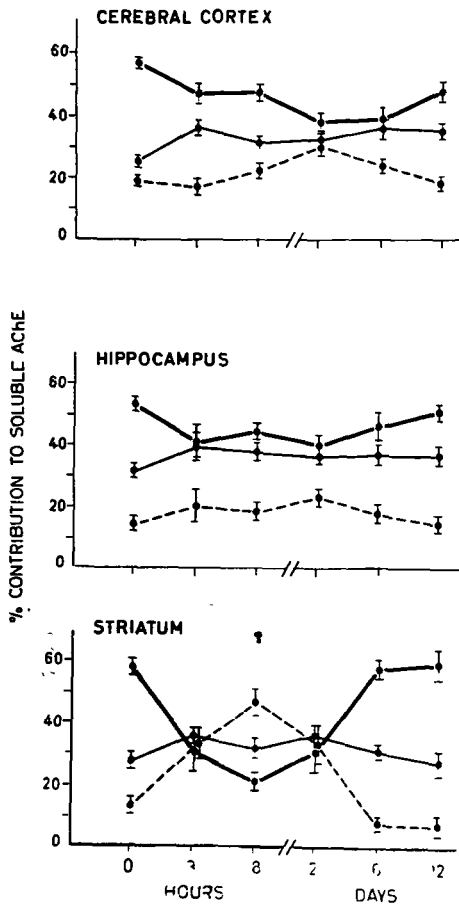


Fig. 4. Percentage contribution of individual molecular forms to soluble AChE during the intoxication by DFP in various brain regions. For symbols see Fig. 2.

ADDITIONAL COMMENTS:

During the course of this investigation other series of experiments have been performed whose results are not included in the present report. a) A countercheck on the reliability of the results obtained by gel electrophoresis was provided by gel filtration (on Sephacryl 300) experiments allowing to separate three main peaks of soluble AChE. Each of these, when subsequently processed by gel electrophoresis, behaved as a single band corresponding to the molecular forms detected under standard experimental conditions. The molecular weights of the three forms, estimated in experiments on gel electrophoresis and confirmed by gel filtration were of over 700,000, 340,000 and 115,000.

b) Some experiments were performed on whole brain of un-

treated rats during early post-natal development (from birth up to 1 weeks) when an intense biosynthesis of AChE occurs. The results indicated that a 6-fold rise of enzymatic (mainly membrane-bound) activity was accompanied by important modifications in the percentage contribution of individual molecular forms, consisting in a considerable increase of high-molecular-weight-forms at the expense of the other two forms between the 14th and 28th day. These modifications appeared similar to those observed during an acute intoxication by DFP in adult rats (publications 2 and 3).

c) Preliminary experiments on the effects of DFP, administered to rats in the last days of pregnancy and sacrificed at delivery, indicated that in offspring soluble and total brain AChE appeared considerably less inhibited than in mothers. (publication 7).

Contributors: G. Bignami, G.M. Bisso, A. Meneguz, H. Michalek and M. Bastianelli.

LIST OF PUBLICATIONS AND COMMUNICATIONS ON CONTRACT RESEARCH:

1. H. Michalek, A. Meneguz, G.M. Bisso, G. Carro-Ciampi, G.L. Gatti and G. Bignami. Neurochemical changes associated with the behavioural toxicity of organophosphate compounds. Advances in Pharmacology and Therapeutics, Vol.9 Toxicology (Proceedings of the 7th International Congress of Pharmacology, Paris - July 1978) Ed. Y. Cohen, Pergamon, Oxford, 1979, pp. 187-201.
2. G.M. Bisso, R. Nemesio, H. Michalek: Early post-natal changes in the pattern of molecular forms of acetylcholinesterase in the rat brain. Multidisciplinary Approach to Brain Development. (Proceedings of the International Meeting on Multidisciplinary Approach to Brain Development, Selva di Fasano, April 1979). Eds. C. Di Benedetta, R. Balazs, G. Gombos and G. Porcellati, Elsevier-North Holland, Amsterdam, 1980, pp. 235-236.
3. G.M. Bisso, A. Meneguz, R. Nemesio and H. Michalek. Polyacrylamide gel electrophoresis as an analytical tool for the study of the alterations of rat brain acetylcholinesterase isoenzymatic pattern in some physiological and pathological conditions. (Third International Symposium on Affinity Chromatography and Molecular Interactions, Strasbourg, June 1979.) Colloque: Chromatographie d'Affinité et Interactions moléculaires, Ed. J.M. Egly, Inserm, Paris, 1979, p. 438.
4. G.L. Gatti: Analysis of the mechanisms of the neurotoxicity of organophosphorus pesticides in relation to the inhibition of the isoenzymatic profiles of acetylcholinesterase. Environment and Quality of Life, Second Environmental Research Programme 1976-80. Ed. Commission of European Communities, ECSC-EEC-EAEC, Brussels 1980. Part 1,2 of the Contract 268-77-1 ENV I, pp. 290-29 .
5. A. Meneguz, G.M. Bisso, H. Michalek: Regional differences in brain soluble acetylcholinesterase and its molecular forms after acute poisoning by isoflurophate in rats, (International Congress of Neurotoxicology, Varese, September 1979), Clinical Toxicology in press, 1981.
6. H. Michalek, G.M. Bisso, A. Meneguz: Comparative studies on rat brain soluble acetylcholinesterase and its molecular forms during the intoxication by DFP and Paraoxon. Cholinergic Mechanisms: Phylogenetic Aspects, Central and Peripheral Synapses, and Clinical Significance (Proceedings of the International Symposium on Cholinergic Mechanisms, Florence, March 1980), Eds. G. Pepeu and H. Ladinsky, Plenum, New York 1981; in press.
7. G.M. Bisso, H. Michalek and A. Meneguz. Effects of DFP administration to pregnant rats on brain acetylcholinesterase and its molecular forms of mother and offspring at birth. First Meeting of the International Society for Developmental Neuroscience, Strasbourg, July 1980, P. 95, p. 214.
8. G.M. Bisso, V. Giardini, A. Meneguz and H. Michalek. Brain acetylcholinesterase recovery in rats as a function of treatment order after a second organophosphate intoxication. Second International Congress on Toxicology, Brussels, July 1980), Tox.Lett., Special Issue, ISSN,1980,p39
9. A. Meneguz, G.M. Bisso and H. Michalek. Soluble acetylcholinesterase and its molecular forms in brain regions of various animals species, (Fourth European Neuroscience Meeting, Brighton, September 1980), Neuroscience Letters, Suppl. 5, S 244, 1980.
10. G. Bignami and Hanna Michalek. Cholinergic mechanisms and aversively motivated behaviors. Psychopharmacology of Aversively Motivated Behavior Eds. H. Anisman and G. Bignami, Plenum, New York, 1978, pp.173-255.

PART-2. Objective of the research: While many studies have been done on the biochemical characteristics of acetylcholinesterase (AChE) molecular forms from nervous and muscular tissues of different species and from human erythrocytes (1,2,3), little information is available about the molecular forms of rat erythrocyte AChE. This species on the other hand is largely used as an experimental model of human exposure to organophosphorus pesticides. Aim of this research is to fill this gap and in particular to compare the molecular characteristics and sub-unit composition of the two forms of rat and human erythrocyte AChE, previously revealed by gel electrophoresis (4).

Materials and methods: For the extraction of rat and human AChE and for the determination of enzymatic activity and isoenzymatic patterns, the same procedures have been used as described in a previous report (5). Rat and human red cell AChE preparations have been purified by affinity chromatography on Affigel 202, in the presence of the specific inhibitor N-methyl-3-aminopyridium iodide (6), according to a batchwise procedure. The purified enzymatic samples were subjected to analytical electrophoresis on polyacrylamide gel in the presence of SDS and compared, for molecular weight determination, with calibration proteins. Density gradient centrifugation was performed on sucrose gradients with bacterial beta-galactosidase and beef liver catalase as markers for molecular weight determination. Isoelectric focusing of enzymatic preparations was performed on thin layer of polyacrylamide gel, with ampholines at pH ranges 3.5 to 10 and 4 to 6, in the presence of Triton X-100. The AChE activity was revealed according to Koelle (7).

Results: The two forms of rat and human erythrocyte AChE were identified by means of gel electrophoresis at different polyacrylamide concentrations (in comparison with proteins of known molecular weights) as isomers of different molecular size: approximately 150,000 and 245,000 for forms I and II respectively. Both for rat and man the differences in molecular weight between the two forms were statistically significant; on the other hand rat and man did not differ significantly between themselves with respect to the molecular weights of two isoenzymatic forms. As previously reported (5) both for rat and man form I is not present in all individuals therefore single-banded or double-banded preparations of red cell AChE can be obtained.

SDS polyacrylamide gel electrophoresis of rat and human double-banded red cell AChE preparations purified by affinity chromatography, revealed a single band with an apparent molecular weight (S.E.±) of 130,000 ± 2,000 for man and of 131,000 ± 1,000 for rat.

A single peak of enzymatic activity at 5.3 S was obtained by density gradient centrifugation in the presence of Triton X-100 of double-banded rat and human red cell AChE preparations, while a peak at 6.3 S was obtained from single-banded preparations.

Isoelectric focusing resolved the human and rat erythrocyte AChE into a complex pattern of molecular forms: eleven AChE activity bands were revealed by inspection, with isoelectric point values from 4.6 to 5.3 for human and from 4.8 to 5.5 for rat AChE, while six components were detectable by means of



densitometry. An analysis of these patterns, for both rat and human red cell AChE, did not reveal any difference between single-banded and double-banded preparations.

Conclusions and additional comments: The two molecular forms of rat red cell AChE resulted to be similar to the human AChE, with regard to a series of biochemical characteristics; therefore rats represent a good experimental model for chronic intoxication studies with anticholinesterase agents regarding the effects on red cell AChE isoenzymes.

The sedimentation coefficients obtained by density gradient centrifugation for rat and human AChE are in agreement with the data reported by Ott and co-workers (3) for human erythrocyte AChE. The presence of a single band with a molecular weight of approximately 130,000; obtained by SDS gel electrophoresis of double-banded rat and human AChE preparations, suggests that the faster moving form (form I) is a protomer with a molecular weight of about 130,000 and the slower one (form II) a dimeric form (130,000 x 2). These values are not too far from the ones as mentioned above, and obtained by a different method. Both methods are particularly effective in separating size isomers.

The heterogeneity of rat and human preparations observed by means of isoelectric focusing could be caused by the presence of sialic acid residues, as suggested by Ott (8). The similarity of the isoelectric patterns obtained from single and double-banded rat and human red cell AChE preparations, is in agreement with the hypothesis suggested above, i.e. the two components of erythrocyte AChE are predominantly size isomers.

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Contributors: Biagioni S., Frontali N., Scarsella G., Settini L., Traina M.E.

List of publications and communications on contract research:

1. S. Biagioni, G.F. Scarsella, L. Settini, M.E. Traina, D. Trucchi. Forme molecolari dell'acetilcolinesterasi negli eritrociti umani e di ratto. Riunione del Gruppo per lo Studio dell'Eritrocita, Ferrara, June 1979.

2. G. Scarsella, L. Settini, M.E. Traina. Confronto tra acetilcolinesterasi eritrocitaria umana e di ratto. Bollettino della Società Italiana di Biologia Sperimentale, 56, 1957-1961, 1980.

3. S. Biagioni, G. Scarsella, L. Settini, G. Toschi, M.E. Traina, D. Trucchi. Molecular forms of rat and human erythrocyte acetylcholinesterase. Italian Journal of Biochemistry, 29, 362-364, 1980.

Contractor : British Food Manufacturing Industries Research Association

Contract no ENU-359-UK

Project leader : Dr C L Walters

Title of project : The influence of water-borne nitrate on the availability of nitrite and N-nitroso compounds in the saliva of consumers

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Objectives of the research :

To determine the effect of water-borne nitrate on the formation and incidence in vivo of nitrite, N-nitroso and related compounds

Materials and methods :

Determination of N-nitroso compounds. The method of Walters, Downes, Edwards & Smith (1978) for such compounds as a group has been employed in which N-nitroso compounds are selectively converted to nitric oxide for measurement in a chemiluminescence analyzer. It is applicable to all types of N-nitroso compounds investigated (N-nitrosamines, -amides, -guanidines, -urethanes, -sulphonamides) and for their differentiation from nitrite and the great majority of compounds potentially derived from it in a biological matrix with the exception of nitrolic acids and S-nitrothiols (Walters, Hart & Perse, 1978).

Use has been made of a Thermal Energy Analyzer (Thermo Electron Corporation, Waltham, Mass., U.S.A.) for two purposes. This instrument is designed for the selective catalytic cleavage of N-nitroso compounds into nitric oxide which is determined using a chemiluminescence analyzer. It has been employed firstly for the characterization and determination of volatile N-nitrosamines in the manner of Fine, Ruffe, Lieb & Rounbehler (1975). Such compounds were separated selectively from biological sources by the method of Telling, Bryce, Hoar, Osborne & Welte (1974). Secondly the TEA has been employed for the detection of more complex N-nitroso compounds which can be derivatized by methylation with diazomethane into forms suitable for separation and characterization by gas chromatography.

Determination of nitrite. This was undertaken by the IUPAC method or, where necessary on the grounds of its greater sensitivity, that of Walters, Downes, Hart, Perse & Smith (1978), making use of a chemiluminescence analyzer for nitric oxide. Semiquantitative estimations of nitrite have been made in fresh biological samples by means of Ames N-Labstix reagent strips.

Determination of S-nitrosothiols. The method employed was based on that of Saville (1958) for the measurement of thiols by nitrosation.

Collection of biological specimens. Samples of saliva were collected from volunteers into 10 ml beakers over the course of a few minutes. An endoscope was employed for gastric juice samples, its aspiration channel and polythene tubing being sterilized with glutaraldehyde and subsequently rinsed thoroughly with water. Every effort was made to avoid contamination with saliva during collection. An aliquot of each fresh gastric juice was used for nitrite determination and microbiology whilst the remainder was stabilized with sulphamic acid at a pH value of  $< 2.0$ . Such samples were stored deep frozen before extraction for the determination of N-nitroso compounds.

#### Results:

It has been established that interference in salivary nitrite levels from nitrate ingested from normal dietary sources is minimal during the morning period in subjects who use distilled water for the preparation of their beverages and who abstain as completely as possible from nitrate rich foods at breakfast.

#### Salivary nitrite levels

The nitrite levels in the salivas of 28 subjects, both males and females, smokers and non-smokers, have been determined at intervals on at least ten occasions during morning periods. Records have been maintained of the eating habits of volunteers and of other factors of possible concern such as drug therapy. From the results obtained, a selection of ten individuals has been made for subsequent investigations based on the following factors:

- 1 Endogenous nitrite levels in the saliva. Fig I illustrates that the subjects covered a wide range of values.
- 2 Sex and smoking. The volunteers included both males and females, smokers and non-smokers.
- 3 Production of saliva. All of the individuals chosen were able to produce reasonable volumes of saliva within a short period.

Incubation of saliva with nitrate in vitro:

Following the incubation at 37°C of samples of saliva from the ten volunteers selected with and without the addition of nitrate to a level of 1000 mg NO<sub>3</sub><sup>-</sup> l<sup>-1</sup> the formation of nitrite and the production of N-nitroso compounds as a group and S-nitrosothiols are being monitored. By the end of 1980, it was already apparent that the capacities of the salivas of volunteers to convert nitrate into nitrite cover a considerable range with at least a tenfold difference between the least and the most efficient converters. The variations in the activities of salivas expressed by an individual donor from day to day were not nearly so marked. The mean production of nitrite by the saliva of a volunteer has been approximately linear up to at least 60 minutes at 37°C. Thiocyanate has been found in all samples of saliva to date and the difference reported previously (Densen, Davidow, Bass & Jones, 1967; Walters, Dyke, Saxby & Walker, 1976) between smokers and non-smokers has been confirmed. So far, no S-nitrosothiols have been detected in salivas incubated with or without added nitrate. N-nitroso compounds have been detected from time to time at levels of hundreds of µg l<sup>-1</sup> as N-nitrosopyrrolidine in incubations both with and without added nitrate but the distribution cannot be evaluated as yet.

The formation of nitrite in the saliva leads to its release into the stomach with the potential formation of N-nitroso compounds in this organ. Thus, studies have been made of the contents of N-nitroso compounds in the fasting gastric juice of 50 normal healthy individuals and of 320 patients with disorders such as pernicious anaemia, atrophic gastritis, reflux oesophagitis, gastric ulcer, gastric cancer, etc. or having been subjected to operative procedures such as vagotomy, partial gastric resection (Bilroth I & II). Nitrite levels were monitored in the fresh gastric juices by the collaborating clinical authorities who also cultured them for total and faecal type nitrate reducing bacteria.

The data results obtained have been analysed on an ICL 2980 computer, the variates examined including age, sex, smoking habit, presence of nitrite, pH, N-nitrosamine concentration and bacteriology.

Thus, concentrations of N-nitroso compounds in fasting gastric juice rose progressively with age in both sexes both in normal controls ( $p = 0.028$ ) and in pathological conditions ( $p = 0.56 \times 10^{-6}$ ), in which the elevation of pH with age was also highly significant ( $p = 0.00087$ ). There was, however, no significant difference in levels of N-nitroso compounds between the sexes after taking account of all of the analysable factors. Similarly, cigarette smoking had no significant effect on levels of N-nitroso compounds overall although the nitrite content increased significantly ( $p = 0.047$ ).

The most significant relationship to emerge was, however, that between pH and the concentration of N-nitroso compounds in the fasting gastric juice. After taking into account the other factors and their interactions, concentrations of N-nitroso compounds as a group rose progressively with elevation of pH from a mean value of  $0.11 \mu\text{mole l}^{-1}$  at pH 1.0 - 1.5 to a mean of  $1.33 \mu\text{mole l}^{-1}$  at pH 6.5 - 9.0 ( $p < 10^{-6}$ ). This correlation was especially pronounced in several diagnostic groups including duodenal ulcer ( $p < 10^{-6}$ ), vagotomy ( $p = 0.21 \times 10^{-5}$ ), gastric ulcer ( $p = 0.0011$ ), pernicious anaemia ( $p = 0.05$ ), gastric cancer ( $p = 0.0016$ ) and oesophagitis ( $p = 0.018$ ). The overall relationship of extractable N-nitroso compounds to the pH of the fasting juice is shown in Fig. 2.

In those subjects with a low nitrite concentration (above 0.01 mM) in the fasting gastric juice, an increase in nitrite concentration was associated with a significant elevation in the concentration of N-nitroso compounds ( $p = 0.0033$ ). Similarly, in those whose nitrite concentration was higher (0.2 mM), the correlation with N-nitroso compound levels was even better ( $p = 0.00019$ ). Highly significant correlations were also demonstrated between the rise in pH and the elevation in nitrite concentration ( $p < 10^{-6}$ ), both at low and high nitrite levels.

Highly significant correlations were also demonstrated between the rise in pH and the elevation in nitrite concentration ( $p < 10^{-6}$ ), both at low and high nitrite levels. Overall, a highly significant association was observed between pH, nitrite and concentrations of N-nitroso compounds.

In terms of bacteriology, a highly significant relationship has been demonstrated between the growth of nitrate reducing organisms and elevation in N-nitroso concentration ( $p = 0.00035$ ). The presence of such organisms was associated significantly also with an increase in pH ( $p = 0.034$ ). Taking all diagnostic groups into account, it was found that nitrate reducing micro-organisms were significantly more often cultured from gastric juice in the older age groups ( $p < 10^{-6}$ ).

Studies have been commenced on the nature of the N-nitroso compounds detected as a group in biological samples. Only very small amounts of precursors to the simpler volatile N-nitrosamines are generally available; in fasting gastric juice, for instance, only about 0.2% of N-nitroso compounds formed from endogenous precursors comprised volatile N-nitrosamines, the predominant compounds of this type being N-nitrosodimethylamine and N-nitrosopyrrolidine, as determined by gas chromatography with a Thermal Energy Analyzer as detector. Much greater amounts are available in biological systems of precursors leading to more complex N-nitroso compounds which cannot be separated from a matrix by distillation and are not amenable to gas chromatography. After derivatization, however, it has been found that considerable proportions of such complex N-nitroso compounds can be separated by gas chromatography and detected using the Thermal Energy Analyzer. From fasting gastric juice, for instance, more than twenty individual peaks have been resolved on the gas chromatogram, corresponding in total to approximately 40% of the N-nitroso compounds determined by the group selective procedure of Walters, Downes, Edwards & Smith (1978). The corresponding value for the nitrosatable precursors of saliva is approximately 33%. Thus, a Thermal Energy Analyzer is being obtained to facilitate future studies along these lines and should be available in the first half of 1981.

**Conclusions and comments:**

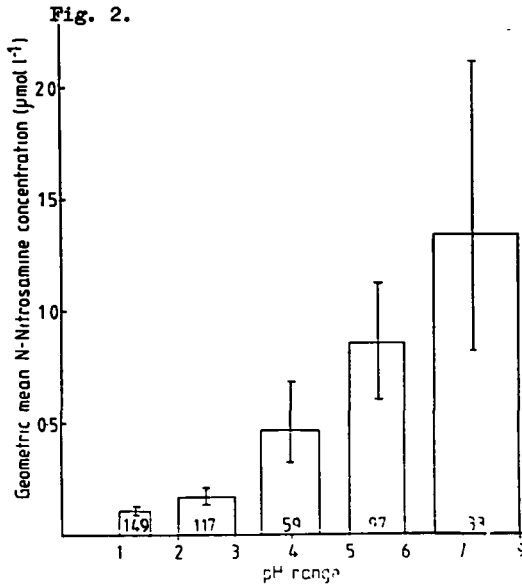
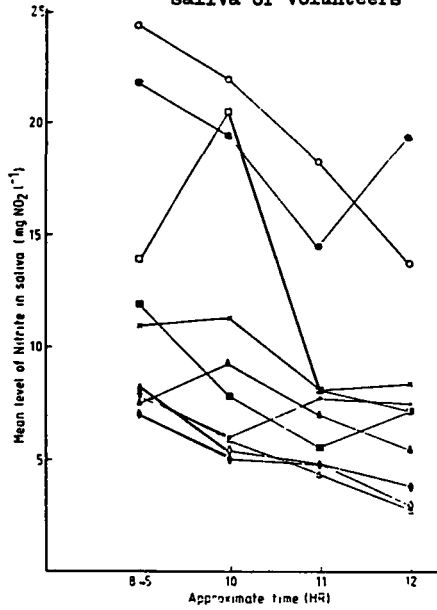
1. The levels of nitrite in the saliva of human subjects who have restricted their input of dietary nitrate overnight vary greatly from one volunteer to another. This is reflected in the considerable variations seen in the capacities of salivary samples to form nitrite from added nitrate. It is also apparent that N-nitroso compounds can be formed during such incubations but their occurrence has not yet been related to any of the other factors involved. As yet, therefore, the pathways involved in the synthesis of N-nitroso compounds in the saliva are not understood.
2. Since the formation of N-nitroso compounds in the fasting stomach is significantly associated with pH, the presence of nitrite and the availability of nitrate reducing organisms, it is presumably the nitrate ingested in water and food from which they are derived; major endogenous sources of nitrate have now been discounted (Tannenbaum, 1981). This finding is thus of considerable importance in that gastric cancer is often preceded and/or accompanied by a hypochlorhydric condition of the stomach which results in elevated counts of nitrate reducing organisms (Ruddell, Bone, Hill, Blendis & Walters, 1976).
3. The finding that the great majority of N-nitroso compounds formed from biological precursors are not simple volatile N-nitrosamines emphasizes the need for procedures for the separation, determination and characterization of the individual more complex N-nitroso compounds formed biologically. The success obtained for proportions of these compounds by gas chromatography with the Thermal Energy Analyzer as detector after their derivatization needs therefore to be extended.

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**Fig I.** Variation of nitrite concentrations in the saliva of volunteers



**Fig II** Relationship of the geometric mean of the concentration of N-nitroso compounds in the fasting gastric juice with pH range. Each mean value is accompanied by its 95% confidence limits.





Contractor : Gesellschaft für Strahlen- und Umweltfor-  
schung, Institut für Genetik, Neuherberg,  
Federal Republic of Germany

Contract No. : 136 - 77 - 1 ENV D

Project Leader : Dr. U.H. Ehling

Title of Project : Chemically-Induced Mutations in Mice

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The main scope of this project is a) the mutagenicity testing in vivo with mammals, b) the evaluation of factors affecting mutagenesis in mammals, and c) the assessment of the genetic risk to man.

#### Dominant Lethal Mutations

The results of mutagenicity testing are summarized on page 6.

In toxicology the determination of the median lethal dose (LD50) is usually the first experiment performed with a new chemical. If the lethal dose is divided into 2 or 4 equal parts applied in intervals of 24 h a pronounced reduction in mortality can be observed.

A single i.p. injection of 20 mg/kg of methyl methanesulfonate (MMS) induces in the mating interval 5-8 days postinjection 9.4% dominant lethal mutations. A fractionation of the total dose into 4 injections of 5 mg/kg of MMS given 24 h apart induces in the same interval 17.7% dominant lethal mutations. In another experiment a single i.p. injection of MMS induces 14.1% dominant lethal mutations in the mating interval 5-8 days post-treatment. The application of 4x5 mg/kg of MMS with an injection interval of 48 h induced in the same mating interval 10.2% dominant lethals. Similar results were obtained when a total dose of 40 mg/kg of MMS was fractionated. In all MMS experiments there is no indication that fractionation of the dose reduced the yield of the induced mutations.

Fosfestrol and procarbazine hydrochloride in contrast to MMS, require metabolic activation. Independent of the basic difference

between the compounds the results of the fractionation experiments lead to the same conclusion that fractionation of the dose in 4 equal parts given 24 or 48 h apart does not reduce the yield of dominant lethal mutations.

The fractionation of the dose affects the yield of mutations differently in specific locus (Ehling, Arch. Toxicol. 46, 123, 1980) and dominant lethal experiments. Several assumptions could explain these results:

1st The distinct genetic endpoints.

2nd The differences between the germ cell stages (differentiating spermatids and spermatozoa versus the dividing spermatogonia). After additional experiments it will be possible to decide which assumption is the correct one or if it is necessary to invoke an additional explanation, such as, the influence of a repair process.

Chromosome analysis and micronucleus test in mouse bone marrow

These experiments were continued with the new chemicals chosen by the Contact Group: Benzo-a-pyrene and ethylnitrosourea. Also, the experiments with mitomycin C, procarbazine hydrochloride and atrazine were completed (page 6).

For mitomycin C and procarbazine hydrochloride the doses were lowered stepwise to 0.08 and 1.56 mg/kg, respectively. The lowest positive dose in both tests for mitomycin C was 0.16 mg/kg and for procarbazine hydrochloride it was 3.12 mg/kg. The common test protocol used consisted of single treatments and preparation of the bone marrow specimen 24 h later. When the highest dose, 5 mg/kg of mitomycin C, was used in the common test protocol it produced a decline in the aberration frequency as compared to 3.75 mg/kg. However, given 30 h instead of 24 h before preparation, the aberration yield with 5.0 mg/kg was in accordance with the expected aberration rate from the linear-quadratic dose-response relationship that existed between 0.08 and 3.75 mg/kg. Therefore, it is assumed that this relatively high dose prolongs the cell cycle such that the maximum effect which reflects the majority of first post-treatment mitoses occurs at 30 h instead

of 24 h after treatment.

Atrazine had previously given a positive response in a replicate experiment with chromosome analysis. Even though it has since been extensively tested in the micronucleus test the outcome was negative. In analogy to the chromosomal test, atrazine was first suspended in corn oil and given orally at a dose of 2000 mg/kg. Erythrocyte preparations were obtained 24 h later. Since the application of a dose is rather incorrect for a suspension, the experiments were repeated using dimethylsulfoxide (DMSO) as a solvent and oral atrazine doses of 100, 500 and 1000 mg/kg. Preparation intervals were varied from 24 to 120 h. Throughout the experiment none of the mean micronucleus frequencies exceeded 0.2% and the variability between means was similar in treatment groups and the controls. For the first time, a negative result in the micronucleus test was obtained while the compound was found to be mutagenic in chromosome analysis, the dominant lethal test, the mammalian spot test and the host mediated assay with *E. coli*.

The newly selected compounds benzo-a-pyrene and ethylnitrosourea both gave a positive response in the chromosome test and the micronucleus test. The maximum aberration rate with benzo-a-pyrene was observed 30 h after treatment with oral doses of 25 to 150 mg/kg. In the micronucleus test the maximum response occurred 36 h after treatment. At the peak time of response the lowest positive dose was 25 mg/kg in both tests. The dose-response curve for micronuclei was linear and for chromosome aberrations it had a logarithmic shape. Ethyl nitrosourea has so far only been tested at 24 h after treatment. The lowest positive dose in both tests was 30 mg/kg. These experiments will be continued.

The conclusions drawn from the results of the

6 compounds tested so far can be summarized as follows:

- 1st For the in vivo cytogenetic tests, chromosome analysis and micronucleus test, it is most important to determine the time of maximum response after a single application of the test compound.
- 2nd With 2 out of 5 extensively studied chemicals the chromosome

analysis proved to be more sensitive. These chemicals were . . . . .  
methyl methanesulfonate and atrazine.

#### Transplacental effects in mouse embryos

Cytogenetic preparations were obtained from whole mouse embryos on the 12th day of gestation. The method was applied to five chemical mutagens which are known to cause chromosomal damage in adult animals. Three of the chemicals belonged to the group of compounds chosen by the Contact Group: Benzo-a-pyrene, mitomycin C and procarbazine hydrochloride. Additionally bleomycin and triethylenemelamine (TEM) were used as model mutagens because of their known effects on the DNA.

All five mutagens tested were able to cross the placenta and produced chromosomal aberrations in the post-implantation embryo. The times of maximum aberration yields depended on the cell cycle, which in 11-12 day old embryos averages 10 h, and on the chemicals' mode of action. For bleomycin the maximum occurred already 3 h after treatment, while for TEM it was observed after 18 h when most cells were in their second mitosis after treatment. There was a smaller peak also at 9 h. With mitomycin C and procarbazine hydrochloride the maxima were not very pronounced and occurred at 12 h after treatment. Similar aberration yields were obtained after 15 and 18 h after treatment with benzo-a-pyrene. The dose response-curves established at the time of maximum response were linear except for mitomycin C which showed a distinct quadratic component.

The test for transplacental cytogenetic effects, similar to the mammalian spot test, provides valuable information for possible teratogenic, carcinogenic and mutagenic effects in embryos.

#### Specific Locus Mutations

The results for mutagenicity testing of germ cell and somatic mutations are summarized on page 6.

From the point of view of genetic risk to humans the differential spermatogenic response to the induction of mutations is of great

importance. The time required for the development of spermatozoa from type A spermatogonia is about 35 days in the mouse and 72-74 days in man. The time for post-spermatogonial development of mature male gametes constitutes less than 0.7% of a human generation time. Therefore, this germ cell stage is less important than spermatogonia for the assessment of the genetic risk of chemical mutagens in man. MMS does not significantly increase the frequency of specific locus mutations in spermatogonia, however, it is a very potent inducer of specific locus mutations in spermatozoa and spermatids of mice (Ehling, Progress in Environmental Mutagenesis, 1980). Procarbazine hydrochloride induces specific locus mutations in postspermatogonia and spermatogonia, whereas ethylnitrosourea (ENU) induces specific locus mutations primarily in spermatogonia.

If spermatogonia and oocytes are equally sensitive to the induction of specific locus mutations by procarbazine hydrochloride, one would expect 5.2 mutants in 14 863 offspring after treatment of females with 1x600 mg/kg. The expected frequency is significantly different from the observed zero mutations ( $P = 0.03$ ). This result indicates that oocytes are less sensitive than spermatogonia to the induction of mutations.

The yield of gene mutations induced by chemical mutagens in mice depends on the spermatogenic stages, the sex and the different treatment conditions. The knowledge of these factors is the foundation for the assessment of the genetic hazard in man. The experimental approaches available and the suppositions for the estimation of the genetic risk in man were discussed in several publications of this project.



Summary: Mutagenicity in Mice

Compound	Test systems					
	Dominant Lethals	Heritable Translocations	Micronuclei	Bone marrow Chromosome aberrations	Somatic Mutations in vivo	Specific Locus Mutations
MMS	+	+	+	+	+	+
Procarbazine hydrochloride	+	+	+	+	+	+
Mitomycin C	+	(-)	+	+	+	+
Atrazine	+	n.t.	-	+	+	n.t.
Benzo(a)pyrene	(+)	n.t.	+	+	+	n.t.
EthylNitrosourea	+	n.t.	+	+	+	+

n.t. = not tested

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Contractor: University of Freiburg, GFR, Forstbotanisches Institut  
Contract n°: 144-77-1ENVND  
Project leader: Dr.D.Siebert  
Title of project: Further improvement of a genetic prescreening test  
pattern for carcinogenic effects of environmental  
chemicals

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### Introduction and methods

During the last years a test pattern of genetic tests with yeasts and cytogenetic methods with Chinese hamsters has been developed to obtain mutagenicity and additional pharmacokinetic data. Using selected environmental chemicals, mainly compounds of heavy metals, this pattern should be examined for its possibility to draw conclusions concerning (1.) the correlation between genetic effects at the molecular level and cytogenetic activity, (2.) between the induction of micronuclei and sister chromatid exchanges (SCE) in vivo and (3.) between mutagenic and carcinogenic effects of a given substance.

The genetic test systems were (1.) induction of suppressor mutations (reverse mutation) in the *ilv1-92* locus of a haploid *Saccharomyces cerevisiae* in the fluctuation test, (2.) induction of mitotic gene conversion in the diploid strain D4-RDII of the same yeast with the treat and plate technique, on the cytogenetic level (3.) the micronucleus test and (4.) the sister chromatid exchange test in vivo both in the bone marrow of Chinese hamsters.

### Results and discussion

#### 1. The fluctuation test

The commonly used treat and plate technique with yeasts and bacteria allows to estimate the mutagenic activity of a chemical accurate at rather high concentrations which are often much higher than in the human environment. To determine small increases over the spontaneous mutation frequency which will be expected after treatment at low concentrations the fluctuation test of Luria and Delbrück designed to measure the spontaneous mutation rate is used in mutagenicity experiments. The mutation rate itself is not estimated but the number of turbid tubes which show growth after treatment of an auxotrophic strain. Since diploid yeasts

are able to undergo meiosis during the needed treatment time of about 14 days one has to pay attention to avoid this process which would falsify the results.

In the first experiments we used the respiration and meiosis deficient strain D4-RDII in a fluctuation test. For unknown reasons it turned out to be not suitable in this test. To prevent meiosis therefore we used the haploid strain  $\alpha$ 92-5a which can be reverted at the locus *ilv1-92* by suppressor mutations. A cell titre of  $10^4$  cells/ml and a supplementation of the nutrient isoleucine of 6  $\mu\text{g/ml}$  allowed the growth of about 25% of untreated tubes - enough to apply the  $\chi^2$ -test to measure the level of significance. The application of the fluctuation test to the known mutagens EMS and hycanthone gave clear positive results at concentrations of 0.001 to 1  $\mu\text{g/ml}$  resp. 0.001 to 1  $\mu\text{g/ml}$  (Table). In the case of hycanthone this test is by a factor of  $2.5 \times 10^6$  more sensitive than the treat and plate technique using the concentration of the test agent as criterion. Because of contradictory results in carcinogenicity testing of different cadmium compounds (IARC monographs, Vol.2) cadmium acetate and sulfate were first examined of the heavy metal program but they did not show any activity. Of the chromium compounds only potassium chromium (III) sulfate was tested and was found to be active at concentrations from 0.01 to 1  $\mu\text{g/ml}$ . Cis-dichlorodiammine-Pt (II) (cis-PDD) which was active in other organisms was not able to induce reverse mutations in the fluctuation test in the concentration range from 0.0001 to 2.0  $\mu\text{g/ml}$ . This substance seems to be not stable enough during the treatment time of 14 days and shows one limitation of this system.

## 2. The treat and plate technique with the strain D4-RDII

All cadmium salts tested in the standard gene conversion test were toxic to yeasts but turned out to be genetically inactive at concentrations from 10 to 1000  $\mu\text{g/ml}$ . However, cis-PDD acted in this endpoint in the concentration range from 10 to 1000  $\mu\text{g/ml}$  as a potent mutagen with increasing factors of about 70 over the control value. The chromium (VI) compounds potassium chromate, potassium dichromate and calcium chromate all gave positive results at concentrations given in the table. In the case of potassium chromium (III) sulfate only the more sensitive fluctuation test reacted positive. A clear positive correlation to carcinogenicity data only exists in calcium chromate. For the other compounds

there is not information enough about their carcinogenic activity. To clear up the question of the correlation between mutagenicity and carcinogenicity in the case of chromium perhaps the soluble compounds are not of high relevance.

### 3. The micronucleus test

Because of its low toxicity hycanthone is suitable to act as a positive control in in vivo cytogenetic experiments. In both the micronucleus test and the sister chromatid exchange test in Chinese hamsters it was active in a concentration range from 75 to 200 mg/kg and 2.5 to 200 mg/kg, resp. (Table). The nuclear base analog BudR, necessary to get differentially stained chromatids in the SCE test, gave positive results at concentrations from 25 to 50 mg/kg in the micronucleus test. The two cadmium salts tested of which the sulfate is a known carcinogen induced micronuclei of the control level. Out of the three chromium compounds tested only potassium chromate and dichromate gave positive results. At doses of 10 to 40 mg/kg the carcinogenic calcium chromate was not able to induce micronuclei in polychromatic erythrocytes. The cytostatic platinum compound showed a strong activity in this test system.

### 4. The SCE test

In order to verify a correlation between the induction of micronuclei and SCE in vivo all substances of the micronucleus program were examined for the induction of SCE. Differences in the cytogenetic activity could be obtained with the two cadmium salts which induced SCE at one concentration (see table). The potassium chromates showed nearly the same activity like in the micronucleus test and the micronucleus negatives calcium chromate and mercury chloride gave significant increases of SCE against the control. A positive correlation between these two cytogenetic test systems again showed hycantone and the cis-platinum compound.

Out of the eight substances which were tested both in the gene conversion system (D4-RDII) and in the SCE test five compounds showed a simultaneous induction of these different effects. With the other three agents only the more sensitive SCE test gave positive results; no convertogenic compound was inactive in inducing sister chromatid exchanges. Compared to the micronucleus test the SCE test turned out to be the more sensitive one.



Table

Induction of genetic effects in *Saccharomyces cerevisiae* and cytogenetic effects in Chinese hamsters bone marrow by selected chemicals.

Substance	mutagenicity test system			
	Saccharomyces treat and plate (D4-RDII)	<i>cerevisiae</i> fluctuation test ( $\alpha$ 92-5a)	Chinese hamster micronucleus test	SCE- test
Ethyl methane sulfonate (EMS)	+	+		
	(1000 $\mu$ g/ml)	(0.001-1 $\mu$ g/ml)		
Hycanthone	+	+	+	+
	(10-100 $\mu$ g/ml)	(0.0001-2 $\mu$ g/ml)	(75-200 mg/kg)	(2.5-200 mg/kg)
5-bromodesoxy- uridine (BUdR)			+	
			(25-50 mg/kg)	
Colchicine			-	
Cadmium- acetate . 2H <sub>2</sub> O	-	-	-	+
				(2.5 mg/kg)
Cadmium sulfate . 8H <sub>2</sub> O	-	-	-	+
				(1.5 mg/kg)
Cadmium chloride . H <sub>2</sub> O	-			
Potassium chromate (VI)	+		+	+
	(500-2000 $\mu$ g/ml)		(40 mg/kg)	(10-40 mg/kg)
Potassium dichromate (VI)	+		+	+
	(100-1000 $\mu$ g/ml)		(20-50 mg/kg)	(10-40 mg/kg)
Potassium chro- mium (III)sul- fate. 12H <sub>2</sub> O	-	+		
		(0.01-1 $\mu$ g/ml)		
Calcium- chromate (VI)	+		-	+
	(500-2000 $\mu$ g/ml)			(10-40 mg/kg)
Mercury chloride(HgCl <sub>2</sub> )	-		-	+
				(1-10 mg/kg)
cis-Dichlorodi- amine-Pt (II)	+	-	+	+
	(10-100 $\mu$ g/ml)		(1-40 mg/kg)	(10 mg/kg)

In parentheses the effective dose/concentration is given. All positive effects given are significant increased over the respective control : at  $p \leq 0.01$  (Kastenbaum and Bowman for treat and plate and micronucleus test,  $\chi^2$ -test for fluctuation test and Mann-Whitney-U-test for the SCE-technique).

Contractor : Medizinische Einrichtungen der Universität  
Düsseldorf, FRG

Contract no. : 175-77-1 ENV D

Project leader : A. Basler, G. Röhrborn

Title of project: Effects of chemical mutagens in embryonic  
stages of mammals

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### Cytogenetic effects of Mitomycin C in zygotes of mice

#### Material and methods:

Female mice of the strain NMRI were treated with 0.5 ml physiological NaCl-solution (negative control), and 1.25, 2.5 or 5.0 mg Mitomycin C/kg at the preovulatory phase. The compounds were injected intraperitoneally. The females were caged with untreated males. Next morning the females were checked for vaginal plug. At 10<sup>30</sup> h zygotes of fertilized females were flushed out of the oviduct and cultured for 24 hours according to Payne and Jones (Mutat. Res. 33: 239-250, 1975). The preparation of metaphase chromosomes at the pronucleus stage (zygotes) was performed using the method of Röhrborn and Hansmann (Humangenetik 13: 184-198, 1971).

#### Results:

Structural chromosome aberrations could be analysed at the first cleavage stage (Tab.1). The number of induced chromosome aberrations was dose dependent. The frequency of metaphases with aberrations increased from 3.0% in the control group to 43.0% in animals treated with 5.0 mg Mitomycin C. The dose of 1.25 mg/kg induced only gaps, breaks and fragments, whereas exchanges and multiple aberrations were found with higher doses, only.

#### Conclusions:

In earlier experiments (Progress report 1.1.1977-31.12.1978) Mitomycin C was injected to female mice at the preovulatory phase and chromosome aberrations were analysed in unfertilized

oocytes at the metaphase II stage. The number of metaphases with aberrations was low (Tab.2), compared to the data presented here. The frequency of aberrations in metaphase II oocytes was 2.5%, whereas 43.0% of zygotes were affected, when 5.0 mg Mitomycin C was injected.

The analysis of metaphase II oocytes is an appropriate test system to analyse numerical chromosome aberrations (non-disjunction). The analysis of structural chromosome aberrations, however, might lead to fals-negative results, as demonstrated. It is known that some chemicals require a DNA replication for the maximum clastogenic effects. During oogenesis, however, there is no replicative DNA synthesis between treatment at the preovulatory stage and analysis of metaphase II oocytes. Therefore, the analysis of structural chromosome aberrations should be performed at the first cleavage stage of zygotes, following the first postmeiotic DNA replication.

Table 1: Chromosome aberrations in zygotes after treatment of female mice with Mitomycin C at the preovulatory stage

dose mg/kg	No. of females	No. of zygotes analysed	metaphases with aberrations	
			No.	%
0	43	100	3	3.0
1.25	89	100	9	9.0
2.50	93	100	37	37.0
5.00	98	100	43	43.0

Table 2: Chromosome aberrations in metaphase II oocytes.

dose mg/kg	No. of females	No. of oocytes analysed	metaphases with aberrations	
			No.	%
0	100	362	0	0
5.0	50	243	6	2.5
10.0	50	322	15	4.6
20.0	50	441	27	6.1

## Transplacental cytogenetic effects of chemical mutagens

### Material and methods:

Female and male mice were caged in the proportion 2:1. The following morning the females were examined, and those with vaginal plugs were assumed to have mated successfully.

Mitomycin C, dissolved in physiological NaCl-solution, was injected intraperitoneally at day 15 past conception. The applied doses were 1.25, 2.5, 5.0 or 10.0 mg/kg.

The females were killed either at 12 hours or 24 hours after the treatment with Mitomycin C to prepare bone marrow chromosomes of the dams and embryonic liver cells. The dams received an intraperitoneal injection of 1 mg colcemide/kg 1 hour prior to preparation. Chromosome preparations were made in the conventional way.

### Results:

In bone marrow cells of the dams, a dose-dependent linear increase of the chromosome aberration frequency was observed (Tab. 3). It is also obvious, that Mitomycin C has transplacental effects in embryonic cells (Tab.4). The maximum of metaphases with aberrations was found if chromosomes were prepared 12 hours after the injection of Mitomycin C. 12 hours later the mutagenic response was not as distinct as in bone marrow cells of the dams.

Table 3: Chromosome aberrations in bone marrow cells of mice  
24 hours after the injection of Mitomycin C

Dose mg/kg	No. of mice	No. of metaphases	Metaphases with aberrations	
			No.	%
0	8	800	1	0.1
1.25	8	800	28	3.5
2.50	8	800	91	11.4
5.00	8	800	367	45.9
10.00	8	800	702	87.8

Table 4: Chromosome aberrations in embryonic liver cells of mice, transplacentally exposed to Mitomycin C

Dose mg/kg	12 h after the application		24 h after the application	
	No. of metaphases	% metaphases with aberrations	No. of metaphases	% metaphases with aberrations
0			1000	1.9
1.25	500	8.6	1000	3.0
2.50	500	8.8	400	5.3
5.00	500	40.2	400	8.3
10.00	500	81.6	400	23.3

#### Discussion

To analyse transplacental mutagenic effects we prefer the restriction to embryonic liver cells. The embryonic liver is the tissue with the highest enzymatic activity. Late in gestation, hepatocytes which metabolize mutagens to active forms develop in the fetal liver. Therefore it is to suppose, that the highest mutagenic effects will occur in this tissue.

The different expression of chromosome aberrations in bone marrow cells of the dams and in embryonic cells depends on differences in the cell kinetics. The main expression of structural chromosome aberrations occurs if chemical mutagens reach the target cell during the penultimate cell division before chromosome preparation. Due to the short embryonic cell cycle the maximum of cytogenetic effects occurred still 12 hours after the application of Mitomycin C. Furthermore, completely fragmented chromosomes occurred in embryonic cells only 12 hours after the application. It has to be supposed that damaged cells with such severe aberrations do not reach further cell divisions. Thus these aberrations are not detectable if the preparation was performed 12 hours later, and at this time the mutagenic response was not as distinct as in the experiments before and not as distinct as in the bone marrow of the dams prepared 24 hours after the injection of Mitomycin C.

Sister chromatid exchanges in embryonic liver cells transplacentally exposed to chemical mutagens

We developed a method to demonstrate SCEs in transplacentally exposed embryos (Basler, 1979).

Material and methods:

At day 17, past conception, a 50 mg tablet of pure BrdU was implanted subcutaneously into the neck of Chinese hamsters. Two hours later, the test compound, 5 mg cyclophosphamid (CP), or 20 mg methyl methanesulfonate (MMS), was injected intraperitoneally. The females were killed 20 hours after the implantation of the BrdU tablet. The bone marrow of the dams and the liver of the embryos were transferred to centrifuge tubes, containing 5 ml culture medium (McCoy's 5A medium) and 0.5 ml of a 0.005% colcemide solution. The liver was cut into 3 pieces and a cell suspension was obtained by gently pipetting. The cells were incubated for 2 hours at 37°C. After fixation of the cells in a mixture of glacial acetic acid : ethanol = 1 : 2.5, chromosome preparations were made in the conventional way. Differential staining of chromatids was carried out with Hoechst 33258 dye and Giemsa.

Results:

The baseline SCE frequencies averaged 3.3 in bone marrow of adult hamsters and 4.1 in embryonic liver cells.

5 mg CP/kg increased these values in both tissues. The mean number of SCEs in bone marrow cells, as well as in liver cells of transplacentally exposed embryos was 8.8 per cell.

The mean number of MMS induced SCEs in bone marrow was 10.4 per cell and 11.8 per embryonic liver cell.

Discussion:

As demonstrated, the method described here may prove useful for investigating whether subtoxic doses of chemical mutagens or their metabolites pass the placental barrier and affect embryonic cells.

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Eurotox 79, 11.-13.6.1979 in Dresden
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- Basler, A.: Diaplazentare Induktion von Chromosomenaberrationen und SCEs.  
SCE-Workshop; GUM; 6.-10.10.1980 in Basel

CONTRACTOR : Association pour le Développement de la Recherche sur le Cancer (ADRC), 94800, Villejuif, France.

CONTRAT : n° 176-77-1 ENV F

PROJECT LEADER : Ivan CHOUROULINKOV

TITLE OF PROJECT : Study on the Mutagenic and Carcinogenic Effects of Environmental Chemicals using short term tests.

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- OBJECTIVE OF THE RESEARCH :

Chemical carcinogenesis and carcinogenesis in general is a multi-stage and plurifactorial phenomenon. The existence in our environment of complete carcinogens, of initiators and of promoters is actually well established. Each one has its importance in cancer development. The initiators induce cell lesions which remain in cell memory but are not sufficient to provoke cell transformation. Only after repeated intervention of the promoters, the initiated state is expressed as visible tumour.

From the pragmatic point of view, this concept implies studies concerning all potentialities : - carcinogenic, initiating and promoting. Present knowledge indicates that mutagenic effect is closely related to initiation. Consequently, the use of mutagenesis tests for detection of carcinogens and initiating effects is justified. This is not the case for the detection of promoting potential which importance should not be neglected. At the present time, the improvement of the mutagenesis and of the *in vitro* cell transformation systems take a special importance. Our objective was to study the mutagenic and the carcinogenic effects of different compounds and to look for correlation between those effects and for the validity of the methods.

- MATERIALS AND METHODS :

- Cells : V-79 established line from chinese hamster lung cells
  - Human lung diploide fibroblasts
  - Normal chinese hamster kidney cells.
  - Syrian hamster embryo cells from primary or secondary culture.
  - C3H/10T1/2 mouse cells.



- Compounds : The studied compounds are given in the table with the results.
- SCE-s test. In V-79 cells :  $1.0 \times 10^6$  cells were plated and incubated for 16 h in 10 mm dishes with 10 ml Eagle's MEM with 10 % inactivated FBS in humidified incubator at 37°C in presence of 5 % CO<sub>2</sub>. The medium is then replaced with serum free MEM medium containing the studied compound. After 1 h treatment, the BrdU (5 µg/ml) with complete medium is added to the cells for 7 hours to cover one S-phase. After that, the BrdU was replaced by complete medium for another 18 h. Then 6 µg/ml of Colcemid was added for 2 h. The cells then was collected for preparation.

With the Syrian hamster embryo cells (secondary culture), the technic was the same. The number of the seeded cells was  $2.5 \times 10^6$ /dish, the treatment 17-18 h and the BrdU was kept for 9 h.

- Induction of forward gene mutation in V-79 cells. The induction of 6-thioguanine resistant mutants was carried out according to the method of Abbondandolo et al. (Mut. Res., 46, 365-373, 1977).
  - Cell transformations essays. The transformation effect was evaluated by the foci formation in Syrian Hamster embryo cells following the technic described by Casto et al. (Cancer Res., 37, 3568-3575, 1977) and in C3H/10T1/2 mouse cells according to the method of Mondal et al. (Cancer Res., 36, 2254-2260, 1976).
- RESULTS :
- Sister chromatides exchange system : The results from table 1 indicate that compounds such as acetic, butiric, crotonic and valeric acids which are not known to be mutagen or carcinogen may increase the SCE frequency. However those compounds did not show any dose response as the mutagens and carcinogens do, and the interpretation of the results should be carefully done.

Table 1 - SCE-S and 6-Tg<sup>F</sup> induction in V-79 cells except indicated (+ = positives, dr = dose response).

Compounds	SCE-test		6-Tg <sup>F</sup> -test	
	- S9	+ S9	-S9	+ S9
Benzo (a)pyrene	- Chin. H.c.	+ dr		
	- V-79 c.	+ dr	-	+ dr
	- Human c.	+ dr		
	- Syr. H.c.	+ dr		
Mitomycine C .....	+ dr		+ dr	
Procarbazine (Natulan) .....	+ dr	+	-	-
Atrazine .....	-	-	-	-
Cis-platinum .....	+ dr		+ dr	
N-methyl-N-nitrosourea . V-79	+ dr			
<i>in vivo</i>	+ dr			
Methyl-Methane-Sulfonate .....	+ dr		+ dr	
Ethyl-methane-sulfonate .....			+ dr	
MNNG .....			+ dr	
Acetic acid .....	+ no dr		+ no dr	
Formic acid .....	-		-	
Propionic acid .....	-		-	
Butiric acid .....	+ no dr		-	
Crotonic acid .....	+ no dr		-	
n-Valeric acid .....	+ no dr		-	
Iso-Valeric acid .....	+ no dr		-	
Cig. smoke condensates (four) ..	+ dr			-
Organic water micropolluants ..	+ dr		-	

From pragmatcal point of view, the sensitivity and the quantitative dose-response of the SCE-system autorise investigations for detection and for evaluation of relative adverse cell effect in general and for indication of a possible mutagenic and/or carcinogenic potential. This system seems very appropriate for complex compounds such as condensates, extracts (air pollutant, water pollutant ...).

- Induction of forward gene mutation in V-79 cells : This system is with more value, in respect to the mutagenesis than SCE. But it is less sensitive and more laborious. The lake of metabolic capacity of the V-79 cells and the problems linked to the specie variations, types and the manner of preparation of the enzymatic systems complicate the task.

Nevertheless in association to SCE-test the results from

this system (gene mutation in mammalian cells) have the decisive position. So we should consider the studied acids (table 1) as non mutagenic. Acetic acid (neutralized with NaOH) should be analysed closely to explain the low effect in the two systems.

- Batterie test study for detection of mutagenic and carcinogenic potentials.

Epidemiological study showed that esophageal cancer incidence in China (Linxian) is in relation to the consumption of Pickled vegetable (PV). To elucidate the causality of this cancer, a batterie test studies was performed with dichloromethan PV extract and with an identified compound (Roussin red). The results are shown in table 2 and 3.

Table 2 - Study on the mutagenic and carcinogenic effect of DCM-PV-extract. (+ positive, - negative).

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<u>Mutagenesis</u>		<u>Conclusion</u>
. Ames (+ S9)	: +	} presence of indirect mutagen(s) and probably presence of carcinogen(s). The SCE seems to be induced by other compound(s)
. Tg <sup>F</sup> (V-79) (+ S9)	: +	
. SCE (V-79) (- S9)	: +	
. SCE (Syr.H.c.(- S9):	: +	
<u>Cell Transformation</u>		
. Syr. H.E.c.	: + :	presence of carcinogen(s)
. C3H/10T1/2 c.	: - :	no direct acting carcinogen(s)
. Initiated C3H/10T1/2	: + :	présence of promotor(s)

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*CONCLUSION : PV-extract contain mutagen(s) carcinogen(s) and promotor(s).*

Table 3 - Study with Roussin red (Rr).  
(+ posit. - neg).

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<u>Mutagenesis</u>		<u>Conclusion</u>
. Ames	+	
. Gene Mut.	or S9	: - Rr in not mutagen
. SCE	-	

<u>Cell Transformation</u>	
. Syr. H.c. ....	:
. C3H/10T1/2 .....	: - Rr is not carcinogen
. Initiated C3H/10T1/2 .....	: + promoting propriety
. Short term skin t .....	: + promoting propriety

---

*CONCLUSION : Roussin red seems to be one of the promoters present in PV - extract.*

These results conform the role of the PV in the development of esophageal cancer in China, and suggest a multifactoriel and a multi-stage environmental cancer in Man.

Moreover, these results show the possibility to use short term tests for study the mutagenic and carcinogenic potentials of simple and complex substances.

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Contractors: International Agency for Research on Cancer  
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69372 Lyon Cédex 2, France

Contract No.: ENV-368-79 F

Project Leaders: Dr H. Bartsch  
Dr R. Montesano

Title of Project: Prescreening of Environmental and Industrial Chemicals  
in a Series of Short-Term Tests for the Detection of  
Potential Carcinogens

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#### Objective of the research

One major goal of this activity was to contribute to a long-term programme of primary cancer prevention aimed at identifying and minimizing human exposure to environmental or industrial carcinogens or mutagens (either pure chemicals or complex mixtures) by systematic screening in a series of short-term tests. The chemicals that were considered were selected from those for which some suspicion of carcinogenicity existed, for which there was evidence of their use, production and social importance, thus implying human exposure. In view of the large number of environmental and industrial chemicals for which long-term animal testing could not be implemented, it was hoped that the outcome of testing these chemicals in a battery of short-term tests would help to select among those for which further testing in animals should be considered as priority.

Special attention was also given to factors involved in the efficiency and reproducibility of the mutagenicity tests.

#### I. BACTERIAL MUTAGENICITY TESTS

##### Materials and Methods

###### (i) Chemicals

Test compounds were selected either because of their socio-economic importance and lack of data on carcinogenicity or because of interest in studying their mechanism of action in chemical carcinogenesis or mutagenesis. Sources from which the chemicals were obtained and data on the purity of many of the chemicals are given in published articles referred to in this article and Bartsch *et al.*, 1980c.

(ii) Mutagenicity assays

*Salmonella typhimurium* strains G-46, TA1535, TA98, TA1530 and TA100 were provided by Professor B.N. Ames, Berkeley, Ca, USA. The presence of the R-factor in TA98 and TA100 strains was checked by seeding bacteria on ampicillin-containing agar; the mutability of TA1530 and TA1535 strains was confirmed using MNNG, and of TA100 with MMS, respectively; TA98 strain was checked by using 4-NQO. Three procedures for bacterial S9-mediated mutagenicity assays were used, depending on the chemical to be tested: the plate incorporation assay (method P), the gaseous exposure assay (method G) and the liquid incubation assay (method L), as described in detail elsewhere.

Results

1. Validation studies of screening tests on 180 chemicals

Results from testing a total of 180 compounds in the *Salmonella*/microsome assay and its adapted procedures have been summarized and the full data have now been published (Bartsch *et al.*, 1980a, 1980b). The following specific problems were analysed: the *predictive value* of the test; frequency distribution of chemicals (classes) according to their mutagenic activity; quantitative relationship between mutagenicity *versus* electrophilicity *versus* carcinogenicity of some selected carcinogens; chemicals which were activated into mutagens by human liver enzymes; compounds which have been tested in the presence of rodent hepatic *versus* extra-hepatic tissue fractions, and some factors involved in the efficient detection of mutagens *in vitro*, i.e., the source and concentration of liver microsomal protein required for maximal mutagenic activity. As 34 chemicals have also been tested in microsome- or cell-mediated mutagenicity assays using V79 Chinese hamster cells, an intercomparison of test results obtained in bacterial and mammalian assays has been made.

Figure 1 shows the frequency distribution and range of mutagenic activity for 101 mutagenic chemicals which were tested in plate assays. The mutagenic activity on the abscissa is expressed as the logarithm of the number of revertants in the most sensitive *Salmonella* strain per  $\mu\text{mol}$  of the test compound. The mutagenic activities for these compounds varied over a 100 million-fold range; this finding was also reported previously by other investigators, on the basis of lists of different chemicals. In the (nearly) Gaussian distribution curve, one-third of the chemicals (29/101) displayed mutagenic activity ranging from 100-1000 revertants per  $\mu\text{mol}$  of test compound; and about nine-tenths of the chemicals (89/101) showed mutagenicity varying only by four orders of magnitude ( $10$ - $10^5$  revertants per  $\mu\text{mol}$ ).

2. Some factors for the efficient detection of mutagens in the *Salmonella*/microsome test

The role of the concentration and the source of the liver postmitochondrial supernatant used as a metabolic activation system in the efficient detection of carcinogens in *in vitro* mutagenicity assays was also investigated. Results obtained in our laboratory indicated that the concentration of liver postmitochondrial supernatant required for maximum mutagenicity is affected by numerous variables, including the lipophilicity of the test compound. This phenomenon seems to be a general one and to be independent of which genetic indicator organism is used (see Part II). The amount of liver S9 from phenobarbital treated rats required to demonstrate appreciable mutagenicity of *N*-nitrosodimethylamine (a water-soluble precarcinogen) to *Salmonella* in plate assays is at least seven times higher (28% by volume of S9 in the soft agar overlay) than that for benzo(a)pyrene (a lipophilic compound), for which optimal mutagenicity is reached with about 4% of liver S9 from 3-methylcholanthrene treated rats. With concentrations as low as  $\leq 6\%$  of liver S9 (equal to about 150  $\mu$ l S9/plate, amounts normally used for routine screening of lipophilic compounds), no mutagenicity was observed for the *N*-nitroso compound. These data and those from V79 cells (see Part II) re-emphasize the fact that chemicals should be assayed for their mutagenicity in the presence of several S9 concentrations, taking into account their lipophilicity.

3. Screening of chemicals of socio-economic importance

We have used short-term assays to screen the possible mutagenic effects of chemicals that are of socio-economic importance but for which there are no adequate carcinogenicity studies in rodents. Since it has been shown that an appropriate combination of short-term assays can provide a higher level of reliability for predicting the carcinogenic potential of chemicals belonging to different classes, a new, effective antischistosomal drug, Praziquantel, was tested in a collaborative study in 14 different short-term assay procedures that have shown a predictive value for the detection of carcinogens. The absence of any genetic DNA-damaging activity of Praziquantel that was observed uniformly in this battery of tests (a) supports the assumption that the antischistosomal effectiveness of this drug is not related to mutagenic activity, and (b) has encouraged the implementation of extended clinical and field trials.



#### 4. Quantitative comparisons between mutagenicity and carcinogenic activity of some direct-acting alkylating agents

Since the mutagenicity and carcinogenic activity of chemicals can vary over a one million-fold range (this study, Figure 2), it would be extremely helpful in estimating the carcinogenic risk of chemicals or complex mixtures in the human environment (in the absence of sufficient animal data) if a quantitative relationship between carcinogenic activity and mutagenic activity *in vitro* were established; we chose direct-acting alkylating agents for comparison of their biological activities.

A comparison was attempted of the mutagenic activities in TA100 (plate assays) *versus* the carcinogenic potencies of seven alkylating agents (see legend to Figure 2) for which carcinogenicity studies permitted calculation of TD<sub>50</sub> values (the daily dose of carcinogen in mg/kg bw required to reduce by one-half the probability of animals being tumour-free when administered over a standard life time) (Figure 2).

The following conclusions may be drawn from the limited data presented in Figure 2. Most of the studies could not be used because they were inadequately described; the reporting of animal carcinogenicity data should thus be improved. The TD<sub>50</sub> values for a given compound varied considerably according to route, mode of administration and animal species, as, for example, in the case of 1,3-propane sultone. For several compounds, there was a rough proportionality between carcinogenicity in rodents and mutagenicity in *Salmonella*; but there were several exceptions, e.g., ENU, which appeared to be a potent carcinogen in rats, but which was only weakly active as a mutagen; in contrast, glycidaldehyde was much more mutagenic but only weakly carcinogenic.

It thus appears that a quantitative relationship between carcinogenesis and mutagenesis in *Salmonella* is not sufficiently established for the class of carcinogens studied to allow the confident prediction of carcinogenic potency of new compounds.

## II. MAMMALIAN MUTAGENICITY TESTS

### Materials and Methods

Mutagenesis assays were performed on V79 Chinese hamster cells using 8-azaguanine and ouabain as genetic markers. Two systems of metabolic activation, namely microsome and cell mediation, were used according to the procedure reported elsewhere (Kuroki *et al.*, 1977).

## Results

### 1. Mutagenic activity in V79 hamster cells of compounds with different lipophilic properties in the microsome-mediated assay

Using the experimental procedure of the microsome-mediated mutagenesis assay in V79 Chinese hamster cells standardized with NDMA as a model compound (Drevon *et al.*, 1977; Kuroki *et al.*, 1977), we failed to detect the mutagenic activity of benzo(a)pyrene (BP), 7,8-diol benzo(a)pyrene (7,8-diol BP), and aflatoxin B<sub>1</sub>. In these assays, V79 cells were treated with the chemicals in the presence of a S15 fraction from rat liver and the cofactors needed for the enzymic reactions. The concentration of the S15 fraction in the reaction mixture was 30%. As shown in Figure 3 when cells are treated with BP, 7,8-diol-BP or aflatoxin B<sub>1</sub>, no mutagenicity can be observed with S15 concentrations higher than 10%; however, these compounds were mutagenic to V79 cells when the S15 concentration was lowered to 1%-5%. In the case of NDMA, the mutagenic activity increased with increasing amounts of S15 fraction (up to 40%). Similar requirements for maximum mutagenicity were found with NDMA and BP in *S. typhimurium*. NDMA a hydrophilic compound, requires a high concentration of S15 for an optimum mutagenic effect, while for lipophilic benzo(a)pyrene and aflatoxin B<sub>1</sub>, optimal mutagenesis is achieved with lower concentrations of S15. The possibility that optimal concentrations of postmitochondrial fraction in the assay are related to the lipophilicity of the compounds has been investigated, using a series of *N*-nitrosamines with different lipophilicity i.e., NDMA, *N*-nitrosamorpholine, *N*-nitrosodiethylamine and *N*-nitrosodi-*n*-propylamine. A reverse relationship was found between the log ratio of the mutagenic activity of the nitrosamines with 30:2% S15 and the log of their partition coefficients for octyl alcohol (Kuroki *et al.*, 1979). These results suggest that the optimum concentration of the post-mitochondrial fraction depends in part on the lipophilicity of the chemicals tested. The lack of mutagenicity at high concentrations of S15 may also be explained, at least in the case of benzo(a)pyrene, by the rate at which the compound is metabolized (Kuroki *et al.*, 1979).

### 2. Cell- and microsome-mediated mutagenicity

Two systems of metabolic activation i.e. cell and microsome mediation are used in the *in vitro* test for mutagenicity with mammalian cells, and it is important to determine the efficiency and specificity of these metabolic activation systems in relation to the metabolic pattern observed in the intact animal. The mutagenic activities of NDMA and BP were examined using

these two different metabolic activation systems; the results are shown in Table 1 and Figure 4.

Benzo(a)pyrene was only slightly mutagenic in the presence of liver S15 fraction from methylcholanthrene-pretreated rats but was much more mutagenic in cell-mediated assays using embryo fibroblasts and the liver cell line. *N*-nitrosodimethylamine, a hepatocarcinogen was mutagenic to V79 cells only in the presence of liver S15 and primary hepatocytes but not in the presence of fibroblast or the liver-cell line. Our findings indicate that primary liver cells can metabolize NDMA into a mutagenic metabolite that interacts with the DNA of V79 cells. The rat liver-cell line and the secondary cultures of rat embryo cells do not appear to be able to carry out this metabolic activation, as indicated by the lack of mutagenic response. The pattern of mutagenic response described above, involving the same substrate but different metabolic activation systems, is probably the consequence of qualitative and quantitative differences in the metabolic capacity of the various cell types and of intact cells and S15 fraction to convert the BP and NDMA into mutagenic metabolites.

### 3. Screening for mutagenic activity of sex hormones

Drewon *et al.*, 1981, in press. In view of the extensive use of sex hormones by the human population, either as therapeutic agents, or in the composition of contraceptive pills, it is important to investigate more thoroughly the adverse biological effects of synthetic hormones. Diethylstilboestrol, ethinyl oestradiol, oestradiol-17 $\beta$  and oestrone were chosen for our experiments. Evidence of carcinogenicity in rodents has been reported for each of these compounds, but so far very few studies have been carried out with *in vitro* tests. Since it has been demonstrated that isolated liver-cells in suspension are able to metabolize efficiently steroid hormones, we have tested these chemicals in V79 cells with a cell-mediated system using primary hepatocytes from male and female rats as the metabolic layer. The incubation in the presence of the chemical to be tested was carried out for 48 h before plating the V79 cells to score for mutagenicity or toxicity. In the absence of hepatocytes all of these compounds were very toxic, but not mutagenic. The cocultivation of V79 cells with primary hepatocytes decreased the toxic effect induced by the sex hormones, except in the case of ethinyl oestradiol. However, no mutations, determined as 8-azaguanine- or ouabain-resistance, were induced under these conditions by any of the hormones tested. The lack of mutagenic activity of the hormones in our assay has been confirmed by the use of primary liver cells originated from a rat treated with Aroclor, an inducer of drug metabolizing enzymes.

CONCLUSIONS:

Results from testing a total of 180 compounds in the *Salmonella*/microsome assay and its adapted procedures are summarized. The following specific problems were analysed: the predictive value of the test; frequency distribution of chemicals according to their mutagenic activity; quantitative relationship between mutagenicity and carcinogenicity of some selected carcinogens; compounds which have been tested in the presence of rodent hepatic *versus* extra-hepatic tissue fractions, and some factors involved in the efficient detection of mutagens *in vitro*, i.e., the source and concentration of liver microsomal protein required for maximal mutagenic activity. As 34 chemicals have also been tested in microsome- or cell-mediated mutagenicity assays using V79 Chinese hamster cells, an intercomparison of test results obtained in bacterial and mammalian assays is made.

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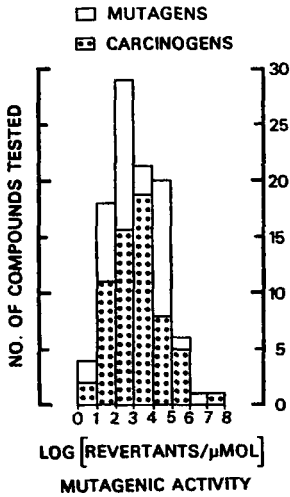


Fig. 1 - Frequency distribution of 101 chemicals tested in *Salmonella typhimurium* by plate assays according to their mutagenic activity (revertants/ $\mu$ mol) in a semi-logarithmic plot

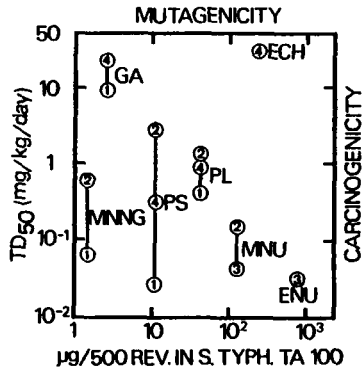


Fig. 2 - Comparison of mutagenic activity and carcinogenic potency of 7 alkylating agents. The  $TD_{50}$  values (the daily dose of carcinogen in mg/kg bw required to reduce by one-half the probability of animals being tumour-free when administered over a standard life time) were plotted (ordinate) against mutagenic activity, expressed as  $\mu$ g of compound necessary to induce 500 revertants in *S. typhimurium* TA100 in plate assays (abscissa). The numbers 1-4 in open circles refer to different types of experiments: (1) subcutaneous, repeated, rats; (2) oral, continuous rats; (3) intravenous, repeated, rats; (4) subcutaneous, repeated, mice. ENU: *N*-nitroso-*N*-ethylurea; MNU: *N*-nitroso-*N*-methylurea; MNNG: *N*-nitroso-*N*-nitro-*N*-methylguanidine; ECI: epichlorohydrin; GA: glycylaldehyde; PL:  $\beta$ -propiolactone; PS: 1,3-propane sulfone.

Table 3 Microsome- and cell-mediated mutagenesis in V79 Chinese hamster cells by *N*-nitrosodimethylamine

Metabolic activation <sup>a</sup>	Dose (nM)	No. of mutants/10 <sup>5</sup> survivors <sup>b</sup> AZA <sup>a</sup>	OUA <sup>a</sup>
<b>Microsome-mediated</b>			
Rat liver	10	33	5
PB-pretreated rat liver	10	184	30
MC-pretreated rat liver	10	62	N T
<b>Cell-mediated</b>			
Rat embryo cells	30	2	N T
Rat liver-cell line	30	8	0.4
Primary rat liver cells	13.5	71	18
None	10	2	0.5

<sup>a</sup> Microsomal fractions were obtained after centrifugation at  $15,000 \times g$  of liver homogenates from BD rats that were untreated or treated with phenobarbitone (0.1% in drinking-water for 1 week) (PB) or methylcholanthrene (40 mg/kg administered 48 hrs before the test) (MC). The rat embryo cells were secondary cultures from BD rats, the liver-cell line originated from 10-day-old BD rats and was maintained in culture for 19 weeks. The primary liver cells were obtained after collagenase perfusion of BD rats and utilized 3 hrs later.

<sup>b</sup> AZA<sup>a</sup> and OUA<sup>a</sup>, 8-azaguanine- and ouabain-resistant mutants, N.T., not tested

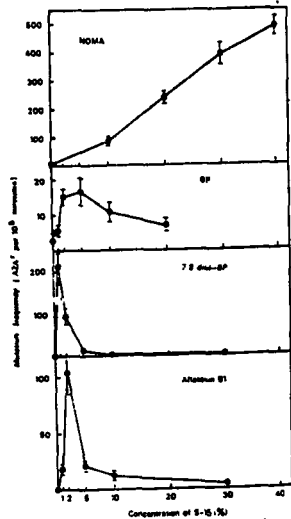


Fig. 3 Mutation frequency in V79 Chinese hamster cells (measured as AZA<sup>a</sup>) of NOMA, BP, 7,8-diol-BP and aflatoxin B<sub>1</sub>. Relationship with concentration of 15 000  $\times$  g supernatant of rat liver (S15)

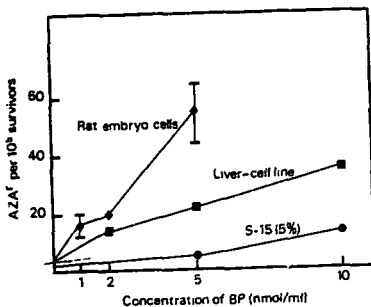


Fig. 4 MICROSOME- (●) AND CELL-MEDIATED (○,■) MUTAGENESIS IN V79 CHINESE HAMSTER CELLS BY BENZO(a)PYRENE (BP). AZA<sup>a</sup>, RESISTANCE TO 8-AZAGUANINE. S-15, 15,000  $\times$  g SUPERNATANT OF RAT LIVER

Table 1. Microsome- and cell-mediated mutagenesis in V79 Chinese hamster cells by *N*-nitrosodimethylamine

Metabolic activation <sup>a</sup>	Dose (mM)	No. of mutants/10 <sup>5</sup> survivors <sup>b</sup>	
		AZA <sup>r</sup>	OUA <sup>r</sup>
<b>Microsome-mediated</b>			
Rat liver	10	33	5
PB-pretreated rat liver	10	184	30
MC-pretreated rat liver	10	62	N.T.
<b>Cell-mediated</b>			
Rat embryo cells	30	2	N.T.
Rat liver-cell line	30	8	0.4
Primary rat liver cells	13.5	71	18
None	10	2	0.5

<sup>a</sup> Microsomal fractions were obtained after centrifugation at 15,000 x *g* of liver homogenates from BD rats that were untreated or treated with phenobarbitone (0.1% in drinking-water for 1 week) (PB) or methylcholanthrene (40 mg/kg administered 48 hrs before the test) (MC). The rat embryo cells were secondary cultures from BD rats; the liver-cell line originated from 10-day-old BD rats and was maintained in culture for 19 weeks. The primary liver cells were obtained after collagenase perfusion of BD rats and utilized 3 hrs later.

<sup>b</sup> AZA<sup>r</sup> and OUA<sup>r</sup>, 8-azaguanine- and ouabain-resistant mutants; N.T. not tested

Contractor: Istituto Superiore di Sanità, Rome, Italy.

Contract n° 177-77-1 ENV I

Project leader: A. CARERE

Other responsible of the project: G. MORPURGO and I. CAMONI

Other persons involved: D.BELLINCAMPI, R.BENIGNI, M.BIGNAMI, A.CALCAGNILE, O.CERVELLI, G.CONTI, L.CONTI, R.CREBELLI, E.DOGLIOTTI, E.FALCONE, G.GUALANDI, M.FABRIZI.

Title of project: Mutagenicity of pesticides as pure compounds and after plant metabolism

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### Objective of the research

The general purpose of the project was to analyse the genotoxic potential of environmental chemicals with special regard to pesticides by using a battery of short-term tests as wide as possible (mutation, recombination, DNA-damage repair, transformation) giving more attention to the tests at cellular level. Moreover, among the original objectives efforts were concentrated on the following points: a) to try to improve the predictive value of the A.nidulans tests; b) to test several chemicals for induction of mitotic non disjunction in A.nidulans with quantitative tests and to study their mechanism of action by analysing physiological parameters involved in non disjunction and the relationship with the mutagenic activity; c) by using "in vitro" mammalian cell cultures to analyse the genetic activity of pesticides previously tested in microorganisms.

### Materials and methods

The following mutation, recombination, DNA-damage repair and transformation tests have been so far set up:

Prokaryotic systems: induction of back-mutations (AMES test) and forward-mutations (8-AG-resistance) in S.typhimurium; induction of forward-mutations in S.coelicolor; the methodology relative to the application of the AMES test to rat and human urine concentrates was also set up.

Eukaryotic systems: induction of two types of gene mutations (8-AG-resistance and methA1 suppression) and somatic segregation (crossing-over and non disjunction) in A.nidulans; induction of gene mutations (6-thioguanine and ouabaine resistance) in Chinese Hamster cell cultures (V79); stimulation of UDS in human cell cultures (EUE, MRC-5, lymphocytes) with autoradiographic and liquid scintillation counting techniques; induct-



ion of transformation in the mouse embryo fibroblast established cell line (C3H10T1/2)(Focus assay).

### Results

#### In vitro microbial mutational studies with pesticides

A comparative mutational study on five structurally related pesticides was began during the previous programme and concluded with this project; the chemicals analysed are the following: three saturated short-chain haloalkanes-ethylene dibromide (DBE), ethylene dichloride (DCE), propylene dichloride (DCP)-used in agriculture as insecticidal fumigants, one unsaturated hydrocarbon -allyl alcohol-employed as herbicide and Sulfallate, a thio-carbamate containing in its molecule a 2-chloroallyl group and used as herbicide. The five chemicals were tested for their ability to induce gene mutations in S.typhimurium, S.coelicolor and A.nidulans. DBE was positive in all the genetic systems employed; sulfallate was also positive, at different degree, in all microorganisms; DCE was weakly active in S.typhimurium following microsomal activation; DCP was detected as direct acting mutagen in S.typhimurium and A.nidulans but not in S.coelicolor; allyl alcohol was completely negative in all test systems. The results of this study are in publication.

#### In vitro mutational studies with trifluralin and trifluorotoluene derivatives

An environmental contamination by trifluorotoluene derivatives-4-chloro-trifluorotoluene(CTT), 4-chloro-3-nitro-trifluorotoluene(NCTT) and 4-chloro-3,5-dinitro-trifluorotoluene(DNCTT) was detected in 1977 in the water bearing stratum in the area of Vicenza (North Eastern Italy); responsible of this pollution was a plant producing several fluoro-toluene derivatives among which DNCTT, an intermediate in the synthesis of dinitroaniline herbicides, such as Trifluoro-2,6-dinitro-trifluorotoluene(Trifluralin). The three pollutants as well as Trifluralin were tested for their ability to induce gene mutations in S.typhimurium (AMES test), mitotic crossing-over in A.nidulans and UDS in in vitro EUE cell cultures by autoradiography. CTT, NCTT and DNCTT failed to exert any mutagenic or recombinogenic activity; CTT and NCTT stimulated unscheduled DNA synthesis(UDS); Trifluralin turned out to be unable to induce point mutations and UDS but induced a significant increase of mitotic crossing-over in A.nidulans.

The results obtained are in publication.<sup>10</sup>

#### Mutagenicity of Trichloroethylene in S.typhimurium TA100

A Trichloroethylene(TCE) sample of technical grade was fo

und to be directly mutagenic versus S. typhimurium TA100 when tested as vapour in sealed dessiccators. Purified TCE, free of epoxides, was found to be borderline mutagenic only when tested with metabolic activation. Microsomal fractions from Arochlor induced male rats were effective in converting TCE into very weak mutagenic metabolites, while preparations from uninduced rats, mice and Hamster were not. The mutagenic activation of TCE turned out to be NADP dependent and insensitive to the addition of powerful epoxide hydase inhibitor trichloropropane, 2-3-oxide (TCPO).

These results are in publication.<sup>11</sup>

#### Growth-mediated metabolic activation of procarcinogens in A.nidulans

In order to try to improve the predictive value of A.nidulans tests, seven procarcinogens- dimethylnitrosamine, diethylnitrosamine, nitrosomorpholine, dimethylhydrazine, Natulan, cyclophosphamide and 2-aminoanthracene- were tested for their ability to induce gene mutations and mitotic crossing-over; with the only exception of 2-AA all chemicals were clearly positive when tested by using the so called "Growth-mediated assay" procedure. The apparent inability of A.nidulans to activate the aromatic amine is at the moment under test: it is not clear if it is attributable to the lack of specific enzyme activities, to the inadequacy of the genetic systems used or to the low permeability of the A.nidulans cell wall to large molecules. Obviously further work is required to assess the real activating potential of A.nidulans and our future goal will be the assay of an heterogeneous set of chemicals representative of the most important categories of promutagens and procarcinogens and by looking for optimal conditions of metabolic activation. These results are published.

#### Studies on mitotic non disjunction in A.nidulans

In the period 1979-80 we focused our attention on the process of induced non disjunction or missegregation which leads, via aneuploid condition, to newly acquired diploidy or to aploidy in A.nidulans. We preferred the permissive genetic system of homozygous segregants for the colour marker ya2 instead of the selective p-fluorophenylalanine (FPA)-resistance test previously used. In fact the first one resulted to be more sensitive<sup>4</sup> allowing the growth of the potential aneuploid segregants so that they can evolve toward a stable haploid or diploid condition; in addition the fact that FPA itself induces non disjunction and haploidization and the possible interference with inducing agents thrust us to discard the system. Both qualitative (plate test) and quantitative (liquid test) methods were used.

The relationship between damage and physiological parameters which can influence the action of the tested compounds was investigated also in relation to the potential target of such agents. The results obtained showed that DNA-damaging agents (i.e. MMS and 4-NQO) could induce point mutation and non disjunction on quiescent and germinating conidia as well; conversely benomyl, FPA, ethanol, Chlorpropham (CIPC), amphotericin B and other agents acting on the spindle during mitosis or microtubules or membranes were able to induce non disjunction only on growing spores. Membrane as potential target in mitosis of the chemicals inducing non disjunction has been also investigated. Pharmaceutical and agricultural fungicides as polienic antibiotics (Amphotericin B, nistatin and pimarinin) which interfere with membrane sterols directly in situ and chemicals inhibiting the ergosterol synthesis (fenarimol and miconazole) turned out to be highly effective in inducing non disjunctional haploid and diploid segregants. The totality of the compounds found as non disjunctional in A. nidulans and which are known to affect structures different from DNA resulted to be ineffective to increase the gene mutation induction. The possibility that some agents (i.e. damaging membrane drugs) could produce false results in virtue of an enrichment process due to a preferential killing of normal fast growing conidia with respect to the abnormal ones, was tested by using some chemicals (DMSO, phenol, SDS) which can damage in an unspecific way membrane integrity. These compounds resulted especially toxic on germinating conidia but did not produce increase in non disjunction level also when low survivals were considered.

#### DNA-damage repair studies on diallate, triallate and sulfallate

Three structurally related S-chloroallyl thiocarbamates - diallate, triallate and sulfallate - used in agriculture as herbicides, were tested for their ability to induce UDS in in vitro EUE cell cultures by autoradiography as well as by liquid scintillation counting of tritiated thymidine incorporation. The three chemicals are known to be mutagenic in Salmonella, Streptomyces and Aspergillus; they also induce mitotic crossing over in Aspergillus; furthermore, diallate and sulfallate are known to be carcinogenic. The three herbicides turned out to be able to stimulate UDS only when tested by autoradiography; negative results were obtained by using the liquid scintillation counting procedure. This method needs suppression of replicative DNA synthesis by hydroxyurea, but because S-phase cannot be completely arrested, the technique has a less degree of sensitivity. This fact can explain why rather weak DNA damaging agents such as diallate, triallate and sulfallate, gave false negative results in this test.

The results obtained have been submitted to Mutation Res. Letters for publication.<sup>12</sup>

DNA-damage repair and mutagenicity studies on dichlorvos, trichlorfon and dichloroacetaldehyde

Two organophosphorus insecticides - dichlorvos and trichlorfon - as well as dichloroacetaldehyde, one of their major metabolites, were tested for their ability to stimulate UDS in in vitro EUE cell cultures by autoradiography and to induce gene mutations (6-thioguanine and ouabaine resistance) in Chinese Hamster cell cultures (V79). The three chemicals are known to be mutagenic in various genetic systems (Ramel et al. Mutat. Res. 76, 1980, 297-309). The results obtained have shown that dichlorvos is able to induce UDS while trichlorfon is only borderline positive in this test; dichloroacetaldehyde was unable to stimulate UDS. The three chemicals resulted negative in the V79 system for induction of gene mutations. A manuscript containing these results is in preparation for publication.

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Contractor : Istituto di Ricerche Farmacologiche 'Mario Negri'

Contract N. 191-77-1 ENV-I

Project Leader : Prof. Silvio GARATTINI

Principal Investigator : Dr. Mario SALMONA

Title of project : THE CAPACITY OF LIVER AND OTHER TISSUES FROM DIFFERENT ANIMAL SPECIES TO FORM AND METABOLIZE EPOXIDES

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#### OBJECTIVE OF THE RESEARCH

The presence of cytochrome P-450 in the nuclear membrane has been stressed by several authors for its possible interest in genotoxic effects. In fact even though this metabolism is quantitatively much less important than microsomes, its close contact with nuclear DNA enhances its qualitative importance as a target site for carcinogen activation. So far there are no data available in the literature to clearly indicate that the nuclear membrane can metabolize a large spectrum of foreign compounds, comparable to microsomal preparations. In fact there are two main questions still to be answered in order to have a clear idea of the relative importance of this metabolism : (i) the qualitative and quantitative similarities between microsomes and nuclear preparations ; (ii) the nuclear membrane's real contribution in the onset of toxic effects caused by chemicals.

#### RESULTS

The first achievement obtained during our contract period was to define the relevance of microsomal contamination in the nuclear preparations. In fact a quantitative study of the level of nuclear styrene monooxygenase after artificially increasing the native microsome to nuclei ratio showed that if any microsomal contamination is present, it cannot account for more than 30% of the total enzymatic activity found in nuclear preparations.

The apparent  $K_m$  and  $V_{max}$  values of styrene monooxygenase and epoxide hydrase were determined in intact nuclear preparations from male rat liver after in vivo treatment with phenobarbital and beta-naphthoflavone, which are known to induce microsomal cytochrome P-450 and cytochrome P-448 respectively. Treatment with phenobarbital does not alter the apparent  $K_m$ , but greatly increases the  $V_{max}$  of both enzymes. Almost the same pattern is observed for styrene monooxygenase after treatment with beta-naphthoflavone, whereas the same treatment slightly increases both  $V_{max}$  and  $K_m$  values of epoxide hydrase.

The influence of different modifier (i.e. SKF-525A, metyrapone, 1,2 epoxy-3,3,3 trichloropropane, cyclohexene oxide) on nuclear epoxide hydrase was also determined. Cyclohexene oxide and 1,2-epoxy-3,3,3 trichloropropane oxide, two well known inhibitors of the microsomal epoxide hydrase caused a marked inhibition, metyrapone had a strong activating effect whereas SKF 525A had no effect. In vivo pretreatment with phenobarbital significantly induced the nuclear epoxide hydrase whereas beta-naphthoflavone caused a lower degree of induction. This pattern is quantitatively different but qualitatively very similar to the microsomal one. Moreover a toxifying to detoxifying enzymatic balance was attempted for the metabolism of the alkenic double bond of styrene, taking into account the ratio between styrene monooxygenase (toxifying enzyme) and styrene 7,8-oxide hydase (detoxifying enzyme) after the above mentioned pretreatments, both in the microsomal and nuclear fractions.

### CONCLUSIONS

Nuclear monooxygenase activity has always been measured using classical assays such as benz[a]pyrene non specific hydroxylase, aminopyrine N-demethylase, benzphetamine N-demethylase and ethylmorphine N-demethylase, but no data were so far available on styrene epoxidation. Styrene oxide is an important substrate because of its widespread use in the assay of epoxide hydrase activity. Thus the possibility of measuring simultaneously the enzymes involved in the formation and hydration of styrene oxide may be useful in comparing the toxifying and detoxifying ability of microsomes and nuclei.

LIST OF PAPERS PARTIALLY SUPPORTED BY THIS CONTRACT

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Contractor : Laboratorio di Genetica  
                  Università di Pisa

Contract no. : 267-77-1 ENV I

Project leader : Prof. N. LOPRIENO

Title of project : Investigations on the relationships between metabolic  
                  conversion and mutagenic activity of environmental  
                  chemicals with potential cancerogenic activity

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### Objective of the research

It has been demonstrated that genetic risk may be the consequence of the exposure of human population to industrial pollutants, such as vinyl chloride, chloroprene, etc. (J.K. WAGONER, 1978) : these compounds are known to produce all kind of mutagenic events in laboratory organisms and animals, such as point mutations, chromosome mutations, mitotic recombinations, etc. It is therefore relevant in mutation research to develop different experimental procedures to detect all types of genetic end points which may be produced by a chemical agent : these procedures should include all possible types of exposure protocol for investigate the potential mutagenic activity of chemico-physically different agents and all possible variables of biological factors which might influence the metabolic fate of the compounds.

By developing different short-term tests for the mutagenic evaluation of chemical pollutants, it is moreover highly important to identify quantitative criteria for a comparison of the results obtained with different test-organisms.

### Materials and methods

In vivo and in vitro mutagenicity methodologies, which have employed the yeast Schizosaccharomyces pombe as genetic indicator (a forward gene-mutational system which allows the detection of mutations induced in five genes), have been utilised to investigate : (a) the mutagenicity of two trichloroethylene (TCE) samples of pure and technical grade, and on two epoxidic stabilisers present in the TCE's technical sample, namely epichlorohydrine and 1,2-epoxybutane ; (b) the correlation of the chemical structure to the genotoxic activity of a series of alkene oxides (propylene oxide; 1-chloro-2,3-propylene oxide; 1,1,1-trichloropropylene oxide; 1,2-epoxybutane; 2,3-epoxybutane); (c) the in vivo formation of N-nitroso compounds derived from a chemical reaction of nitrous ion with secondary amine, and the influence of other diet factors.

### Results and discussion

1. in the in vitro studies the metabolic conversion system to analyse the potential biotransformation of TCE into an electrophilic compound was supplied by liver homogenate (S-9 mix) from mice and rats untreated, or pretreated with phenobarbital and/or B-naftoflavone.

In the in vitro studies two different host mediated assays, namely intraperitoneal methodologies, were performed on different mice breeds treated by oral administration.

Epichlorohydrin and epoxybutane were tested singularly or combined in a mixture with the same ratio as in the technical grade TCE sample.

Both TCE samples have produced negative results, whereas the two stabilisers have been found mutagenic above the concentrations at which they were present in the TCE's technical grade sample.

On the basis of the experimental and literature data it has been assumed that TCE can be converted into a mutagenic metabolite, which is, however, not available as an active reactive for the genetic target sites.

2. A comparison of the mutagenic power of the epoxides under analysis has provided the following specific mutagenic activity :

1-chloro-2,3-propylene oxide	0.238/10 <sup>4</sup> /mM/h/gene
propylene oxide; 1,2-epoxybutane	0.039 " " " "
1,1,1-trichloropropylene oxide	0.036 " " " "
2,3-epoxybutane	0.001 " " " "
cyclohexeneoxide	0.0002 " " " "

Mutagenicity of epoxides has resulted to be related to their electrophilicity and their reactivity towards nucleophilic sites of cellular macromolecules.

3. By combining a mutagenicity test procedures with yeast cells inoculated into the blood system of mice and incubated in the liver for various times (minutes or hours) a model methodology has been devised which allows the detection of the formation of N-dimethylnitrosamine (NDMA) at a level lower than 1 mg/kg. This methodology has been examined for its use in the study of inhibitors of the nitrosation, such as ascorbic acid and tannic acid.

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Contractor : Istituto di Genetica, Università, Milano

Contract n. : ENV/345/I

Project leader : G.E.Magni

Title of the project    CHANGES IN CHROMOSOME NUMBER DURING MEIOSIS  
                              IN Saccharomyces cerevisiae  
                              S.Sora,G.Lucchini,G.E.Magni

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#### OBJECTIVE OF THE RESEARCH

The analysis of variations in chromosome number during meiosis is important not only for a basic understanding of the meiotic process but also for environmental mutagenesis.

It is recognized as mandatory to obtain informations about two types of abnormal meiotic events : nondisjunction of one or few chromosomes (proper non-disjunction) and gamete diploidization, i.e. the failure of complete sets of chromosomes to separate.

The aims of this research were : 1) to set up fast procedures for investigating meiotic non-disjunction and diploidization of meiotic products in Saccharomyces cerevisiae, and 2) to obtain detailed quantitative informations about the occurrence of the two phenomena and their timing during the meiotic process.

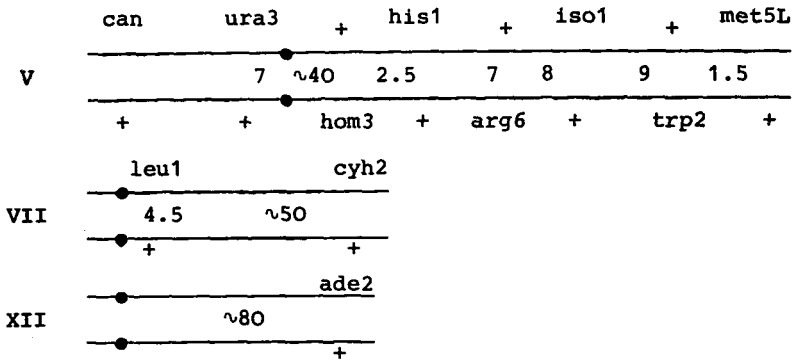
#### MATERIAL AND METHODS

The major requisites for a proper analysis of non-disjunction were : a) the possibility of proving by genetic analysis that the proposed non disjunctants are really  $n+1$  clones; b) the possibility of distinguishing between I and II division non-disjunctions. Our investigation was limited to the non-disjunction of chromosome V.

The isolation of diploid clones on selective media was not considered a reliable proof that diploidisation of meiotic products had occurred, since some unsporulated cells still persist in the spore suspension and, at least in theory, copulation between haploid spores could occur on selective media.

The strain : a diploid strain DIS13 has been constructed with the following genotype :

Chromosome



The procedure is based on the selection of clones derived from spores showing resistance to cycloheximide and prototrophy for the markers of chromosome V.

It consists of: a) sporulation of the diploid strain carried on for 10 days in order to measure the maximal sporulation frequency even after chemical treatment, b) ascus wall lysis and sonication of necked spore; this procedure give rise to a spore suspension containing, less than 1% of surviving unsporulated cells even when the starting sporulated culture contained up to 50% of cells; c) seeding on the selective medium containing cycloheximide, uracil, leucine and adenine.

It is expected that on this medium growing colonies are :

- a) aneuploids diplo-V and at least haploid for chromosome VII.
- b) diploid clones.

RESULTS

1. Control experiments

The genetic analysis of a large number of events occurring during normal untreated meioses allowed the following conclusions :

1. Selected colonies can be grouped in two classes : copulating (C) and non-copulating (NC) clones.
2. "C" colonies constitute about 80% of the entire selected population. On a sample of 30 of them, analyzed by tetrad analysis, it was demonstrated that all such colonies are haplo-III, VII and XII and diplo-V. This means that they derive from a non-dis-

junction event of the V, although not excluding that same other chromosome could be in a diploid state too. In any case the results seem quite satisfactory for the diagnosis of non-disjunction of chromosome V.

3. "NC" colonies were all able to sporulate and a good number of them were genetically analyzed proving that they are fully diploid. Proper experiments, which will not be reported here, exclude that among "NC" clones there is any one derived from unsporulated cells or from copulation on the selective medium. Our conclusion is that "NC" colonies derive from diploid spores.
4. I vs II division non disjunction. The presence of marker *ura3* in hetherozygous state on the V chromosome allows a distinction between non-disjunctions (n.d.) occurring during the I or the II meiotic division. In fact, assuming a constant rate of exchanges (about 14%) in the region *ura3*-centromere, I division n.d. will yield 3.5% of *ura -/-* while II division n.d. will produce 43% of uracil dependence. It is obvious that, after treatment with chemicals, this estimate could be affected by variations in X-over frequency. This has been taken into account by means of the estimate of exchanges in regions hom-his on the same sample of spores.

Experiments repeated several times on untreated meioses have shown a very low variability among each other. The frequencies of the investigated events (7 experiments) and their variability are reported in the following table.

Spontaneous frequencies of numerical chromosome changes

Aneuploids diplo V		Spore diploidization	
Frequency $\times 10^4$	I division %	II division %	Frequency $\times 10^4$
0.95 $\pm$ 0.12	66.3	33.7	0.54 $\pm$ 0.068

## 2. Treatment with chemicals

Rather complete data were sofar obtained treating sporulating cultures with : Benomyl, MMS, Epichlorohydrin and Cyclophosphamide. Treatment in each case was started at the time the cells were trans

ferred into sporulation medium and continued for the entire sporulation process.

a) Benomyl

The commercial composition called Benlate was used, which contains about 50% of Benomyl. Data reported in Fig.1 are referred to Benlate and consequently the doses of the abscissa must be divided by two.

It appears quite clearly that Benomyl is much more efficient in inducing aneuploidy than diploidization.

Among aneuploids diplo V it was also observed an increase of II division events with the dose as it appears from the following table.

Benlate ug/ml	II Division aneuploids %
0	.33
20	.32
30	.48
40	.55

b) MMS, shows an effect much stronger on spore diploidization than on single chromosome non-disjunction (Fig.2)

c) Epychlorohydrin causes practically no numerical chromosome changes even at doses which inhibit 74% of meioses. Data are reported in the following table.

EPICHLOROHYDRIN			
Dose mM	Sporulation Inhibition %	Aneuploids <sub>4</sub> diplo-Vx10 <sup>4</sup>	Diploids <sub>4</sub> x10 <sup>4</sup>
0	0	.96	.60
.25	0	1.44	.53
0.5	0	.80	.70
1.0	6.3	1.02	.25
1.5	52	1.54	.65
2.0	74	1.07	1.17

d) Cycloposphamide has an effect qualitatively comparable with that of MMS, (see following table) i.d. a much more significant increase of 2n spores than of n+1 meiotic products. It has to be mentioned that no external metabolic activation was performed during this treatment.

CYCLOPHOSPHAMIDE

Dose ug/ml	Sporulation inhibition %	Numerical changes x 10 <sup>4</sup>		
		Total	2n	n+1
0	0	1.6	0.7	0.9
250	20	3.2		
1000	29	4.9		
1250	30	4.8	3.44	1.41
2500	32	15.2	13.30	1.91
4000	41	23.0	20.10	2.90
5000	42	29.00		

CONCLUSIONS

As a general conclusion we believe that our method is suitable for analyzing from a quantitative point of view meiotic chromosomal numerical changes either spontaneous or induced.

The procedure is now refined enough to allow a clear distinction between true non-disjunction and diploidization and a discrimination between I and II division events.

The analysis of the first four chemoclasts seems to indicate that their effect on numerical changes is not necessarily correlated with their mutagenicity. This is indicated by the good results obtained with Cyclophosphamide (a poor mutagen in yeast) and the lack of effect demonstrated by Epychlorohydrin which on the other hand is a very powerful mutagen in many yeast systems.

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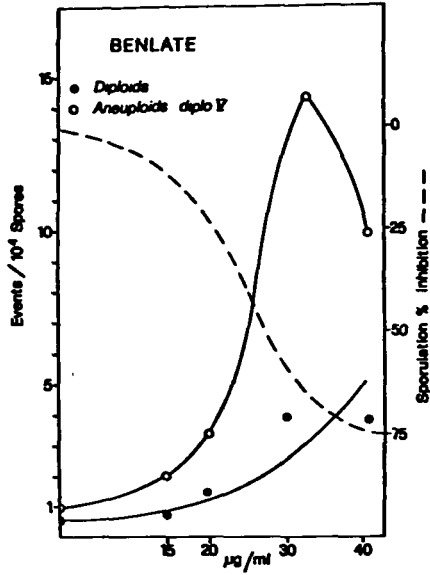


Fig. 1

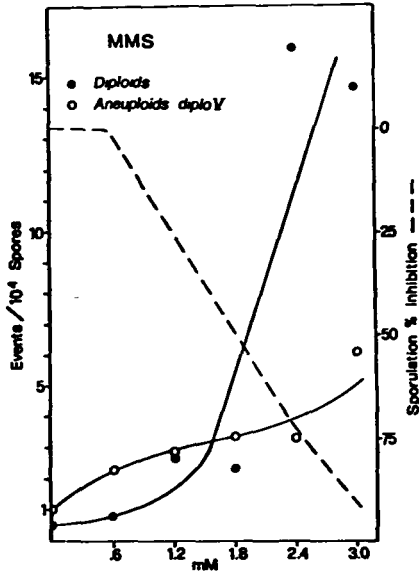


Fig. 2



Contractor : Università degli Studi di Pavia (Istituto di Genetica)

Contract n° ENV/346 I

Project Leader : G. Mazza

A bacterial test system using repair deficient strains of Bacillus subtilis:  
A quantitative assay for DNA damaging agents

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### Objective of the research

Recombination and repair deficient mutants in bacteria are more sensitive, as compared to wild-type cells, to the killing action of DNA damaging agents. Based on this relation, a simple assay for screening chemical mutagens of DNA-damaging type was devised in Salmonella, in Escherichia coli and in Bacillus subtilis. Among the repair tests, the most extensively used is that involving B. subtilis rec mutants, named "rec-assay" (Kada et al., 1972 Mut. res. 16, 165-174). Substances showing increased lethal effect on rec mutants over parental cells may damage cellular DNA and generally turn out to be mutagens.

B. subtilis mutants deficient in recombination and/or repair functions (rec, pol, uvr) have been isolated in several laboratories. These mutations have been generally obtained in different parental strains by treatment with NGT, an agent known to produce multiple linked mutations.

The objective of the research was the identification of the mutation/s conferring to the cells of B. subtilis the widest sensitivity to DNA damaging agents in the "rec-assay" test, without interference of unknown mutations affecting, e. g. growth rate, sporulation and cell permeability, and to compare different "rec-assay" procedures for the measurement of the DNA damaging activity of chemicals.

### Materials and Methods

Bacterial strains: The origin and the genotype of the B. subtilis strains, used in this work are listed in Table 1. The construction of isogenic strains was performed by DNA congression in transformation.

Media: Difco Nutrient Broth (NB) added with 0.5% glucose (NBG) was used for overnight cultures. Plates containing 25 ml of Nutrient Agar (NA) (2%

Table 1. List of *E. subtilis* strains used

Strain	Genotype	Origin of <u>rec/pol/uvr</u> marker
PB 1652	<u>trpC2 metB10 lys-3</u>	BR 151 F, Young
PB 1795	<u>trpC2 metB10 recA1</u>	GSY 1025 C, Anagnostopoulos
PB 1796	<u>trpC2 metB10 recB2</u>	GSY 1028 C, Anagnostopoulos
PB 1797	<u>trpC2 metB10 recD41</u>	G. Mazza
PB 1791	<u>trpC2 metB10 recE4</u>	T. A. Trautner
PB 1798	<u>trpC2 metB10 recF33</u>	G. Mazza
PB 1799	<u>trpC2 metB10 recG40</u>	G. Mazza
PB 1800	<u>trpC2 metB10 recH342</u>	A. A. Prozorov
PB 1792	<u>trpC2 metB10 rec-45</u>	NIG-45 T, Kada
PB 1801	<u>trpC2 metB10 polA42</u>	G. Mazza
PB 1802	<u>trpC2 metB10 uvrA1</u>	GSY 1027 C, Anagnostopoulos
PB 1803	<u>trpC2 metB10 uvrB109</u>	N. Munakata

lys, met, trp indicate respectively requirement for lysine; methionine, tryptophan; rec, deficiency in recombination; polA, deficiency in DNA polymerase I; uvr, sensitivity to ultraviolet radiation.

Agar) were used for the different "rec-assay" procedures. Soft agar (2 parts of NB and 1 part of NA) was maintained at 45°C.

Streaks assay : Cultures of the tester strains, grown overnight in NBG at 37°C with shaking, were streaked with a sterile toothpick or with an 0.1 ml pipette on dried NA plates. Sterile paper disks were placed on the streaks and soaked with a solution of the chemical to be tested (max 40 µl). Plates were incubated for 24 hrs at 37°C and growth inhibition measured.

Inhibition halo assay : 0.1 ml of an overnight culture in NBG medium of the tester strains, grown at 37°C with shaking, were added to 2 ml soft agar tubes maintained at 45°C. The tubes were mixed and the soft agar distributed onto the surface of a well dried NA plate. When the soft agar was solidified, a sterile paper disk was placed in the center of the plate. Increasing concentrations of the chemical to be tested were added to the paper disk in a volume of 10-40 µl. The plates were then incubated at 37°C for 12-20 hrs and the inhibition halo measured. The DNA damaging activity is calculated by the ratio of the diameter of inhibition measured on rec and parental strains.

Fractional survival assay : Tubes containing 2 ml of soft agar, maintained at 45°C, were additioned with, in the order; increasing concentrations of the chemical to be tested (usually in 0.1 ml or less), 0.1 ml of a  $5 \times 10^{-6}$  dilution of an overnight culture in NBG of the tester strain. The contents of each tube was distributed onto the surface of an agar plate. Plates were incubated at 37°C for 12-24 hrs.

## Results

To identify the mutations affecting recombination and/or repair functions, able to confer to B. subtilis cells the widest sensitivity to DNA damaging chemicals in the "rec-assay" test, an isogenic set of strains was constructed by DNA congression in transformation. The inhibition halo test was used to determine the DNA damaging activity of several known active chemicals on the isogenic strains.

Strains PB 1791 (recE4) and PB 1792 (rec-45) share the highest and widest sensitivity to the different agents considered. All the isogenic strains display the same sensitivity to antibiotics as the parental one. PB 1652 (parental) and PB 1791 (recE4) were used as tester strains to determine the optimal conditions for "rec-assay" procedures (see Materials and Methods).

The original procedure for the DNA damaging test, as devised by Kada et al. (1972, Mut. Res. 16, 165-174) is a disk diffusion procedure in which the differential sensitivity is measured on NA plates, by radial streaks to a centered sensitivity disk containing the chemical. Due to non homogeneous cell distribution along the streaks, this test offers only a quantitative measure of the DNA damage.

The measurement of the inhibition halo produced on a lawn of cells distributed by the soft agar technique (see Materials and Methods) allows a more accurate determination of the activity of DNA damaging chemicals. The results obtained with several DNA damaging agents are reported in Table 2; a ratio of 1-1.1, generally observed with common antibiotics, is indicative of the absence of DNA damaging activity. Also for this assay the major disadvantage is that the inhibition zone depends upon the diffusion of the chemical from the disk in the agar. Charge, molecular weight, solubility, may affect diffusion of the chemical and produce a false negative result.

A quantitative measurement of the DNA damaging activity of a chemical in the "rec-assay" can be obtained by the determination of the efficiency of plating in the presence of increasing drug concentrations (fractional survival assay). This test does not suffer from a limited diffusion rate since chemicals are directly mixed in the top agar (see Materials and Methods) with bacteria.

Table 2. Inhibition halo produced by known DNA damaging agents on PB 1652 (parental) and PB 1791 (recE) strains

Agent	Amount /disk	Diameter of zones of inhibition		Relative activity <u>rec</u> / <u>rec</u> <sup>+</sup>
		PB 1652	PB 1791	
MMS	800-1000 µg	11-13	43-45	3.9-3.46
EMS	6-12 mg	10-13	28-31	2.8-2.4
MC	0.12-0.25 µg	11-14	20-22	1.8-1.6
MEV	5-10 µg	12-15	32-35	2.6-2.3
4 NQO	10-20 µg	10-11	19-20	1.9-1.8
NTG	20-40 µg	10-12	25-28	2.5-2.3
Nitrofurantoin	100-200 µg	10-13	21-24	2.1-1.8
Epichlorohydrin	4-8 mg	0-0	b	
Nitrofurazone	50-100 µg	18-22	29-32	1.61-1.45
Ethylene bromohydrin	20-30 µl	10-13	20-24	2.0-1.84
4-Nitroso-morpholin	2-4 mg	11-17	19-25	1.73-1.47
Neocarzinostatin	2.5 µg	12	21	1.75
Adriamycin	5-10 µg	11-12	19-22	1.73-1.83
Phleomycin	1-2.5 µg	13-15	20-21	1.54-1.4
ICR 191	125 µg	10	15	1.5
Hycanthone	200-400 µg	11-12	15-18	1.36-1.5
Kanamycin	30 µg	15	16	1.06
Novobiocin	30 µg	20	21	1.05
Tetracycline	30 µg	25	16	1.06
Chloramphenicol	30 µg	21	22	1.04
DMSO	40 µl	0	0	

- a) Inhibition measured after direct incubation at 37°C for 12 hrs.  
 b) Complete inhibition of growth.

### Conclusions

The Bacillus subtilis "rec-assay" test, was studied in detail, using an isogenic set of strains carrying different mutations in repair and/or recombination functions. recE4 and rec-45 mutations turned out to confer to the cells the highest sensitivity to known mutagens.

A strong limitation of the "rec-assay" is the lack of a protocol for efficient metabolic activation of premutagens other than nitrocompounds.

Although the "rec-assay" is not a mutation assay and is not applicable to all kinds of chemical mutagens, it is very useful, in addition to mutagenic assays, for preliminary screening programmes.

As compared to other microbial repairs tests with E.coli or Salmonella, the "rec-assay" offers several advantages: increased permeability of the cells to various chemicals due to the gram<sup>+</sup> organization of envelopes, easy culture maintenance of the strains and the possibility to use purified spores instead of vegetative cells. The applicability of the "rec-assay" does not suffer from the limitation imposed on other microbial assay by strong bactericidal compounds and may provide good evidence for DNA interaction and damage.

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Contractor : Association EC - University of Leiden

Contract no.: 139-77-1 ENV N

Scientific Director : Prof.Dr. F.H. Sobels

Title of project : Comparative studies on the induction of genetic damage  
by chemical mutagens in Drosophila, mammals and  
mammalian cells in culture

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I. STUDIES ON THE INDUCTION OF GENETIC DAMAGE BY CHEMICAL MUTAGENS IN  
DROSOPHILA

Scientists: Prof.Dr. E.W. Vogel (Project Leader)  
W.G.H. Bligjeven; M.Sc.  
Dr. P.M. Klapwijk  
J.A. Zijlstra; M.Sc.

A. Objective of the research

The major objective is to use the Drosophila system for an integrated analysis of both biochemical and genetical parameters relevant for the expression of genetic alterations produced by promutagens in higher eukaryotic systems.

B. Methods and approach

This project involves: (i) the analysis of the various components of drug-metabolizing enzymes in Drosophila; (ii) the identification of strain-dependent variations in response to promutagens (procarcinogens); (iii) the investigation of the ability of Drosophila homogenates to activate promutagens into ultimate mutagens, using the Salmonella tester strains TA 98 and TA 100 as the genetic detection system; and (iv) the screening of both promutagens and directly acting mutagens for the induction of genetic damage in the germ line and of mutation and recombination in somatic cells.

C. Results and conclusion

1. Benzo(a)pyrene hydroxylation constitutes a standard reaction used for measuring the activity of the cytochrome P-450 system. When either males or females were pretreated with phenobarbital, a 7-fold enhancement in the specific benzo(a)pyrene hydroxylating capacity was found in both sexes. Enzyme induction was about equal in various parts of the body except the testis, where it was lower. This observation is in keeping with the failure

of attempts to enhance by phenobarbital the mutagenic activity of aromatic amines and polycyclic hydrocarbons in germ cells.

2. Analysis of the drug-metabolizing enzymes in *Drosophila* larvae as well as in adult flies revealed that the spectral and enzymic features of the larvae cytochrome differ considerably from that present in adult flies, i.e. in the peaks of the cytochrome P-450 and in its content per gr. body weight. A difference between larvae and adult flies was also observed in paranitroanisole demethylation, whereas this was not the case for benzo(a)-pyrene hydroxylation.

3. It is only recently that studies have been initiated to explore genotype-dependent variations in mutagenic response to procarcinogens. Distinct strain differences in dosage-mortality characteristics and mutation induction were found when two *Drosophila* populations, one resistant and the other susceptible to several insecticides, were examined for their response to several nitrosamines and dialkylaryltriazenes. Evidence for the existence of pronounced strain-dependent variations in mutagenic response was also obtained for the polycyclic hydrocarbon 7,12-DMBA.

4. Much attention has been drawn to the ability of *Drosophila* to activate carcinogenic aromatic amines and polycyclic hydrocarbons. Usage of special fat emulsions for injection of adult males and well-defined brood-fractionation experiments after treatment of larvae enhance the yield of mutations. As a result of this improved protocol, 3-methylcholanthrene, 2-acetylaminofluorene, 9,10-dimethylanthracene, 7,12-dimethylbenzanthracene and benzo(a)-pyrene were mutagenic in the recessive lethal test.

Several of these promutagens (2-acetylaminofluorene; benzo(a)pyrene and 9,10-dimethylanthracene) have also been shown to be mutagenic to *Salmonella* TA 98 and TA 100, depending on the activation by *Drosophila* enzymes. Several alterations were made in the original test protocol (e.g. the plates are incubated at 25° C instead of 37° C).

5. Somatic assay systems seem valuable as a complement to the recessive lethal test on germ-line cells for several reasons: they may cover a different level of genetic organization and DNA-repair and can be conducted in one generation. Five procarcinogens, 3-MC, AAF, DA, DMBA and BP, were tested for the induction of somatic mutation and recombination in larvae, and all five were active in this test.

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II. STUDIES ON INDUCTION OF CHROMOSOMAL ABERRATIONS IN MAMMALIAN GERM CELLS AND SOMATIC CELLS BY CHEMICAL MUTAGENS

Scientists: Dr. A.D. Tates (Project Leader)

Prof. Dr. A.T. Natarajan

A. Objective of the research

The prime objective of the study with mammalian cells in vivo and in vitro is to correlate the production of chromosomal aberrations by chemical agents with specific DNA lesions.

B. Methods and approach

This project involves: (i) in vivo studies on time-dependent repair of chromosomal damage induced by chemical mutagens in hepatocytes and lymphocytes of rats; (ii) measurement of in vivo repair of chemically induced DNA lesions in rat liver with biochemical methods; (iii) comparison of sensitivities of in vitro techniques for measuring SCE's and conventional chromosomal aberrations as quick indicators for clastogenic effects of chemicals; (iv) development of new techniques for measuring chromosomal damage in mammalian cells.

C. Results and conclusions

1. An in vivo method has been developed for detecting chromosomal damage (measured as micronuclei) in hepatocytes isolated from partially hepatectomized rats. Experiments with diethylnitrosamine (DEN) and dimethylnitrosamine (DMN) clearly showed that the method is especially suitable for detection of clastogenic effects of compounds, or their metabolites, that are too short-lived to reach classical target cells (bone-marrow, lymphocytes) in cytogenetic studies. In these experiments chemicals were given after partial hepatectomy but before the first wave of DNA synthesis. Results of another series of experiments showed that the same compounds were also positive when animals were treated at different time intervals prior to hepatectomy. An advantage of the latter procedure is that chemicals are administered to hepatocytes that normally undergo little or no cell proliferation (analogous to liquid holding systems for lower organisms and mammalian cells in vitro, or the sperm storage system in *Drosophila*). This makes the test system suitable for in vivo studies of: (i) storage effects; (ii) time-dependent repair of DNA lesions resulting in chromosomal damage, and (iii) effects of chronic exposure to chemicals.

Using this system evidence was obtained for a storage effect following treatment with Mitomycin C. However, this effect was of a transient nature to the extent that an initial increase of chromosomal damage was later followed by a decrease and a return to the control frequency of chromosomal damage. Furthermore studies were made of time-dependent induction of chromosomal damage in hepatocytes in relation to alkylation damage of DNA caused by DEN, DMN and EMS administered at time intervals of 1, 6, 28 and 56 days prior to hepatectomy. At all time intervals after DEN injection, frequencies of micronuclei (MN) were significantly increased and remained constant. Following DMN treatment MN frequency dropped to control level between days 28 and 56 whereas EMS was inactive at all time intervals tested. Results of parallel biochemical studies showed different extents of alkylation at the 7-position of guanine: DMN > EMS > DEN, and at the O<sup>6</sup>-position of guanine: DMN > DEN > EMS. These findings and others on other alkylated bases seem to exclude N-alkylation of DNA-bases as an important event in micronucleus formation. DNA of treated hepatocytes was also assayed for alkylphosphotriesters by means of alkaline sucrose gradient centrifugation. The data point to the possible involvement of alkylphosphotriesters in micronucleus formation, but it remains possible that other long-lived DNA-adducts (e.g., O<sup>2</sup>- or O<sup>4</sup>-alkylthymidine), which have not yet been investigated, are responsible for the observed cytogenetic damage.

2. Studies on the nature of Mitomycin C induced chromatid aberrations in mice revealed that chromatid breaks predominated as the major class of aberrations in bone-marrow cells, whereas symmetrical chromatid exchanges predominated in spermatogonial cells. The exchanges were mainly localised in centromeric heterochromatic regions. Further analysis of the exchange events in these regions with the Giemsa banding technique showed that 85% of the exchanges were between non-homologous chromosomes. This finding stands in sharp contrast with observations on *Vicia faba* root tips and human peripheral lymphocytes which showed that exchanges occurred mainly between homologous chromosomes.

3. It has been reported in the literature that the technique for detecting SCE's is more sensitive for demonstrating clastogenic chemicals than that for measuring conventional chromosomal aberrations (CA). We believe that such a conclusion may not be valid because it is based on comparisons of frequencies of SCE's and CA's in M2 cells. In our laboratory a new method has been worked out to compare frequencies of SCE's and CA's in the same population

of treated cells. Using Mitomycin C as model compound we compared frequencies of SCE's and CA's obtained with the "old" and "new" method. Results with the "new", and more reliable, method indicated the SCE's and CA's are equally sensitive indicators for the clastogenic effects of MMC. This observation stands in contrast with results of the "old" method which revealed that SCE's are more sensitive indicators for chromosomal damage than CA's.

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III. STUDIES ON THE INDUCTION OF MUTATIONS BY CHEMICAL MUTAGENS IN CULTURED MAMMALIAN CELLS

Scientists: Dr. J.W.I.M. Simons (Project Leader)  
M. Cupido; M.Sc.  
A.G.A.C. Knaap; M.Sc.  
A.J. de Kok; M.Sc.

A. Objective of the research

The research is directed towards the use of cultured mammalian cells for the assessment and the analysis of the mutational damage induced by mutagens and carcinogens and for the study of cell-transformation.

B. Methods and approach

The approach is related to: (1) the correlation between frequencies of different biological endpoints, induced by agents with different reaction patterns with the DNA; (2) the extension of the number of biological endpoints which can be measured e.g. additional markers, cell transformation, reverse mutation and discrimination between deletions and base changes as cause of observed mutations; (3) the possible synergistic effects of non mutagenic agents on the frequency of induced mutations; (4) the screening of directly acting and indirectly acting agents for the induction of genetic damage in mammalian cells.

C. Results and conclusions

1. Five monofunctional alkylating agents (MMS, DMS, EMS, MNU and ENU) were compared in their induction of different biological endpoints. In collaboration with Dr. Natarajan the induction of (a) cell killing, (b) mutations, (c) SCE's, and (d) chromosome aberrations have been measured. These four biological endpoints were determined simultaneously in V-79 Chinese hamster cells. Subsequently the damage was compared with the chemical reaction pattern of the compounds. As parameter for this reaction pattern the Swain-Scott  $s$  factor was used, which expresses the dependence of the reaction rate on the nucleophilicity of the receptor. Alkylating agents with a low  $s$  value will produce a relatively high ratio of  $O_6 : N_7$  alkylation of guanine and are supposed to be very effective in the induction of gene mutations. No correlation of the  $s$ -factor was found with the concentration of the agents which is needed for 90% killing of the cells nor with the absolute numbers of chromosome breaks, SCE's or mutations. A significant correlation was found with the ratio's of

mutations over SCE's and mutations over chromosome breaks: with decreasing s-factor relatively more mutations are induced than chromosome breaks or SCE's. This indicates that the alkylations, which lead to SCE differ from those, which lead to point mutations and is in agreement with the notion that O<sup>6</sup>-alkylation of guanine is the adduct which is primarily responsible for point mutations.

2. The development of an assay system for the simultaneous determination of the induction of mutations and cell-transformation is pursued with the aim to study the correlation between DNA-adducts, mutation and carcinogenesis. Primary Syrian hamster embryo cells are used for morphological transformation and BHK 21/13 cells for growth in soft agar. Conditions are being sorted out for the quantitative determination of cell-transformation and for mutation at the HPRT and Ouabain-resistance loci.

3. Experiments have been performed with ethionine, which is a potent liver carcinogen but which could not be shown to be mutagenic in a variety of test systems. Hypothetically ethionine could be carcinogenic by an indirect action as it has been shown that ethionine inhibits the methylation of newly synthesized DNA. It has been proposed that the methylation of DNA allows for the discrimination of the parental and the newly synthesized daughter strand and that in this way the correction of mismatched bases in the newly synthesized strands becomes possible. Therefore the effect of ethionine could be a disturbance of methylation instructed mismatch repair which would lead to enhanced spontaneous mutation frequencies and synergistic effects with agents which cause mispairing. Experiments performed on the effect of ethionine on spontaneous mutations, on EMS induced mutations and on HAP (6-hydroxy-amino-purine) induced mutations could not substantiate this hypothesis. On the contrary treatment with ethionine resulted in a decrease of mutant frequencies.

4. Within the framework of the EEC contract the carcinogenic polycyclic hydrocarbon benzo(x)pyrene has been tested for mutagenicity in V-79 Chinese hamster cells with and without rat liver S9. In the presence of S9 BP proved to be clearly toxic and mutagenic.

#### D. List of publications 1979/1980

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Contractor : Organization for Health Research TNO  
Contract no. : 192-77-1 ENV N  
Project Leader : Dr. P.H.M. Lohman  
Title of project : Improvement of the incorporation of liver metabolism in in vitro test systems for mutagenic activity with mammalian cells

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#### Objective of the research

Many compounds which by themselves are not mutagenic are converted by liver oxygenases into substances with genotoxic properties. Therefore, liver extracts are often added to in vitro test systems to take care of the metabolic conversion of the substance to be tested. However, some cell systems react unfavourably to the presence of liver homogenates, the activity of these extracts rapidly decreases with time which forms a problem when prolonged exposures are attempted and frequently the active metabolites are very short lived and are hardly able to reach the interior of the target cells. We have attempted two approaches to overcome one or more of the above mentioned limitations of metabolic conversion systems: (i) the use of porous membranes to separate cells from liver homogenates and (ii) the use of cells with endogenous metabolic capacities. Besides these studies, in the second phase of the 2nd Environmental Research Programme we also contributed to the comparative testing programme of the contract groups. We have applied the following tests with cultured mammalian cells to the study of the chemicals selected within the EEC programme: (i) Unscheduled DNA synthesis, (ii) DNA repair replication (density gradient method) and (iii) inhibition of scheduled DNA synthesis. These tests were applied to collect supplementary data within the framework of the EEC comparative testing programme with the objective to determine the type of DNA lesions induced by the selected chemicals, whether repair occurs, and, if so, what type of repair is induced in relation to the mutagenic action of the compound ("error free" versus "error prone" repair).

#### Materials and Methods

##### A. Porous membranes to separate cells from liver homogenates

Fresh liver homogenate (S9-fraction prepared from rat liver stimulated with Aroclor 1254) was incorporated into a system with mammalian cells by pumping it through capillaries with porous walls, on the outside of which cells are located (Amicon "Vitafiber" tissue culture systems - R.A. Knazek et al., Exp. Cell. Res. 84, 251, 1974). These hollow fibers allow the

transport of compounds (metabolites or xenobiotics) of low molecular weight through the walls of the capillaries, whereas larger molecules (enzymes) remain on the inside. Mammalian cells can grow in high density on the outer surface of these membranes and because of the intensive contact of the growing mammalian cells with the walls of the capillaries it was expected that the testcompound, or its (short lived) metabolites, can reach the cells when this compound, together with fresh liver homogenate, is pumped through the interior of the fibers, whereas the (noxious) direct contact between homogenate and cells is avoided.

B. Cells with endogenous metabolic capacities

B1. The induction of Sister Chromatid Exchanges (SCE) in rat liver cells.

Hepatocytes cultured in vitro would have been the system of choice. However, hepatocytes in vitro do not carry out the necessary two rounds of DNA replication. Therefore, as an alternative, SCE induction was studied in rat hepatoma cells (Reuber Hepatoma H-35, cell line H-4-II-E); these cells are derived from a liver tumor and have maintained (part of) the metabolic capacities of hepatocytes. As control cells without endogenous metabolic capacities we used Rhabdomyosarcoma cells (R1) originating from a non-hepatic rat tumor.

B2. Unscheduled DNA synthesis (UDS)

UDS was measured autoradiography in cultures of rat hepatocytes as described by Williams (G.M. Williams, Cancer Research 37, 1845-1851, 1977), as well as in H-35 and R1 cells (see A1). UDS in H-35 and R1 cells was measured according to the method described in the methodology section of the July 1978 progress report, contract 192-77-1 ENV N.

C. Tests with Chinese hamster and human cells

C1. The induction of forward mutations in Chinese Hamster Ovary (CHO) cells

Mutation of the HGPRT locus of CHO cells was studied according to the protocol described in the methodology section of the July 1978 progress report, contract 192-77-1 ENV N. Forward mutations in CHO cells were used as genetic endpoint to compare the results obtained with DNA repair tests in the same cell system.

C2. Indirect tests for genotoxicity.

Three indirect assays were used to test the genotoxic properties of the selected chemicals in CHO cells and in normal primary human fibroblasts, namely (i) the UDS test, (ii) the measurement of DNA repair replication (density gradient method) and (iii) the DNA synthesis inhibition test. All methods are described in detail in the July 1978 progress report, contract 192-77-1 ENV N. UDS and DNA repair replication are quantitative criteria for the amount of excision repair in mammalian cells. The inhibition of normal DNA synthesis is expected to be a qualitative indicator for interaction of genotoxic chemicals with DNA, because DNA lesions are known to inhibit the semi-conservative DNA synthesis in mammalian cells.

Results

A. The use of porous membranes to separate cells from liver homogenates

In this study hollow fibers with porous, membranous walls were used. On the outer surface of these fibers Chinese Hamster Ovary (CHO) cells were grown. When sufficient numbers of cells were present, fresh liver homogenate was pumped through these capillaries, together with the genotoxic compound to be studied.

First a number of introductory investigations was done, in which it was studied whether liver homogenates could be pumped through the very narrow fibers and whether the chemicals (or their metabolites) could pass through the pores in the walls of the fibers. It was found (in experiments without liver homogenate) that compounds like methyl methanesulphonate can pass through the walls of the hollow fibers and induce mutations in CHO cells growing on the outside. Other compounds, however, such as the directly-acting agent Mitomycin C (MMC), appeared to be adsorbed to the walls as they did not mutate the CHO cells growing on the outer surface of the fibers, although the pore size of the holes in the fibers should, in principle, be big enough to let them pass. Negative results were also obtained with the bio-activation requiring chemicals that were studied (e.g. cyclophosphamide). Therefore, the original goals of using this system for the separation of liver homogenates from mammalian cells by a porous membrane, viz. avoiding direct contact and opening the possibility to study the

effects of a prolonged, constant supply of freshly formed metabolites in rather low concentrations (a situation mimicking the in vivo exposition to chemicals) appeared out of reach. For this reason the experiments with the hollow fibers were terminated during of the 2nd phase of the 2nd Environmental Research Programme.

B. Sister Chromatid Exchanges (SCE's) and Unscheduled DNA Synthesis (UDS) in rat hepatoma cells

In the first phase of the 2nd contract we have demonstrated that rat hepatoma cells (H-35) can detect indirectly acting genotoxic compounds, without the need of a liver extract or an analogous provision, whereas the control rat cells without the endogenous capacity to activate chemical (R1 cells) are unable to do so. These results were obtained with cells treated with diethylnitrosamine (DEN) and 3-methyl-cholanthrene (3-MC). The comparative study with 4-nitroquinoline-1-oxide (4NQO) showed that H-35 cells are able to "detoxify" this genotoxic chemical, in contrast to R1 cells. These results were obtained with the SCE test.

In an attempt to improve the sensitivity of H-35 cells for bio-activation requiring genotoxic chemicals, we have tried to enhance the aryl hydrocarbon hydroxylase (AHH) activity in these cells. It was found that in H-35 cells the AHH activity can be increased 5-10 fold by growth of the cells in 1 mM 3-MC or in 1 ppm Aroclor 1254. (R1 cells have a low basal AHH level, but stimulation does not occur) We have studied the effect of the stimulation of AHH activity in H-35 cells by Aroclor 1254 on the induction of SCE's by exposure to 2-aminofluorene (2-AF) and N-isopropyl- $\alpha$ -(2-methylhydrazino)-p-toluamide (procarbazine, Natulan<sup>R</sup>). Exposition to 2AF and Natulan causes an increase of the SCE frequency in H-35 cells, and not in R1 cells. Unexpectedly, the enhancement of the AHH activity in H-35 cells by Aroclor did not result in a stronger increase in the number of SCE's. This result suggests that an increase in the endogenous AHH activity not necessarily has to lead to an increase in the response to genotoxic agents that require bio-activation. Whether a competition between enhanced bio-activation and bio-degradation is involved, or that other processes are rate-limiting, is not yet clear. It is also possible that stimulation of the AHH activity by Aroclor in H-35 cells failed to increase the SCE induction because other, non-stimulated pathways for bio-activation are required.

In an attempt to analyze the detoxification pathway in cells with an endogenous system for bioconversion in more detail, we not only studied the induction of SCE's in these cells but also measured the induction of UDS following treatment with genotoxic agents. UDS experiments do not require the progression of the cells through the cell-cycle, so in addition to H-35 and R1 cells also freshly isolated rat hepatocytes could be included in this study. Our first experiments were done with 4NQO as genotoxic agent. As indicated above, the induction of SCE's was strongly inhibited in H-35 cells in comparison to R1 cells. To our surprise, however, both cell-lines showed equal induction of UDS at all doses applied, including doses that did not induce SCE's in H-35 cells but which substantially increased SCE's in R1 cells. In freshly isolated hepatocytes the induction of UDS by 4NQO was comparable to that in H-35 and R1 cells. These results suggest that the detoxification process of indirectly acting agents in cells derived from rat liver is much more complicated than was expected: metabolites of 4NQO giving DNA lesions that lead to SCE's appear to be detoxified, whereas products which induce DNA lesions that can be recognized by an excision repair process are not.

#### Comparative Testprogramma

Within the comparative testprogramme of the Contract Group we collected supplementary data on the genotoxic properties of selected chemicals by using three different assays with cultured CHO cells and primary human fibroblasts, namely: (i) Unscheduled DNA synthesis (by autoradiography), (ii) DNA repair replication (by gradient analysis) and (iii) the DNA synthesis inhibition test.

In experiments with CHO cells we studied the possible quantitative relationship between mutation induction at the HGPRT locus and the amount of UDS after exposure to Mitomycine C (MMC), N-acetyl, N-acetoxy aminofluorene (AAAF), 4NQO and, as a reference agent, 254 nm UV radiation.

As a function of dose MMC and, to a lesser extent AAAF, are poor inducers of UDS in CHO cells in comparison to 4NQO. However, dose expressed in equal (molar) quantities is not always a good parameter for a comparison of genotoxic potential. Frequently, a more useful comparison is obtained when doses are compared on the basis of equal cytotoxicity. When the

amount of UDS is plotted as a function of the survival of the CHO cells, not only MMC and AAAF are poor inducers of UDS, but also 4NQO does not induce measurable amounts of UDS at doses allowing a reasonable fraction of the cells to survive, in contrast to UV.

When mutation induction was measured as a function of survival (at doses allowing a survival of at least 10% of the CHO cells), MMC, AAF, 4NQO and UV show comparable positive results. The conclusion to be drawn from these experiments with four powerful mutagenic agents is, that UDS as a test for genotoxic activity of compounds should not be considered as a reliable indicator for the mutagenic capacity of these compounds.

In experiments with human cells we studied UDS, DNA repair replication and the inhibition of the semi-conservative DNA synthesis, after treatment with respectively MMC, AAAF, methyl methanesulphonate (MMS), 4NQO, Dieldrin, dichloromethane, Isoniazid and benzo(a)pyrene. The latter two compounds were also studied in the presence of a rat liver homogenate (S9-fraction), and 254 nm UV was used as a reference agent. 4NQO, AAAF, MMS and UV were found to be strongly positive in all three assays used, with a clear dose-effect relationship. Also Isoniazid was strongly positive in this test, but only when  $Mn^{++}$  was present in the culture medium (independent of the presence of S9). However, when doses were related to cytotoxicity, only UV gave a distinct effect at moderately to hardly toxic doses (survival above 10%). With MMC, extreme high concentrations (20 times LD50) were needed to obtain a weak (2x the background) response in the DNA repair tests. The test for DNA synthesis inhibition, however, was positive at dosages allowing a substantial survival. Dieldrin and dichloromethane showed negative responses at all doses applied. Benzo(a)pyrene served as a model genotoxic compound to study the possibility of using the above mentioned assays with cultured human cells for the detection of indirectly acting agents, by performing the experiments in the presence of rat liver homogenates. Positive responses could be obtained in the DNA synthesis inhibition test and in the UDS test (repair replication assay not performed). However, the responses were found to be very small, especially with the UDS test. Therefore, at this moment these tests with primary human fibroblasts do not look promising for the detection of the genotoxic activity of indirectly acting agents, but before a

definite conclusion can be reached, this impression will have to be confirmed in additional experiments, in which also other indirectly acting agents should be included.

From the experiments with directly working agents it can be concluded that for most agents UDS and DNA repair replication are rather insensitive in comparison to the toxic dose levels of the compounds. The autoradiographical UDS method was somewhat more responsive than the method with density gradients. The DNA synthesis inhibition test was found to be more sensitive. The results of this assay, however, gave difficulties in the interpretation because of the problems how to distinguish between toxic and genotoxic effects.

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Contractor : Etat belge (Ministère de la Santé Publique), Institut d'hygiène et d'Epidémiologie, 1050 Bruxelles.

Contract no 261-77-6 ENV B

Project leader : Dr R.F MOUTON

Title of project : DNA MISREPAIR AND CARCINOGENESIS : Latent cytotoxicity of Repair inhibitors of the human environment

#### Objective of the research

In the perspective already outlined in the CEE report on the first phase of the project (1), we have continued to develop quick screening "syner-tests" by extending application of Painter's DSI test to additional direct and indirect carcinogens.

Parallely, we have initiated a complementary DNA repair induction test by unscheduled DNA synthesis measurement. We developed it using T. pyriformis GL and rat hepatocytes as targets for CEE reference carcinogens plus/minus caffeine, quinacrine and chloroquine as DNA repair inhibitors.

#### RESULTS

##### I. Detection of DNA-damaging agents by extension of the DNA Replication

###### Inhibition (RI/DSI°) test

I-1 After the positive results previously reported (1) for the detection of the direct reference carcinogens MMS, MMC, 4NQO, MNNG with our human KB cell line (mouth carcinoma), distinct but similar to HeLa one (cervix carcinoma) used by Painter, we failed to detect the indirect reference carcinogens B<sub>a</sub>P, DEN, NAT, ATRA using Kuroki's system of activation ± S 15 microsomal fraction from Aroclor pretreated rats. We explored systematically the possible causes of non functioning of the enzymatic activation of three references indirect carcinogens. We used for this purpose exactly Painter's activation conditions with HeLa cells. We succeeded to activate B<sub>a</sub>P and got positive Painter's tests for B<sub>a</sub>P and AAF despite the fact that we used the same S 15 microsomal fraction used in above reported results. We found that dimethylsulfoxide DMSO used as solvent was in fact the main source of the artefactual results. B<sub>a</sub>P was only activated both in the RI (and a control Ames test) when freshly distilled DMSO, protected against air and light, was used. Also the detection of B<sub>a</sub>P activity was significantly affected by the contamination of HeLa culture with PPLO (mycoplasma). The response of cell replication process to DNA insulting agents can then be recovered in those cells cured with high doses of kanamycine and tylosine using PPLO free HeLa cells as control. (°DNA Synthesis Inhibition).



These findings confirm the need and the importance of general study of validation at the level of laboratory practice. Standardisation and organisation of the quality controls remain the aim for each genotoxicological test whatever its novelty (3).

I-2 Effects of DNA-damaging agents on DNA replication of Tetrahymena pyriformis has been extended to the additional CEE reference carcinogens EMS, MNNG (2).

As for the previous carcinogens tested : UV light, MMS, 4NQO (1) characteristic durable inhibition of DNA replication synthesis was observed after removal of the carcinogen.

Similarly to the HU-treated cells (1), previously tested, cycloheximide (Chx) treated cells showed an immediate recovery of DNA synthesis following removal of the drug from the incubation medium (Table 1-A)

The DNA repair inhibitor, caffeine ( $10^{-3}$ M), quinacrine ( $10^{-5}$ M) and chloroquine ( $10^{-3}$ M) appeared non-DNA damaging agents too (Table 1-B).

Synergic effect of posttreatment with caffeine (specific inhibitor of UV early step of photolesions repair (1) (6)) has been investigated in cells treated with UV, MMS, MNNG and 4NQO (Table 2).

We could deduce : - an apparent positive synergic effect of CAF added after 4NQO exposure;  
- an apparent lack of synergic effect of CAF after MNNG exposure;  
- an apparent and unexpected negative synergic effect of CAF added after UV and MMS exposure.

It must be reminded at this point that the global measurement of inhibition of incorporation of tritiated thymidine in DNA may reflect two opposite known effects of caffeine : - inhibition of repair, thus of replication of insulted DNA;  
- stimulation of replication, as confirmed recently by Painter measuring stimulation of replication linked to caffeine mediated increase of the number of growing points in DNA mammalian cells(7).

## II. Detection of DNA-damaging agents using a DNA Repair Induction (DRI/UDS°) test

II-1 We have applied the UDS test to rat hepatocytes primary cultures. This system is expected capable to activate both direct or indirect hepatocarcinogens, activation being in fact considered as a misdetoxylation by this specialized target organ.  
(° Unscheduled DNA Synthesis)

The already known obstacle of high cytoplasmic autoradiographic background interfering with marked nuclei has been solved. However, results from several control experiments, carried out with and without reference direct chemical hepatocarcinogens (MNNG, aflatoxin B1), are still under examination. On other hand, a possibility has been recently opened to us to use an especially selected Chinese hamster cells culture (S 79-CO60) from outside Europe. This system, capable to activate all indirect carcinogens, will become available not only to us but also possibly to interested colleagues from our CEE contact Group (5).

II-2 In our amiconucleate protozoan T. pyriformis GL, UDS was demonstrated autoradiographically by grains over the isolated macronuclei following exposure to  $^3\text{H}$ -Tdr, replicative DNA synthesis being evidenced by nuclei blackened with grains too numerous to count.

Table 3 shows the DNA damaging effect of MNNG, MMC and 4NQO : stimulation of  $^3\text{H}$ -thymidine incorporation due to DNA induced repair amounts to 83 % for MNNG, to 116 % for MMC and to 114 % for 4NQO.

No UDS was observed for the indirect carcinogen DEN.

As positive controls hydroxyurea (HU) and cycloheximide (Chx) did not induced any UDS.

CAF synergic effects with the three selected carcinogens (MNNG, MMC, 4NQO) was shown in Table 3, second column.

We could deduce :  $\hat{=}$  an apparent positive synergic effect of CAF after 4NQO exposure;

- an apparent negative synergic effect of CAF added after MMC and MNNG exposure.

These opposite effects observed with our UDS method confirm so far those observed above (II-1) in the DSI test.

### III Detection of DNA-damaging agents by immunautoradiographic measurements of induced specific DNA lesions

The previously reported detection of UV-induced pyrimidine dimers lesions in DNA of T. pyriformis with specific tritiated antibodies (6) is now extended to other specific lesions like  $\text{O}^6$ -ethyldeoxyguanosine. The advantage of such tests is their high specificity whatever factors influencing their fate. This appears increasingly important when we keep in mind that DNA is simultaneously exposed to multiple insulting and repair inhibiting agents already within the first initiation step of carcinogenesis.

CONCLUSION

Need and importance of general study of validation at the level of laboratory practice have been concluded from a systematic exploration of parameters of enzymatic activation of the indirect carcinogens BxP and AAF carried out using exactly DSI Painter's conditions with HeLa cells line. Our preliminary experiments with UDS test were focussed on rat hepatocytes primary cultures capable to detect both direct and indirect hepatocarcinogens (MNNG, Aflatoxine B1).

The study of effects of several reference direct carcinogens on scheduled and unscheduled DNA synthesis provided a complementary study of response of the ciliate T. pyriformis to such treatments. DNA replication inhibition and DNA repair induction showed a similar level of sensitivity to these DNA insulting agents. On another hand, HU, Chx, CAF, Q and ClQ did not induced any detectable damage to DNA of our test system. Against expectation from our first results (UV ± CAF) with our immunoradiographic test (6), synergic effects observed in both DSI and UDS between direct carcinogens (UV, MMS, MNNG and 4NQO) and a repair inhibitor (CAF) did not yield simple interpretable results (7).

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TABLE 1

TIME in min.	A			B		
	Chx $10^{-5}M$	HU $10^{-2}M$	CAP $10^{-3}M$	Q $10^{-5}M$	ClO $10^{-3}M$	
15	61	--	117	52	104	
30	96	26	121	64	109	
45	107	--	---	115	123	
60	105	54	102	119	119	
90	93	86	92	119	119	
150	---	101	---	---	---	

Reversible DNA Synthesis Inhibition (DSI) with : A. two DNA Replication Inhibitors; B. three instant DNA Repair Inhibitors.

TABLE 2

TIME	UV 450 ergs/mm <sup>2</sup>		MMS 1 $\mu$ l/ml		MMSG 5.10 <sup>-4</sup> M		4NQO 5.10 <sup>-4</sup> M	
	---	+ CAP 10 <sup>-3</sup> M	---	+ CAP 10 <sup>-3</sup> M	---	+ CAP 10 <sup>-3</sup> M	---	+ CAP 10 <sup>-3</sup> M
15	.87	112	41	49	50	58	50	41
30	70	97	51	62	51	59	67	53
45	66	98	59	76	50	47	61	56
60	48	67	46	66	42	38	44	42
90	49	66	25	56	41	39	42	33

Durable DNA Synthesis Inhibition (DSI) induced by four direct reference carcinogens + three instant DNA Repair Inhibitors.

TABLE 3

	MMSG 5.0 x 10 <sup>-4</sup> M		MNC 1.2 x 10 <sup>-4</sup> M		4NQO 1.0 x 10 <sup>-4</sup> M	
	---	+ CAP 10 <sup>-3</sup> M	---	+ CAP 10 <sup>-3</sup> M	---	+ CAP 10 <sup>-3</sup> M
$\bar{N}$ Grains/nucleus in						
control cells	7.10	6.38	9.80	8.35	12.12	10.50
treated cells	13.00	15.45	21.20	19.77	25.98	12.80
UDS	1.83	2.42	2.16	2.37	2.14	1.22
Caffeine influence		-1.32		-1.10		+1.75

Unscheduled DNA Synthesis (UDS) induced by three direct reference carcinogens + three instant DNA Repair Inhibitors.

Contractor : Université libre de Bruxelles

Contract N° : ENV/355 B

Project leaders : J. Rommelaere and M. Errera

Title of project : Parvoviruses as probes of the regulation of survival,  
mutagenesis and DNA replication in mammalian cells

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### I. Objective of the research

The object of this contract was to investigate the action of environmental chemical carcinogenic agents with the aim of finding out not only fundamental mechanisms, but of investigating a system amenable of giving practical answers on comparative mutagenic or carcinogenic potency and if possible yielding data applicable to human beings and for low dosages.

### II. Material and Methods

a) The system chosen is a single stranded DNA mammalian virus (Parvovirus), some strains of which are infective to human cells and which can only survive by making a double stranded replication form by bypassing a DNA lesion on the invading single stranded DNA, any excision repair of the lesion being lethal. In addition, low doses of some mutagens, those having probably also a carcinogenic effect, are believed to induce cellular mechanisms apparently comparable to the SOS induced repair of microorganisms, mutagenic not only for damaged DNA (targeted mutagenesis) but also to undamaged DNA (untargeted mutagenesis). The independent treatment with chemical or physical agent of host cells and of the virus, enables a thorough understanding of the system, one of the advantages being that the viral DNA can easily be reisolated and sequenced to analyse coding errors induced by the agent.

b) This system not having yet been used for this type of research, the prerequisite of the work was to establish its phenomenology in order to ascertain the optimum conditions. It was also important to be able to compare the present system to similar ones (Herpes simplex, SV40). It was therefore more straightforward to use U.V. light and mouse cells as a first step (with the advantage of being able to quantitate the damage by well known methods and the cellular molecular processes operating after UV-irradiation being much better known than after chemical carcinogen treatment of cells).

The following combinations of cells and parvoviruses were used :

- 1) A9 cells (a variant of mouse L cells) and clones Cl1, Cl2 and Cl3 (hybrid cells between A9 and L5178 YS obtained by polyethylene glycol fusion) were used as hosts of MVM. L5178YS is a mouse lymphoma cell known for its sensitivity to X and UV radiation. These cells were used for the study of enhanced reactivation of MVM strain - a mouse Parvovirus.
- 2) RL 5E (a Harvey murine sarcoma virus transformed line) and NBE, a new born human kidney cell line were used for the study of mutation of H-1 ts6 to H-1 wt. For details of culture conditions see publications. Nitronaphthofuran derivatives were a kind gift from R. Royer (Institut Curie, Paris).

### III. Results

#### A. Phenomenology of Enhanced Reactivation (ER) and Mutagenesis (EM) with U.V. light

##### a) Enhanced reactivation of Minute virus of mice (MVM) in para-synchronous mouse A9 cells treated with U.V. light prior to infection.

Mild UV-irradiation of the host cells blocked in G<sub>0</sub> by starvation, enhances after various growth periods in starving media the survival of UV irradiated virus grown in normal medium. The magnitude of ER is a direct exponential function of the UV dose to the virus, while virus survival is inversely proportional to U.V. dosage. The expression of ER requires de novo protein synthesis and is maximal when the cells are irradiated 30 hours before onset of viral DNA replication which is synchronous with cellular DNA replication. Optimal cell irradiation was of 3 to 5 J m<sup>-2</sup> which gives a cell survival of 85 p.c. in these conditions.

##### b) Enhanced reactivation in cell hybrids of various sensitivities to lethal effects of X and U.V. rays

Hybrids between normal cells and cells demonstrating an increased sensitivity to a foreign agent gives the hope of analysing cell responses on a genetical basis. When U.V. irradiated and challenged with normal MVM virus, the three cell hybrid lines showed an enhanced capacity (EC) for the growth of this virus. However only one of the cells lines used was capable of Enhanced Reactivation (ER) of UV damaged virus although all 3 lines when non irradiated gave identical surviving curves for the irradiated virus, indicating a deficiency in the induced mechanism rather than a constitutive behavior. Both E.C and E.R require de novo protein synthesis after U.V. treatment - both processes being therefore likely to share common regulatory

and/or functional steps - but the fact that only one line responded to ER suggests that EG and ER involve different proximal effectors.

c) Indirect induction by an irradiated virus of mutagenesis of intact parvovirus H-1

Irradiation of host cells or preinfection of the cells with UV irradiated parvovirus H-1 or SV40 in conditions in which they invade the host cells but do not multiply, induce a cellular process which is mutagenic to superinfecting intact thermosensitive H-1. Mutagenesis was assayed by determining the reversion rates of the wild type phenotype at the restrictive temperature. The cellular events leading to an enhancement of the frequency of mutants among the descendants of unirradiated H-1 requires de novo protein synthesis suggesting that a mutagenic cellular process could be induced. Doses to the cells below  $1 \text{ J m}^{-2}$  already gave a significant enhanced mutagenesis, a plateau (or perhaps even a maximum) being already reached for doses of  $2 \text{ J m}^{-2}$ .

B. Phenomenology of Enhanced reactivation (ER) and mutagenesis (EM) with 2 Nitronaphtofuran derivatives

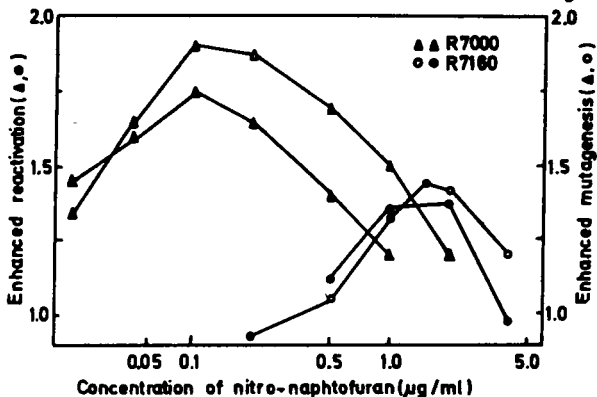
Nitronaphtofuran derivatives were chosen as experimental chemicals because Royer and his group (Institut Curie, Paris) had shown that one of them, R7000, was among the most potent mutagens in the Ames test. If chemical carcinogens did induce ER and EM, this compound gave a much better chance of obtaining an effect if chemical agents had any effect at all.

Two different Nitronaphtofuran derivatives were used : R7000 and R7160, the latter being a very weak mutagen in Ames test. Fig. 1 demonstrates the results obtained with RLSE rat cells and H-1 ts6 parvovirus : ER and EM were maximum for  $0.1 \mu\text{g/ml}$  of R7000 which is non toxic, but it takes 10 times more R7160 for the same effects. In addition, increased concentration of the drug demonstrates that the system is highly sensitive to it. The fact that ER and EM are inhibited by  $5 \mu\text{g/ml}$  cycloheximide suggests that the chemical mutagen used induces a cellular process, mutagenic to the normal infecting DNA of parvovirus H-1 ts6.

An interval of 14 hours between cell treatment and infection is required for optimal triggering of ER and EM by both chemicals, which is of the same order of magnitude than for ER studied with the A9 variant of mouse L cells used in I a. With both agents the "induced" process has decayed when 30 hours or more elapse between cell treatment and virus infection.



Dose response of enhanced reactivation and enhanced mutagenesis



#### IV. Conclusions and further plans

A) Non lethal (UV) and non toxic (R7000) doses of a strong mutagen to cells induce a transient cellular reaction responsible for enhanced capacity (for a normal parvovirus), enhanced reactivation (for a UV treated virus) and enhanced mutagenesis (for an untreated virus). Both agents have a maximum effect when the virus is added at 10-15 hours post treatment - the effect having completely disappeared by 30 hours. With both agents the effect is inhibited by cycloheximide.

B) Planned research : (not included in the former proposal)

- Induction of ER and EM with aflatoxin (in collaboration with W. Thilly, Dept. Nutrition and Food Sciences, MIT, Cambridge, USA)

The involvement of the viral EM process in cell mutagenesis will be tested using low doses of aflatoxin as a treatment to human lymphoblast cultures. Dose responses will be compared with respect to the induction of (i) DNA adducts, (ii) cell mutagenesis and (iii) enhanced untargeted mutagenesis of H-1 parvovirus. Estimating dose response curves for mutagens at very low doses is of primary importance for risk assessments.

- Induction of EM with benzopyrene (in collaboration with I.B. Weinstein and V. Ivanovic, Columbia University, New York)

The role of the cytosolic aromatic hydrocarbon receptor in the induction of viral EM by benzopyrene will be investigated.

- Search for early proteins induced during ER and EM and their relationship with DNA replication (in collaboration with P. Yot, Institut Curie, Paris)

Nothing is known of the enzymology of ER and EM : it is expected that the identification and characterization of early induced proteins constitute an early cellular response which could probably be used to identify chemical compounds of possible carcinogenic properties.

### Seminars

- "Les Parvovirus, sonde virale pour la réparation SOS dans les cellules de mammifères irradiées aux UV" (CNRS, Biophysique Moléculaire, Orléans, 22/4/80)
- "Indirect induction of mutagenesis of intact Parvovirus H-1 in mammalian cells" (Brookhaven National Laboratory, Upton, 19/11/80)
- "idem" (Columbia University, Cancer Center, New York, 28/11/80)
- "Utilisation des parvovirus comme sonde des mécanismes de cancérogénèse et de mutagénèse dans les cellules de mammifères (ICP, Bruxelles, 28/1/81)

### Congresses

- Keystone, 22-28/2/81  
ICN-UCLA symposium on Mechanism of Chemical Carcinogenesis "Indirect Induction of mutagenesis of intact parvovirus H-1 in mammalian cells"

### Publications

- J. Rommelaere, J.M. Vos, J.J. Cornelis and D.C. Ward (1981) UV-enhanced reactivation of Minute-Virus-of-Mice : stimulation of a late step in the viral life cycle.  
Photochemistry and Photobiology, in press
- J.M. Vos, J.J. Cornelis, S. Limbosch, F. Zampetti-Bosseler and J. Rommelaere (1981) UV-irradiation of related mouse hybrid cells : similar increase in capacity to replicate intact Minute-Virus-of-Mice but differential enhancement of survival of UV irradiated virus.  
Mutation Research, in press
- J.J. Cornelis, Z.Z. Su, D.C. Ward and J. Rommelaere (1981) Indirect induction of mutagenesis of intact Parvovirus H-1 in mammalian cells treated with UV-light or UV-irradiated H-1 or SV40 viruses.  
Proc. Natl. Acad. Sci. USA, submitted
- Z.Z. Su, J.J. Cornelis and J. Rommelaere (1981) Mutagenesis in intact parvovirus H-1 is expressed co-ordinately with enhanced reactivation of UV-irradiated virus in mammalian cells treated with 2-nitronaphtofuran. in preparation.

Contractor : University of Sussex

Contract n° : 182-77-1 ENV UK

Project leaders : R.J. Cole and C.F. Arlett

Title of project : Development and comparison of methods for assessment of potentially mutagenic and carcinogenic chemicals using hemopoietic and lymphoid cells

### Objectives

Genotoxic environmental chemicals exert their effects at both gene, and chromosomal level. Screening tests must reflect both types of damage and should be designed to facilitate quantitative comparisons between different end-points. Mouse lymphoma (L5178Y) cells are the basis of efficient in vitro mutagenesis assay systems, and can be used with liver homogenate to provide metabolic activation. As a result of the ease with which these cells can be manipulated, rapid assessments of new selective systems, expression time, stability and phenotype of mutants can be made, as well as providing a comparison of in vitro mutagenesis with in vivo induction of micronuclei, S.C.E.'s and mutations in different cell types.

Cells of prenatal tissues are particularly vulnerable to initiation of genome damage with long-term, deleterious effects on health, including e.g. induction of mutagenic, carcinogenic and teratogenic lesions, and effects leading to functional deficiencies in e.g. the immune and nervous systems. In the 'developed' world diseases attributable to DNA or chromosomal damage are the largest non-accidental cause of illness and death in childhood. We have investigated the suitability of 'short-term' cytogenetic methods, particularly the micronucleus and sister chromatid exchange, to measure prenatal risk from exposure to environmental chemicals.

### Materials/Methods/Results/Conclusions

#### A. Mouse Lymphoma Cells

##### 1. Stability and Properties of Thioguanine resistant (TG<sup>R</sup>) mutants

Thioguanine resistance is one of the most widely used selective systems in mammalian cells and characterization of TG<sup>R</sup> variants is an essential part of the validation of this system. We have extensively analysed 19 TG<sup>R</sup> L5178Y clones: 6 spontaneous, 6 EMS-induced and 7 8-methoxy-psoralen + N UV induced. All clones were cultured for long periods in the absence of TG, and repeated testing has demonstrated that they have the characteristics of stable mutations at the hypoxanthine guanine phosphoribosyl transferase (HGPRT) locus. Thus, all remain resistant to TG, do not grow in HAT medium and have no demonstrable HGPRT activity. In no case could spontaneous reversion to TG sensitivity be demonstrated, nor could any spontaneous or 8-MOP induced mutants be reverted using EMS. 2/5 EMS-induced TG<sup>R</sup> mutants reverted at low frequency ( $\sim 1 \times 10^{-6}$ ) using EMS; in one case HGPRT of the revertant was normal, but in the other, revertants had electrophoretically abnormal HGPRT.

The results suggest that in L5178Y cells, thioguanine is selecting stable HGPRT variants, including point (revertible) and chromosome (non-revertible) mutants.

##### 2. Selective Systems

One disadvantage of TG as a selective system in routine mutagenesis assays is the long period (7-8 days) required between mutagen treatment and full expression of newly induced mutants. An alternative system, ouabain resistance, despite several practical advantages (e.g. short expression time and complete toxicity at high cell density) has one disadvantage in that ouabain selects specific point mutations only. Chromosome deletions and frame shift mutations, which result in absent gene product (cell membrane ATPase) are lethal; thus, qua<sup>R</sup> mutations are not induced by  $\gamma$  radiation, or frame shift mutagens. We have therefore investigated methotrexate (MTX) as an alternative selective system.

Using one mutagen, 4 nitroquinoline oxide, in the absence of exogenous activation, and one, Benzo(a)pyrene, in the presence of rat liver homogenate we have compared the induction of MTX<sup>R</sup> with TG<sup>R</sup>, OUA<sup>R</sup> and cytosine arabinoside resistance. The results indicate that MTX may be a useful addition to the battery of selective systems available for use with L5178Y. MTX<sup>R</sup> is a dominant mutation, has an early expression time (48 - 72h), and a dose-dependent increase in mutants was demonstrated (frequency  $\ll$  TG<sup>R</sup>). Variants tested were MTX resistant in suspension culture, and further characterization is being undertaken.

### 3. 12 - O tetradecanoyl phorbol acetate (TPA)

Following reports that treatment with the tumor promoter TPA increased the frequency of TG<sup>R</sup> and oua<sup>R</sup> mutants following UV or MNNG treatment in V79 chinese hamster cells, we have investigated the effect of TPA on induced mutation in L5178Y cells in conjunction with Dr.C.Lasne (Villejuif).

Using the traditional and fluctuation test protocols, no increase in OUA<sup>R</sup> could be demonstrated for spontaneous, UV or EMS induced mutants, and confirm the suggestion that any effect of TPA on mutagenesis may be by modifying metabolic co-operation and the recovery of resistant cells.

### B. Transplacental Tests

#### 1. The Transplacental Micronucleus Test using Mouse Fetal Liver Erythroblasts

The frequency of polychromatic erythrocytes with acentric chromosome fragments (the result of DNA strand breakage) after chemical exposure of pregnant mice can be measured in the fetal liver, and fetal blood. Since PCE's are also still present in the blood at birth, the method can be extended to exposures late in pregnancy, and their effects monitored in neonatal animals using the same methods. This test is rapid, reliable, and can be used to give quantitative risk-assessment data. Since the target cells develop in close proximity to cells with a wider metabolic competence than those in bone marrow, the 'transplacental' test is sensitive to agents whose clastogenic potential is not detectable by the traditional bone marrow test.

TABLE 1. Induction of micronuclei in maternal bone marrow and fetal liver

Agent & dose range	Damage/unit dose		Rad equivalence	
	(probability of erythroblasts forming acentric fragment)		( $\mu$ M/kg)	
	Maternal bone marrow	15/16 day fetal liver	Maternal bone marrow	fetal liver
irradiation				
2.5 - 50 rad	$1 \times 10^{-3}$ /rad	$1 \times 10^{-3}$ /rad		
Mitomycin C				
1.5-3.0 $\mu$ M/kg	$1.7 \times 10^{-2}$ $\mu$ M/kg	$6.7 \times 10^{-3}$ $\mu$ M/kg	0.06	0.15
3.0-6.0 $\mu$ M/kg	$3.0 \times 10^{-2}$ "	$1.7 \times 10^{-2}$ "	0.03	0.06
Procarbazine				
45.0-135.0 $\mu$ M/kg	$6.7 \times 10^{-4}$ "	$1.5 \times 10^{-3}$ "	1.50	0.77
Cyclophosphamide				
18.0-36.0 $\mu$ M/kg	$2.8 \times 10^{-4}$ "	$1.7 \times 10^{-3}$ "	3.57	0.60
36.0-72.0 $\mu$ M/kg	$2.8 \times 10^{-4}$ "	$3.5 \times 10^{-3}$ "	3.57	0.29
Methyl methanesulphonate				
91.0-273 $\mu$ M/kg	$1.4 \times 10^{-4}$ "	$1.4 \times 10^{-4}$ "	7.00	7.00
273.0-910.0 $\mu$ M/kg	$1.4 \times 10^{-4}$ "	$5.0 \times 10^{-4}$ "	7.00	2.00
Benzo(a) pyrene				
300.0-1190.0 $\mu$ M/kg	$3.3 \times 10^{-5}$ "	$1.7 \times 10^{-5}$ "	30.00	58.00
Diethylnitrosamine <sup>+</sup>	no effect	$1.4 \times 10^{-5}$ "	no effect	77.00
100.0-500.0 $\mu$ M/kg				

+ Preliminary estimate

## 2. Sister Chromatid Exchange in Mouse Fetal Liver Erythroblasts

Fetal liver erythroblasts have been explanted into medium containing BUdR and erythropoietin (a specific growth factor for erythroid cells) after in vivo exposure to chemicals, and SCE's measured in 2nd metaphase cells. The selective culture conditions allow direct numerical comparisons between the initiation of acentric fragments (micronuclei) and SCE's in fetal erythroblasts. (Table 2). Since both in vitro and in vivo cell kinetics are well characterized, the method can be designed to give high yields of 2nd metaphase cells, and the cell cycle phase exposed to initiation of lesions in vivo can be identified by appropriate time courses. The technique is a very sensitive indicator of in vivo transplacental interactions between genotoxic chemicals and chromosomes, and may be useful in further investigations of the nature and significance of SCE's.

TABLE 2. Induction of micronuclei and SCE's in fetal liver erythroblasts

Agent & dose range	Induction of micro	Induction of SCE/	SCE/ $\mu$ M
	/nuclei erythroblast/ $\mu$ M/kg	erythroblast/ $\mu$ M/kg	MN/ $\mu$ M
Mitomycin C			
1.5-3.0 $\mu$ M/kg	6.7x10 <sup>-3</sup>	3.9	580
3.0-6.0 $\mu$ M/kg	1.7x10 <sup>-2</sup>	3.9	230
Cyclophosphamide			
18.0-36.0 $\mu$ M/kg	1.7x10 <sup>-3</sup>	4.4x10 <sup>-1</sup>	258
36.0-72.0 $\mu$ M/kg	3.5x10 <sup>-3</sup>	4.4x10 <sup>-1</sup>	125
Procarbazine			
45.0-135.0 $\mu$ M/kg	6.7x10 <sup>-4</sup>	2.0x10 <sup>-2</sup>	30
Methylmethanesulphonate			
91.0-273.0 $\mu$ M/kg	1.4x10 <sup>-4</sup>	2.0x10 <sup>-2</sup>	140
Diethylnitrosamine <sup>+</sup>			
100.0-500 $\mu$ M/kg	1.4x10 <sup>-5</sup>	6.0x10 <sup>-3</sup>	430

+ Preliminary estimate

('Spontaneous' frequencies, micronucleated PCE's in fetal liver 0.3%. SCE's/cell, 7.5

## 3. Sister Chromatid Exchange in Mouse Maternal and Fetal White Blood Cell Precursors.

Committed stem cells of the granulocyte-macrophage lineage and their immediate descendants respond to a specific growth regulator in vitro (GM colony stimulating activity). These cells are found in fetal liver and maternal bone marrow and therefore provide a useful comparison with cells of the erythroid series using the SCE technique after in vivo exposure (Table 3). The GM precursor cells appear to be marginally more sensitive to Procarbazine than the erythroblasts, but this agent is notable for its low effectiveness in inducing SCE's, relative to chromosome breaks. MMS is least effective in the adult bone marrow GM cells, but a significant proportion may not be in S phase when exposed (in contrast to the situation in the fetal liver).

TABLE 3. Induction of SCE's in Fetal and Maternal Erythroblasts and Granulocyte/macrophage Progenitor Cells

Agent & dose range	Fetal liver erythroblasts	Fetal liver GM cells	Maternal bm GM cells
Cyclophosphamide 18.0-90.0µM/kg	$4.4 \times 10^{-1}$	$2.8 \times 10^{-1}$	$3.1 \times 10^{-1}$
Procarbazine 45.0-135.0µM/kg	$2.0 \times 10^{-2}$	$9.6 \times 10^{-2}$	$5.9 \times 10^{-2}$
Methylmethanesulphonate 91.0-273µM/kg	$2.0 \times 10^{-2}$	$1.6 \times 10^{-2}$	$3 \times 10^{-3}$
('spontaneous' SCE's/cell: fetal erythroblast		7.5	
fetal granulocyte/macrophage progenitor		5.4	
adult " " "		6.7	

4. Sister Chromatid Exchange in Mouse Fetal Brain Cells

The rodent fetal brain is particularly sensitive to transplacentally induced DNA damage. In humans, childhood tumors originating in the central nervous system are second only to leukaemias in frequency. The *in vivo/in vitro* SCE test is currently the only short-term cellular assay available to indicate the impact of genotoxic chemicals on the brain *in utero*. Pregnant animals were exposed during the 17th day of gestation. Fetal brains were then isolated, dissociated to single cell suspension without use of enzymes, and cultured in BUdR for SCE analysis. Fetal brain cells show markedly fewer SCE's in response to cyclophosphamide, than the other fetal cell types tested (Table 4).

TABLE 4. Induction of SCE's in fetal brain, fetal erythroblasts, and fetal granulocyte macrophage progenitor cells

Agent	Induction of SCE/cell/µM/kg		
	Fetal brain cells	Fetal erythroblasts	Fetal GM precursor cells
Cyclophosphamide	$7.8 \times 10^{-2}$	$4.4 \times 10^{-1}$	$2.8 \times 10^{-1}$
Procarbazine	$3.7 \times 10^{-2}$	$2.0 \times 10^{-2}$	$9.6 \times 10^{-2}$

5. Monitoring Prenatal Damage in Human Somatic Cells

The SCE and micronucleus techniques have been applied to human fetal lymphocytes obtained from cordblood at birth. The 'spontaneous' frequency of SCE's in cord lymphocytes from 50 'normal' pregnancies is 4.17 (range 2.9 - 6.6). A method for scoring micronuclei in intact PHA stimulated cord lymphocytes has also been developed. The 'spontaneous' frequency of micronucleated PHA responsive cord lymphocytes is 0.26% (range 0.05 - 0.6) and the frequency of micronucleated descendants of G<sub>0</sub> irradiated cells is  $2.5 \times 10^{-4}$ /rad (10 - 200 rads). These data will provide a basis from which to evaluate 'at risk' pregnancies.

6. In vivo/in vitro mutagenicity Assays

We have measured the frequency of variant granulocyte/macrophage colony forming cells (GMCFUC), which are 'spontaneously' resistant to 6-thioguanine, (60µg/ml) from mouse fetal liver and adult bone marrow. These variants (which include HGPRT<sup>-</sup> clones from autoradiographic evidence) occur at a frequency

of  $c \times 10^{-6}$ . We have not observed increased frequencies of 6 TG<sup>res</sup> GMCFUc after in vivo exposure to Procarbazine. Other organs have been surveyed for their content of cells with high primary cloning efficiency and fetal lung appears to offer a good basis for a transplacental mutagenesis assay using 1<sup>ary</sup> clones. The cells are also suitable for the micronucleus test and SCE analysis.

Publications

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- Rogers, A.M., Hill, R., Lehman, A., Arlett, C.F., and Burns, V. (1980) Induction and characterization of mouse lymphoma L5178Y cell lines resistant to 1-B-D arabinofuranocytosine. *Mutation Res.* 69, 139-48



Contractor: The Middlesex Hospital Medical School

Contract no: 184-77-1 ENV UK

Project Leader: Dr. S. Neale

Title of Project: Development of rapid, economical screening tests to detect carcinogenic or mutagenic compounds of environmental significance

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### Objective of the research

Mammalian metabolism is a factor of prime importance in estimating the potential genetic toxicity of chemicals released into man's environment. Both activation and detoxification may occur following uptake of a chemical by mammals. The purpose of this project is to develop an assay which, while measuring the mutagenic potential of a chemical, will reflect any significant metabolic changes to which the chemical is subjected following administration to mammals.

Prokaryotes provide one of the cheapest and quickest methods for measuring mutagenicity and in this project Escherichia coli is used in conjunction with whole-body mammalian metabolism. A complement-resistant strain, E.coli D494, has been chosen which will survive following intravenous injection, and forward, antibiotic-resistance, genetic markers have been selected to provide the sensitive system used to study the interaction of metabolism and secondary chemicals on the mutagenic potential of chemicals in our environment.

### Materials and Methods

Tuck T0 female mice, 22 gm, were used in all experiments. Natulan<sup>R</sup> and cycasin were gifts from Roche Products Ltd and Dr. P. Swann respectively, other chemicals were obtained from commercial sources.

Broth-grown, stationary-phase, complement-resistant E.coli K12 D494 (met<sup>-</sup> pro<sup>-</sup> pan<sup>-</sup> thi<sup>-</sup>, serotype 08) were injected intravenously ( $7 \times 10^8$  cells/mouse). Except where otherwise stated the required chemicals were administered by oral intubation immediately prior to, or by i.v. injection immediately after, injection of bacteria. After a suitable interval, generally 5 h, the animals were killed and the

livers removed, washed and homogenized in 1.5 volumes sterile saline. Crude homogenate was plated on agar containing ampicillin (6 µg/ml) or naladixic acid (5 µg/ml) to determine resistant mutants while suitable dilutions of each homogenate were plated onto minimal agar to determine the total number of survivors.

### Results

We have previously demonstrated that i.v. injection of 0.05-10 mg/kg of the potent carcinogen dimethylnitrosamine (DMN) causes a linear dose response of induced mutations in E.coli D494 recovered from mice. More detailed study of DMN shows that above 10 mg/kg a higher mutagenic response is obtained following i.p. administration but that at low doses the response is reduced by i.p. administration. Optimum mutagenic response to low doses of DMN occurred when cells and chemical were injected simultaneously i.v. If DMN was injected i.v. prior to the bacterial injection the mutagenic response was reduced in proportion to the time lag involved, in accordance with the theory that the mutagenic metabolite of DMN is highly unstable. The decrease in mutagenicity observed after various time lags was used to determine the rate of decay of the mutagenic metabolite and a value of 18 mg/kg/h was obtained for mice administered an initial dose of 5 or 30 mg/kg compared with a figure of 50 mg/kg/h obtained from HPLC measurements of the decay of DMN in blood samples from mice administered 30 mg/kg. Decay of this 'standard' nitrosamine is frequently measured by both physical and chemical methods during research on the potential exposure of humans to nitroso compounds. The discrepancy reported here between our 'biological' method and an established physical method indicates the active metabolite may have a longer half-life than that anticipated by some workers.

The immuno-suppressive and carcinostatic drug Natulan<sup>R</sup> (procarbazine) has been reported to cause chromosome aberrations in mammalian tissues, including man, but did not give a positive result when tested in in vitro bacterial

mutagenicity assays. Natulan (1-40 mM) failed to induce mutations in E.coli D494 in our laboratory in an in vitro liquid assay system containing liver homogenate S9 preparation. However following i.v. administration of E.coli D494 and Natulan (46-455 mg/kg) to mice a linear dose response curve was obtained for induction of mutations in the bacteria recovered 5 hrs later (Table 1), indicating a vital role for the intact animal in metabolic activation of Natulan.

Table 1. Induction of mutations in E.coli D494 following administration of Natulan or DMN to mice

Mutagen	Dose (mg/kg)	Mutagen-induced response (Amp <sup>R</sup> /10 <sup>9</sup> survivors)		
		i.v. injection		oral
		30 min	5h	5h
Natulan	46	60	63	
	137	65	248	96
	228		431	
	278	69	631	94
	455	54	1015	160
	683			194
DMN	0.5		178	130
	2.0		693	902
	5.0		1432	

Comparison of the mutagenicity of i.v. administered Natulan or DMN showed that, for an equal mutagenic response, the dose of DMN required is only 0.5% that for Natulan. A time course for the mutagenic response induced by Natulan showed the rate of decay to active mutagenic metabolite in mice was about 90 mg/kg body wt/h, a rate-limiting factor during measurement of the mutagenic response to high doses after short exposure times. Natulan is most frequently prescribed for oral administration to man, the mutagenic potency of Natulan administered by oral intubation to mice was only 15% of that observed following i.v. injection whereas the response to the positive control, DMN, was unaffected by these two routes of administration (Table 1).

Ingestion of chemicals is a primary route of exposure for man. Aminopyrine (AP), formerly a common antipyretic drug and known to be potentially nitrosatable, was administered to mice by oral intubation, together with nitrite as required. A dose-dependent induction of mutations occurred from 5 to 181 mg/kg AP/NO<sub>2</sub>, no induction occurred in the absence of NO<sub>2</sub> or where NO<sub>3</sub> was substituted for NO<sub>2</sub>. Ascorbic acid is known to inhibit formation of N-nitroso compounds in the stomach and is reported to prolong the life of terminal cancer patients. Ascorbate given by oral intubation prior, and in equimolar concentration, to AP/NO<sub>2</sub> completely inhibited AP/NO<sub>2</sub>-induced mutagenesis. Mutation induction by cycasin (40 mg/kg), a nitroso compound requiring metabolic activation by gut flora, was inhibited only 40% by equimolar ascorbate while even 100-fold excess ascorbate failed to reduce mutation induction by 5 mg/kg DMN, a nitroso compound activated in the liver. The effect of ascorbate on AP/NO<sub>2</sub>-induced mutagenesis was dose dependent, being reduced to 50% inhibition at a molar ratio of 9:1 AP/NO<sub>2</sub>:ascorbate, and was optimal if the ascorbate was added immediately prior to the AP/NO<sub>2</sub>. Ascorbate added 35 min before or 10 min after AP/NO<sub>2</sub> did not affect the induced mutagenesis and even a 2 min interval after AP/NO<sub>2</sub> was added before ascorbate reduced inhibition to 75% (Table 2). Chronic treatment of mice by providing 1% ascorbate in drinking water resulted in a 37% decrease in induced mutations (80 mg/kg AP/NO<sub>2</sub>) after 12 days but no reduction after shorter periods or with 0.1% ascorbate water. Cysteine, administered i.v. or by oral intubation, also caused a reduction in the number of mutations induced by AP/NO<sub>2</sub> treatment. The amino acid contains a SH group, in vitro it does not affect DMN-induced mutagenesis but catalyses the decay of N-methyl-N-nitroso-N<sup>1</sup>-nitroguanidine (MNNG) and causes a profound reduction in the number of mutations induced by this agent. In mice mutations induced by MNNG (66 mg/kg) were reduced only slightly by 225 mg/kg cysteine whereas

mutations induced by DMN (5 mg/kg) were reduced to 50% at 45 mg/kg cysteine. The effect of cysteine does not appear to be proportional to concentration. This observation provides an important indication of the complex nature of the various factors in the human environment which play a role in the final mutagenic potential of chemicals in man.

Table 2. Factors affecting mutation induction by nitroso derivatives.

Treatment	Dose of mutagen (mg/kg, oral)						
	5	10	20	40	66	80	181
AP/NO <sub>2</sub> only	180	400	1200	1800		1880	3100
+ 450							0
90 mg/kg							160
70 ascorbate							545
50							2130
+ 80 mg/kg ascorbate							
35						1790	
20 min before						1440	
10 AP/NO <sub>2</sub>						1090	
5						120	
2 min after						450	
5 AP/NO <sub>2</sub>						1210	
10						1825	
Cycasin only	64		704	1500			
+ 40 mg/kg ascorbate				640			
DMN only	2382						
+ 500 mg/kg ascorbate	2280						
+ 45 mg/kg cysteine	1100						
MNNG only (i.p.)						3990	
+ 225 mg/kg cysteine						2841	

### Conclusions

Evidence has been produced confirming that the potential mutagenic hazard of a chemical towards man cannot be determined from in vitro experiments alone. Modification of the chemical by full mammalian metabolism and by the presence of other chemicals present within the mammalian environment may either activate or deactivate latent mutagenic potential. The route of exposure of the mammal to the chemical may also affect the result profoundly and may even result in a change from negative to positive mutagenicity. The results

obtained show that the assay developed here can be used to elucidate some of these problems, the need for further studies using full mammalian metabolism is emphasized.

Publications and oral communications

- S. Neale. The influence of mammalian metabolism on mutation induction by potential carcinogens, in "Chemical Pathways in the Environment", 1980.
- S. Neale and A.K. Solt. Factors effecting the induction of bacterial mutations by dimethylnitrosamine in mice. Chem.-Biol. Interactions, 31 (1980) 221-225.
- S. Neale and A.K. Solt. The effect of ascorbic acid on the amine-nitrite and nitrosamine mutagenicity in bacteria injected into mice. Chem.-Biol. Interactions, in press.
- A.K. Solt. Use of a host-mediated bacterial test system to study nitrosation and the effect of ascorbic acid. 5th Annual Meeting of the U.K. Environmental Mutagen Society, Canterbury, 1981.
- A.K. Solt and S. Neale. Induction of bacterial mutants in rodents treated with N-nitroso compounds. Mutation Res., 64 (1979) 147.
- A.K. Solt and S. Neale. Natulan, a bacterial mutagen requiring complex mammalian metabolic activation. Mutation Res., 70 (1980) 167-171.
- A.K. Solt and S. Neale. Induction of bacterial mutations by mutagenic metabolites produced in intact mice. Mutation Res., 74 (1980) 214.

Contractor : University of Edinburgh, Department of Genetics

Contract No. : 327-79-1 ENV UK

Project leaders : C. Auerbach, B. J. Kilbey

Title of project : Genetic tests for the detection of chemically induced  
small deletions in *Drosophila*

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Objectives : We assume that, among the so-called point mutations, small deletions of two or more contiguous genes are the greatest genetic risk; this assumption is shared by most geneticists. We have therefore tried to develop tests for assessing the proportion of small deletions among chemically induced point mutations, and we have applied these tests to a number of mutagens.

Material and Methods : *Drosophila* is by far the most suitable organism for this project. Since cytological detection of small deletions is very time-consuming and requires a highly trained observer, we have developed simple genetic tests which seemed likely to give at least a rough estimate of the proportion of deletions among the point mutations produced by a given chemical. We have used three diagnostic criteria.

(1) The ratio between the frequencies with which visible mutations at pre-selected loci on the X-chromosome of treated  $\delta\delta$  are found in the sons of attached-X  $\varphi\varphi$  and in the daughters of multiply-marked free-X  $\varphi\varphi$ . In the hemizygous  $\delta\delta$ , most deletions will be lethal and the visible mutations will wholly or mainly arise from intragenic changes. In the heterozygous  $\varphi\varphi$ , deletions as well as intragenic changes will be detected. The former can be identified by subsequent tests for lethality in  $\delta\delta$ . The higher the proportion of deletions among the visibles, the higher will be the ratio between the mutations detected in  $\varphi\varphi$  and  $\delta\delta$ , and the higher will be the proportion of male-lethals among the mutations found in  $\varphi\varphi$ .

(2) The ratio between sex-linked visibles, which include deletions, and sex-linked viable visibles, which do not do so.

(3) The ratio between the frequencies of sex-linked lethals in spermatozoa and spermatogonia. Because of germinal selection against deletions in the hemizygous spermatogonia, this ratio will increase with the proportion of deletions among point mutations. Since this does not apply to deletions in the diploid autosomes of spermatogonia, the ratio between autosomal lethals in spermatozoa and spermatogonia can be used to determine the intrinsic sensitivities of these two germ cell stages.

These tests were applied to four substances: diepoxybutane (DEB), diethylnitrosamine (DEN), hydroxylamine (HA) and ethylmethanesulphonate (EMS). For a special purpose, mitomycin-C was used in a few experiments. All substances were applied by feeding according to the technique by Lewis and Bacher. In the experiments on the first three substances, visibles were scored at the three loci w(white), sn(singed) and m(miniature); for the experiments with EMS, g(garnet), f(forked) and car(carnation) were added. Mutations in treated spermatozoa, spermatids and spermatogonia were scored in progeny from the first, fourth and 10th day after mating of the treated ♂♂. Results : The results obtained with DEB, DEN and HA are summarized in Table 1.

Table 1

Criterion		DEB	DEN	HA
1. <u>Visibles in ♀♀</u>	Spermatozoa	3.5	0.9	1.3
Visibles in ♂♂	Spermatogonia	1.0	1.8	0.8
Male-lethals among visibles scored in ♀♀	Spermatozoa	9/13	1/7	0/11
	Spermatogonia	0/6	0/5	0/5
2. <u>Lethals</u>	Spermatozoa	47	44	(app. 20)
Viable visibles	Spermatogonia	17 <sup>x</sup>	80 <sup>xx</sup>	(app. 20)
3. <u>Mutations in Spermatozoa</u>				
Mutations in Spermatogonia				
(a) sex-linked lethals		2.7	0.5	(app. 2)
(b) viable visibles		1	0.9	1.6
(c) autosomal lethals		1.1	0.3	

Ratios shown in brackets are in part based on indirect calculations

<sup>x</sup> The difference between 47 and 17 is on the borderline of significance (P = 0.07); it was similar in both experiments with DEB.

<sup>xx</sup> The difference between 80 and 44 is not significant (0.1 < P < 0.2); it was entirely due to a shortage of visibles in one of the two experiments with DEN.

DEB is known to produce chromosome breaks in Drosophila, DEN is known not to do so. HA is a weak mutagen, unlikely to break Drosophila chromosomes. Deletions were therefore expected from treatment with DEB but not from treatment with the other two chemicals. The results of our experiments agreed with expectation. DEB yielded three times as many mutations in ♀♀ than in ♂♂, and the majority of those found in ♀♀ were lethal in ♂♂. This applied only to spermatozoa; in spermatogonia germinal selection had already removed most



or all deletions (3a in Table 1). Row 3c shows that intrinsic sensitivity to DEB did not differ between spermatozoa and spermatogonia. DEN and HA gave quite different responses from DEB: no excess of visibles scored in ♀♀, hardly any male-lethals among them, no germinal selection (intrinsic sensitivity to DEN is about twice as high in spermatogonia as in spermatozoa, but the ratio of autosomal to sex-linked visibles was the same at both stages). HA also yielded a lower ratio of lethals to viable visibles than DEB but, surprisingly, this did not apply to DEN. One possible reason for this, a preponderance of nonsense mutations, is still under test; another possibility would be that DEN produces very small deletions, too small to include a lethal next to a visible.

For EMS, existing evidence suggested a low frequency of small deletions. Our results do not agree with this. In all of 7 experiments, and at doses differing from 5 - 12% sex-linked lethals, visibles were about twice as frequent in ♀♀ as in ♂♂, and more than half of those scored in ♀♀ were lethal in ♂♂. Since EMS does not act in spermatogonia, no tests for germinal selection could be carried out; but the fact that the transmission of visibles was significantly higher through ♀♀ than through ♂♂ suggests that there may be germinal selection. Accurate calculation of the ratio between lethals and viable visibles was made difficult through the very high frequency of mosaics among the visibles, but the ratio certainly was not lower than after treatment with DEB. In view of the discrepancy of our data with many - although not all - of those found in literature, we have also considered the possibility that EMS tends to produce clusters of separate closely linked mutations, a hypothesis for which there is some evidence in literature.

Mosaicism was also a major difficulty in the experiments with HA. This led us to testing whether this substance, which is only marginally mutagenic in the usual sex-linked lethal test, yields more mutations when the tests are carried through to F<sub>3</sub>. This was indeed the case: in two experiments, about 2% sex-linked lethals were found in F<sub>3</sub> as compared with 0.4 - 0.5% in F<sub>2</sub>.

The experiment with mitomycin-C was carried out to test whether its reported specificity for the production of chromosome breaks in spermatogonia was not rather due to a long "storage effect". Experiments in which sex-linked lethals and translocations were scored in spermatogonia and in spermatozoa with and without storage indicated that this is indeed the true explanation: when treated spermatozoa were stored until the time when the spermatogonially treated broods were mated, translocation frequencies were the same.

Conclusions : The first experiments had shown that our diagnostic criteria for the presence of deletions among point mutations agreed with expectation from independent observations. For EMS this is not so clear; many workers have failed to find small deletions after treatment with EMS, and others have found fewer than our data indicate. It would be interesting to analyse this discrepancy further; but for our project this is not important. Whatever the reason for the fact that a high proportion of EMS-induced visibles are lethals, this very fact makes EMS a good candidate for a high-risk mutagen, especially in heterozygotes for treated chromosomes. Temin has indeed shown that this is the case. To finalize our project, it will now be necessary to test whether the classification of chemical mutagens by our technique is indeed a lead to the effects of these mutagens on heterozygotes. We hope to be able to carry out such tests on DEB and DEN and, if the results are encouraging, on other mutagens not yet classified by our technique.

The data for HA show that it is advisable to test marginally effective mutagens for delayed lethals in  $F_3$ . Those obtained with mitomycin-C show that, in brood patterns for breakage, the passage of time has to be considered as a possible factor.

Reports : (1) in Paris, February 1980.  
(2) in Pisa, December 1980.

Publications : P. T. SHUKLA and C. AUERBACH (1979). The delayed mutagenic action of hydroxylamine in *Drosophila*. *Mutat. Res.* 61: 399-400.  
P. T. SHUKLA and C. AUERBACH (1979). The brood pattern of mitomycin-C-induced translocations in *Drosophila melanogaster* males: the effect of time. *Genetics* 93: 403-409.  
P. T. SHUKLA and C. AUERBACH (1980). Genetics tests for the detection of chemically induced small deletions in *Drosophila* chromosomes. *Mutat. Res.* 72: 231-243.  
P. T. SHUKLA and C. AUERBACH (in press). Genetics tests for the frequency of small deletions among EMS-induced point mutations in *Drosophila*. *Mutat. Res.*

Contracter: University College of Swansea, Swansea, U.K.

Contract No. ENV - 357 UK

Project Leader: Dr. James M. Parry

Title of Project: Comparative study of the genetic effects of environmental mutagens and carcinogens during mitosis and meiosis

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#### Objective of the research

It is now well-established that short-term tests may be used to detect the activity of potentially mutagenic and carcinogenic environmental chemicals. However, it is also clear that no single assay can be relied upon to detect all classes and types of chemical mutagens and carcinogens. The international acceptance of these facts is reflected in the design of several proposed guidelines for the detection and regulation of environmental carcinogens and mutagens. Each of these require the use of a range of different assays involving both different species and genetic end-points.

The preliminary screening of environmental chemicals generally involves the use of simple microbial assays and various types of cultured mammalian cells undergoing mitotic or somatic cell division. However, assessment of the genetic risk of human populations requires the assessment of the extent of genetic damage in the gametes which are undergoing meiotic cell division. There are thus two distinct types of cell division events, i.e. mitosis and meiosis involved in the determination of the genetic effects of environmental mutagens and carcinogens. At the present time we have only limited information on the relative effects of DNA damage upon the two types of cell division. It would be of considerable value to have an understanding of the number of important parameters of both mitotic and meiotic cell division, these include: the rates of lesion formation, the rates of repair of lesions and the genetic consequences of such lesions in terms of mutation, chromosome aberration and chromosome aneuploidy.

The yeast Saccharomyces cerevisiae provides us with the simplest model system for the comparative study of the induction of DNA damage and its genetic consequences during both mitosis and meiosis. Saccharomyces cerevisiae has been extensively studied genetically and numerous sophisticated systems are available for the detection of such genetic end-points as mutation, recombination and chromosome aneuploidy. Studies of the regulation of DNA repair activity in Saccharomyces are well advanced thanks to the large and comprehensive collection of repair deficient mutants available. Studies of cells undergoing both mitotic and meiotic cell division may be performed by the use of the appropriate culture media and large numbers of cells may be isolated at specific stages of the cell cycle.

The aims of this project, which commenced in 1980, has been the development of an assay system for the comparative study of the induction and consequences of DNA damage in both mitotic and meiotic cell division in yeast. The intention has been to extend the observations made in the model system to appropriate mammalian systems.

#### Materials and Methods

The project has involved the development of a number of biochemical and physiological techniques and the construction of a variety of strains capable of assaying the induction of a wide range of genetic end-points. These can best be summarised as follows:-

1. The development of physiological parameters for the production of synchronous mitotic and meiotic cell division in genetically marked yeast strains.
2. The construction of yeast strains capable of assaying the induction of a) chromosome aneuploidy, b) point mutation and c) reciprocal and non-reciprocal recombination, during both mitosis and meiosis in the presence and absence of different repair systems.
3. The development of assay systems for the detection of the end-points outlined above.

4. The development of techniques for the detection of chemically induced DNA strand breaks and adducts. These have involved the use of sucrose gradients for the determination of single and double strand breaks and of radiolabelled carcinogens/mutagens and cesium chloride gradients for the detection of binding between test compound and DNA.
5. The development of techniques for the detection of the extent and rates of repair of the lesions detected by 4) during both mitosis and meiosis.

### Results

The major results of the project during 1980 has been the successful development of the techniques and yeast strains described in the Methods section. We are now able to follow the induction and repair of DNA damage and assay its genetic consequences in yeast cells undergoing both mitotic and meiotic cell division.

Yeast cells harvested as discrete fractions from a mitotically dividing culture can be separated to the G<sub>1</sub>, S and G<sub>2</sub> phases of the cell cycle. When such cells are exposed to chemical mutagens/carcinogens they are characterised by a peak of induction of all the genetic end-points assayed in cells treated during the S or DNA synthetic period. This S phase peak of induction is specifically eliminated only in cultures carrying defective forms of the RAD 50 gene of yeast.

Similar experiments with yeast cells treated during meiotic cell division (sporulation) reveal a number of interesting differences with the mitotic event. Meiotic division is characterised by a peak of induction of gene conversion during S phase whereas no such peak of induction can be observed for point mutation (for the base substitution mutations so far studied) even at levels of mutagen treatment that produce > 10 fold increases in mutation across the cell cycle. However, the rates of repair of DNA strand breaks appear to be approximately 50% higher during pre-meiotic S phase. Experiments using the radiolabelled mutagen nitrosoguanidine indicate that the rate of uptake of the

mutagen and the rates of repair of adducts in nuclear DNA is identical across the whole of the cell cycle. In contrast, there is a significant increase of label uptake into mitochondrial DNA during the S phase of cell division.

The results obtained so far clearly indicate that mitotic and meiotic cell division are characterised by different responses to both the induction and genetic consequence of DNA damaging agents.

### Conclusions

The results obtained clearly indicate the potential of our yeast systems for the assessment of the consequences of environmental mutagens/carcinogens upon cells undergoing both somatic (mitotic) and gametic (meiotic) cell division. It is our intention to utilize the systems for the examination of the effects of carcinogen/mutagen analogues and to extend our observations to comparable systems in mammalian cells.

### Publications

Preliminary data from the project were discussed at the following International Meetings.

1. R. S. Tippins, S. Kelly, C. Merrill, R. Waters and J. M. Parry.  
A study of the effects of physical and chemical mutagens during meiosis in yeast.  
Proceedings of the 10th International Conference of Yeast Genetics and Molecular Biology (1980) 115 (Louvain-la-Neuve, Belgium).
2. S. Kelly, R. S. Tippins, C. Merrill, R. Waters and J. M. Parry.  
Some effects of alkylating agents during meiosis in yeast.  
Proceedings of the 10th Annual Meeting of the European Environment Mutagen Society (1980) 42 (Athens, Greece).

Contractor : University of Dublin, Trinity College of

Contract No : 169-77-1 ENV EIR

Project Leader : G.W.P. Dawson (Mice); S. Thompson (Bacteria)

Title of project : Mutagenesis Test Systems in Bacteria and Mice

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Development of the Microbial Assay for screening environmental pollutants

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Objective of the research. In developing microbial assays for the screening of chemicals, either single target back-mutation systems, such as developed by Ames and his co-workers, or multitarget forward mutagenesis systems may be selected for examination. Starting from the legitimate premise that chemicals display high specificities in their interactions with the different DNA bases and base sequences, the theoretical advantage of a good forward mutagenesis system is that a large number of different base-sequence targets would be available in the one gene (or genes) under examination. Thus the base sequence specificity of drug interaction need not constitute the crucial factor in the detection of the drug as a mutagen by the system. As this theoretical advantage is substantial, we chose to examine a number of forward mutagenesis systems in Salmonella typhimurium and Escherichia coli as potential alternatives to test systems utilizing back-mutations.

Materials and methods. Mutants of the galactose operon conferring galactose sensitivity were maintained on nutrient agar and plated for galactose resistance on minimal media containing 0.2% galactose and 0.2% glycerol. Water insoluble mutagens were dissolved in dimethylsulphoxide. Chemicals were added into a soft agar overlay in plate mutagenicity tests together with bacteria and liver microsomal mix when required. All manipulations involving induction and preparations of liver were essentially as in Mutation Res. 31, 347. Pure microsome preparations were according to the technique of Kupfer and Levine (BB Res. Com. 47, 611). Mouse mediated assays were essentially as in Soc. Exp. Biol. Med. 130, 83) and urine metabolite tests were modifications of Proc. Nat. Acad. Sci. 71, 737.

Results. Among several forward mutagenesis systems tested by us previously, the galactose system appeared most suitable for development. 8-azaguanine resistance system (BBA 53, 166) proved a poor detector of cytosine-guanine repeat specific mutagens. L-arabinose resistance (Genetics 47, 416) lactose-resistance (CSHSQB 81, 189) and Sodium chromate resistance (J. Biol. Chem. 239, 2292) all failed to respond to guanine repeat as well as CG repeat specific mutagens. The chromate system was also poor in detecting general base-substitution and frameshift mutagens. Presence of rfa mutations or the plasmid pKM101 both had complicating effects on one or

more of the systems. The presence of liver homogenate resulted in elevated spontaneous rates of mutation in all the systems. However, the galactose resistance system (Genetics 46, 1295) responded to general base-substitution mutagens and to mutagens specific for G repeat and CG repeat targets. Although the spontaneous rate to resistance mutations was found to be high, both in the presence and absence of the pKM101 plasmid, and the presence of liver preparations resulted in even higher levels of resistant mutations, nevertheless, since tests showed that the system could detect some carcinogens as mutagens previously undetectable by the Ames system, and since it had been shown to have all the Ames tester targets, it was considered potentially useful, and selected for development.

Manipulations involving composition of solid media, liquid growth media, cell numbers plated and temperature of incubation, all factors influencing the spontaneous rate of mutation, has shown that the observed spontaneous rate could be reduced, if desired, to as low as 20 for  $10^7$  cells plated. A protocol has been developed for maintaining consistent and reproducible spontaneous mutation rates.

Comparisons have been made between the use of crude 9000g supernatant liver preparations from normal and prestarved animals, pure microsome preparations, and substitutions of NADP and glucose-6-phosphate by NADPH in the homogenate mix preparations, with the objective of reducing the level of spontaneous mutations which are obtained in the presence of liver preparations. In general, preparations from prestarved animals have only marginal effect in reducing observed spontaneous resistant mutants. Pure microsome preparations have significant effect in reducing observed mutants, but show relatively poor activation of chemicals when used at the standard level. Comparable levels of activation may be obtained with pure microsomes by using NADPH and up to sixteen times the level of the standard preparations. Further tests are in progress. When this aspect of the investigation is concluded, attention will be focussed on the spontaneous mutation rate of the system in the presence of the pKM101 plasmid.

Mutagenicity of phenazine derivatives using *in vitro* tests, mouse mediated assays and urine metabolite tests. Phenazine derivatives are potential intercalating agents. Among five synthetic derivatives, one is in clinical use in the treatment of leprosy and analogues are being developed as alternatives for therapy. In our tests, none are mutagenic *in vitro* towards TA98 or TA100 at up to 1mg levels, with or without activation. None are differentially inhibitory towards endonuclease II or polymerase A mutants. The clinically used analogue selected for mouse mediated assays has shown negative results. Two of the compounds have been selected for urine metabolite tests. Despite extensive detailed tests which included daily sample testing for three consecutive days, rat or mouse as the host, two methods of feeding of the chemical and low heat sterilization as alternative to filtration (to avoid possible adsorption of the urine metabolite(s) to filter material), no evidence has yet been obtained of mutagenic metabolites in urine. Mutagenic urine metabolites, however, have been reported from Johns Hopkins University, so the data are in conflict.



### Identification of mutagen target sequences by DNA sequencing

Objectives of the research. One of the advantages of a tester system based on back-mutations is the potential for revealing chemical affinities of mutagens for specific DNA base sequences. For example, the TA1537 and TA1538 tester strains in Salmonella developed by Ames et al. allow the identification of those chemical mutagens which preferentially attack repetitive guanine and repetitive cytosine-guanine (and their complementary sequences) on DNA. For the system to be comprehensive, however, a number of other base sequence target strains need to be developed. Failure to detect some carcinogens as mutagens may be partly due to the absence of appropriate target sequences from presently available back-mutation testers. The objective of this research was to develop the techniques of cloning and DNA sequencing for application to mutants of Salmonella able to detect some false negatives as mutagens.

Materials and methods. The region chosen for cloning and DNA sequencing was the Salmonella trp region. The major restriction enzyme chosen was Eco RI. This enzyme is known to leave the trp region intact (J. Bact. 129, 338) which would be essential for the initial cloning. Other restriction enzymes were HaeIII, HhaI, HpaII, hin fI. These enzymes appear to cleave the trp region frequently (J. Mol. Biol. 121, 153) and are essential to preparations immediately prior to DNA sequencing. The vector chosen for cloning was the E. coli plasmid pBR322 (Nucleic Acids Res. 5, 2721). This vector whose entire 4332 base-pairs are known has a single Eco RI site of restriction and has the advantage of existing at high copy number per cell and replicating in the relaxed mode. For cloning of the trp region, bulk Salmonella DNA was prepared by a modification of the procedure of Marmur (J. Mol. Biol. 3, 208). Salmonella and plasmid DNA were restricted by Eco RI, mixed in specific molar ratios and allowed to ligate in the presence of T4 ligase. Following identification of suitable hybrid plasmids in transformed E. coli recipients, the plasmid DNA was isolated and restriction patterns identified by the technique of slab-gel electrophoresis (J. Mol. Biol. 110, 119). 1% agarose gels allow separation of fragments of 10,000 to 2000 base pairs and 3%, 5% and 8% polyacrylamide gels allow the separation of fragments of 2000 to 30 base pairs.

Results. Salmonella trp DNA from wild-type, two frameshift mutants and a deletion strain spanning the sites of the frameshift mutants have been used in cloning experiments. Using a transformation procedure, whereby the ligated plasmid DNA together with CaCl<sub>2</sub> treated recipient cells is incubated at 45°C, numbers of transformants up to 2x10<sup>5</sup> per µg of plasmid DNA have been obtained. Prolonged treatment of recipients with CaCl<sub>2</sub> increases the transformation rate. However, freezing of cells in CaCl<sub>2</sub> solution containing 15% glycerol (recommended as a method of storage - Nucleic Acids Res. 4, No. 7) greatly reduces viability.

Using the deletion strain spanning the sites of the frameshift mutants, many independent transformants have been made for analysis. To isolate plasmid DNA from large numbers of transformants, a rapid mini-screen method for plasmid DNA isolation has been developed. HaeIII restriction analysis has shown that the amount of Salmonella DNA formed in the different transformants varies considerably. HaeIII restriction pattern of Ø80 DNA also carrying Salmonella trp region confirmed that at least some fragments are common to all DNA digests. Interestingly, only transformants which contained the largest HaeIII fragment found in the Ø80 digest, also checked out on media selective for trp transformants. It thus appears that the transformants may be unstable, with accompanying loss of the cloned trp region. This is clearly shown in the pattern of HaeIII digests following several successive generations of colony growth and replating. Such restriction digests show considerable variation in pattern. In contrast, the pure plasmid digests alone, or the Ø80 trp digests remain consistent in restriction pattern following successive platings.

Conclusions and additional comments. Analysis of cloned trp region using bulk DNA material from Salmonella and the plasmid pBR322 has indicated that the resultant hybrid plasmids show a propensity towards genetic rearrangements and loss of the foreign material. The genetic rearrangements may arise, for example, if more than one type of hybrid plasmid enters the same transformant, followed by recombination events. Loss of cloned DNA region may occur if the presence of particular genes in high copy number (in our case the trp operon genes) confer a selective disadvantage to cells harbouring multi-copy plasmids. Genetic rearrangements and losses can be minimized, however, by following alternative experimental strategies.

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#### Development of the Somatic Mutation Test in the Mouse

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Objective of the research. In the examination of the frequency of induced mutations in the mouse the best established test is to cross animals which are heterozygous for a number of factors with animals homozygous recessive for the same factors. Mutant offspring are scored as a measure of the frequency of mutations at these loci in the germ line. The total numbers of animals to be scored in this test is often high, into the thousands or tens of thousands. In the somatic mutation test, embryos heterozygous for many genes are treated with the potential mutagen and mutations in those genes which affect coat colour are scored as areas/spots of changed coat colour in the mice four weeks after birth. The numbers of mice required are relatively low, in the hundreds, and the objective is to test a wide range of chemicals to evaluate the correlation between the results of this test and of other tests.

Materials and Methods. Female mice of the T strain (non-agouti, aa; pink eye, pp; brown, bb; dilute, dd; chinchilla, c<sup>ChcCh</sup>; spotted, ss; short-ear, sese) were mated to males of the HT strain (aa; leaden, lnl; pearl, pepe; pallid, papa; fuzzy, fzfz; brachypody, bpbp). Pregnant females were treated with the test chemical either orally or intraperitoneally. Two types of alteration were distinguished: a coloured spot which may occur anywhere on the coat and which is presumed to be due to a somatic mutation of a coat colour gene, and white spots on the belly which may be due to cell killing. Analysis of log-likelihood-ratios was performed on the relative frequencies of offspring with non-white spots. Effects were assumed to be additive on the logit scale. The calculations were performed by computer with the GLIM programme.

Results. The test has been used with the following compounds during this period:

1. Succinic anhydride. There were significant differences between the controls and the treated (P=.0002) and there is a clear positive dose response. There were no significant differences between days of injection (P=0.18) and residual deviations were consistent with binomial sampling (P=0.19).
2. Atrazine. There were significant differences between the controls and the treated (P=0.024). There were no significant differences between days of injection (P=0.51) and residual deviations were consistent with binomial sampling (P=0.64).
3. Manganous chloride. There were no significant differences between treated and controls (P=0.73) nor between days of treatment (P=0.48), and residual variation was consistent with binomial sampling.
4. Chromium trioxide. There were no significant differences between the controls and the treated (P=0.52), nor between days of injection and residual deviations were consistent with binomial sampling (P=0.77).
5. Chrome alum. There were significant differences between treated and control mice (P=.004), but not between days of treatment (P=0.88), and residual variation was consistent with binomial sampling (P=0.46).
6. 9-aminoacridine. There were no significant differences between the treated and the controls (P=0.80), nor between days of treatment (P=0.32), and residual variation was consistent with binomial sampling (P=0.59).

As the testing proceeds one result of particular interest is emerging. It is that, of six known carcinogens tested previously in this period, all of which gave negative results when tested for mutagenicity by the Ames test, five have given positive results with the mouse spot test. It seems possible that quite a high proportion of Ames's false-negatives are identified as mutagenic by this system.

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**Contractor:** Gesellschaft zur Förderung der Lufthygiene und  
Silikoseforschung e.V., Medizinisches Institut  
für Umwelthygiene an der Universität Düsseldorf

**Contract No.:** 289 - 76 - 10 ENVD, Project 2

**Project leaders:** Prof.Dr.med. H.-W. Schlipkötter  
Dr. K.H. Friedrichs

**Title of project:** Determination of fibrous particles in ambient  
air and in human lung dust

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#### **Objective of research**

- I. Determination of fibrous particles in lung dusts from deceased  
which had been exposed to different amounts of dust and fibres  
during their lifetime.
  
- II. Determination of fibres in the ambient air.

#### **Methods and results**

Aim of the investigation was the development of a technique suitable  
to determine concentration, size distribution, and chemical composition  
of fibres in dust samples. On account of the fineness of particles  
and the necessity to analyse single fibres for this purpose only  
microscopic methods could be used. The evaluations were performed  
in the following manner:

1. Determination of fibre concentration
  - a) optical microscopy (phasecontrast-technique), magnification 800 x.
  - b) scanning electron microscopy (SEM) using accelerating voltage of 20 KV and magnifications varying from 2 000 to 10 000 x direct.
  - c) transmission electron microscopy (TEM) utilizing accelerating voltage of 100 KV and constant magnification of 22 000 x.
  
2. Estimation of fibre dimensions
  - a) SEM: direct measurement on SEM-screen at a standard magnification of 10 000 x.
  - b) TEM: indirect technique with the aid of microfotos taken at a magnification of 22 000 x.
  
3. Methods of fibre identification
  - a) SEM: by energy dispersive x-ray analysis (EDXA) for fibres of ambient air samples with diameters more than 0.5  $\mu$ m only at a magnification of 10 000 x.
  - b) TEM: by EDXA and selected area diffraction (SAED) technique for all fibres at a magnification of 22 000 x.

**Part I: Evaluation of human lung dust samples**

130 human lung dust samples in total were examined with the SEM. All cases could be classified into following groups:

- 24 samples from lungs without any proved occupational exposure to asbestos (normal lungs = group N),
- 40 samples from lungs of humans who died of mesothelioma without any proved occupational exposure to asbestos (spontaneous cases of mesothelioma = group SM),
- 36 samples from humans who died from a mesothelioma and who had been occupationally exposed to asbestos (occupationally caused mesothelioma cases = group BM),
- 30 samples from lungs of human asbestosis cases, partly with an accompanying bronchial carcinoma, but without mesothelioma (asbestosis cases = group A).

The results of the examinations may be summarized as follows:

1. The average fibre content found in lungs of asbestosis cases is 60 times higher than that of normal lungs.
2. The total fibre content in the lungs of BM-cases in comparison to the SM-group is 8 times higher, if arithmetic means are used. The distribution of single cases per group causes overlapping between them, which does not permit confident distinction.
3. The diameter distribution of fibres of all groups was similar. Lungs of persons who had been occupationally exposed to asbestos contained a higher amount of longer fibres when compared to normal lungs (figure 1). Using this difference as indicator of asbestos/non asbestos exposed cases, one may be able to distinguish between both groups (figure 2).
4. The fibre content in the lungs of SM-cases in comparison to normal lungs is insignificantly higher. This is valid for total fibres as well as for fibres above 5  $\mu\text{m}$  in length.

Only very few TEM-data can be presented at the moment, because the analytical instrumentation was incomplete until the second half of 1980. The results may be summarized as follows:

1. Fibre concentration data are higher, when compared with SEM-values. This result may be due to the influence of microscopic magnification (i.e. resolving power).
2. Identification of fibres by means of EDXA only is 'inconfident', because chemical composition of fibres may have changed during the long time of deposition. In consequence of this, structural characterisation by performing SAED-patterns is inevitable.



## Part II: Evaluation of ambient air samples

Different sampling instruments (impactors, thermal precipitators, and filtration units) were compared in ambient air measurements and under laboratory conditions utilizing dust chambers. It was found that filtration technique seems to be irreplaceable, when sampling for all particle sizes is necessary.

### Results of measurements

#### 1. Optical microscopy

Since 1973 samples were taken at our locations in Düsseldorf, Duisburg, Gelsenkirchen (urban areas), and Krahm (rural area). The results are summarized in Table 1. Measurements indicate fibre concentrations in the range of 1 fibre per liter air in the cities and 0.1 fiber/liter at the remote station respectively.

Seasonal variations of fibre concentration could not be proved. On the other hand, there is a great daily variation of concentration data and, as a consequence of this, standard deviation reaches the magnitude of measuring data. During the sampling period from 1973 to 1978, the annual average of fibre concentration changed only insignificantly.

#### 2. SEM-examinations

SEM-counting results of ambient air samples taken at our locations in Duisburg, Düsseldorf, Gelsenkirchen and Krahm are given in Table 2. The fibre concentration in the cities was found to be in the range of  $10^1$  f/l air, and at the rural station in the range of  $10^0$  f/l, in any case it was about 10 times higher than data calculated from optical microscopy. The standard deviation of data reaches the same order of magnitude as the concentration itself.

From the size distribution of fibres in the ambient air it was to be seen that 50 % of the fibres were thinner than  $1 \mu\text{m}$  and shorter than  $6 \mu\text{m}$ . At the remote location fibres were even smaller (figure 3).

EDXA-analyses of fibres above 0,5  $\mu\text{m}$  in diameter indicated, that 24 % of the fibres in the ambient air consist of asbestos, only 1 % of glass and mineral wool. About 75 % of all fibres were not identifiable.

### 3. TEM-examinations

Only very few samples from Düsseldorf, Duisburg and Krahm were analysed up to now, because of the reasons mentioned earlier. The results may be summarized as follows:

1. Fibre concentrations in the cities range about  $10^3$  f/l air, and in the rural area about  $10^1$  f/l air.
2. The size distributions of fibres of the ambient air samples from locations in Duisburg and Krahm are given in figure 4. As can be seen from the diagram, most fibres are below 1  $\mu\text{m}$  in diameter and 10  $\mu\text{m}$  in length.
3. Results of the combined EDXA- and SAED-analyses are given in table 3. Obviously there is no remarkable difference between TEM- and SEM-data about the chemical composition of fibres in ambient air samples.

### Conclusions

In the second part of this study, further examinations of samples of human lung dust and of the ambient air have been performed by means of SEM- und TEM-techniques. The analyses demonstrated that for morphological examinations scanning electron microscopy is a very useful method, which in comparison to TEM is inexpensive and quick. Quantitative analytical aspects can only be solved by SAED- and EDXA-analyses, i.e. analytical transmission electron microscopy. It is necessary to get further information about the composition of fibres in the ambient air.

Table 1:

Arithmetic annual mean concentration of fibres per m<sup>3</sup> air at locations in Düsseldorf, Duisburg, Gelsenkirchen and Krahm. Evaluation by optical microscopy at 800 x, sampling period 1973-1978.

year	sampling station at			
	Düsseldorf	Duisburg	Gelsenkirchen	Krahm (Bergisches Land)
1973	2690		2510	
1974	2260		4180	
1975	2170			
1976	1800	1800		240
1977				270
1978				230

Table 3:

Results of fibre identification in ambient air samples of locations in Düsseldorf, Duisburg and Krahm. Sampling period: 1976.

location	chrysotile	amphiboles	glass	others
	(%)	(%)	(%)	(%)
Düsseldorf	7.0	9.6	-	83.4
Duisburg	11.2	1.4	10.6	76.8
Krahm	13.5	4.0	-	82.5

Table 2:

Arithmetic monthly mean concentration of fibres per m<sup>3</sup> air at locations in Gelsenkirchen, Duisburg, Düsseldorf and Krahn.

SEM-evaluation at 5 000 x, sampling period: 1975 and 1976.

month	sampling station at			
	Gelsenkirchen	Duisburg	Düsseldorf	Krahn
1/1975	55 270			
2	85 040			
3	27 660			
4	16 120			
5	14 810			
6				
7	15 960		22 250	
8				
9	17 910		13 840	
10	14 880		15 960	
11			49 520	
12	37 200			
1/1976			30 010	
2			27 400	
3			36 990	
4			26 010	
5			18 720	
6				
7				
8				2 640
9		35 920		2 750
10				
11				2 680
12		28 010	17 110	2 960

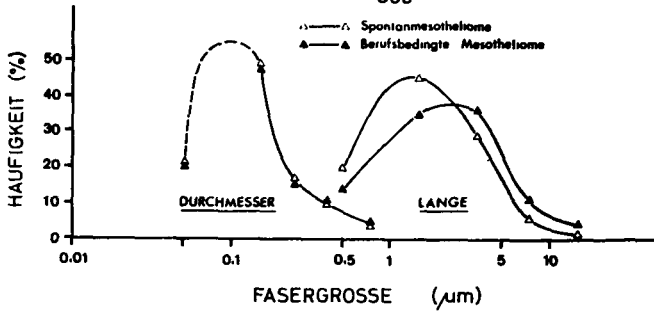


Figure 1: Frequency distribution of fibre size (diameter - and length) in human mesothelioma cases with/ without occupational exposure to asbestos.

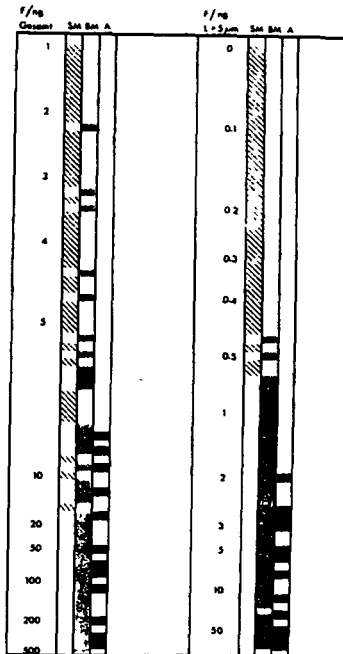


Figure 2: left hand side: total fibre concentration (f/ng) per nanogram lung dust in spontaneous mesothelioma cases (SM) and in occupationally caused mesothelioma cases (BM), partly accompanied by asbestosis (A). right hand side: concentration of fibres above 5 µm in length ( $f > 5 \mu\text{m}/\text{ng}$ ) in mesothelioma cases (see above).

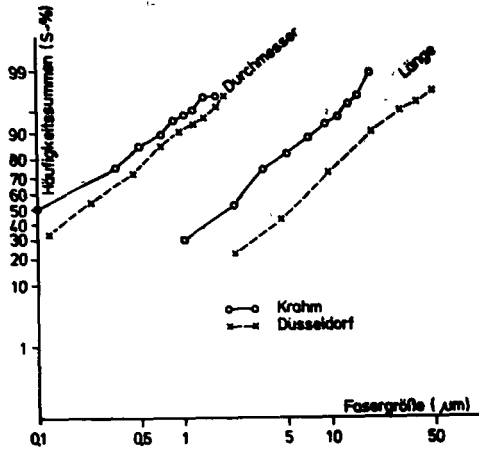


Figure 3: Size distribution of fibres in ambient air samples of locations in Düsseldorf and Krahm (remote station), SEM-evaluation at 10 000 x.

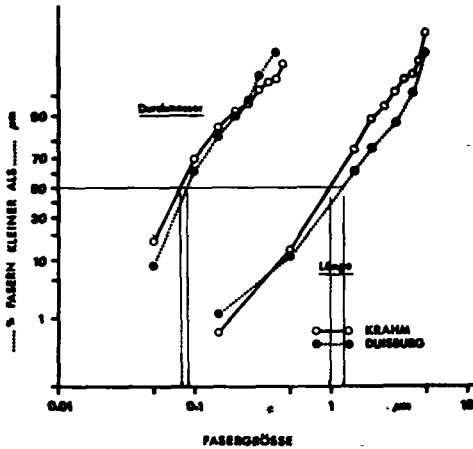


Figure 4: Size distribution of fibres in ambient air samples of locations in Duisburg and Krahm (rural area), TEM-evaluation at 22 000 x.

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**Contract No:** 298-78-1 ENV D  
**Project Leader:** Prof. Dr. H.-J. Weitowitz, Institute and  
Polyclinic for Occupational and Social  
Medicine of the University of Gießen  
**Title of project:** Epidemiological investigations on the fibro-  
genic risks after exposure to asbestos cement  
fine dust on building sites  
**Authors:** H.-J. Weitowitz and K. Rödelsperger

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#### Objective of the research

Besides workers in the asbestos industry and the asbestos cement industry a large number of users of asbestos cement and other asbestos containing products may be exposed to asbestos cement fine dust. For example in the Federal Republic of Germany about 100.000 to 120.000 tons of asbestos are processed into about 1,2 million tons of asbestos cement products which are handled by craftsmen on building sites (1).

Not only chrysotile is used but too amosite for fire insulation and crocidolite for water pipes.

It is the aim of our present epidemiological study to measure the asbestos fine dust and fibre concentration during the handling of asbestos cement products containing chrysotile as well as amphiboles.

Since differences in the different kinds of asbestos are assumed (2), fibre concentrations as well as length and diameter distributions of chrysotile, amosit and crocidolite fibres, emitted simultaneously, are compared. Furtheron it is intended to estimate the asbestos related fibrogenic risk for the relative large group

of craftsmen handling asbestos cement products on building sites.

### Materials and Methods

Dust measurements which in part have already been published (3-8) were done on about 100 building sites during roofing and siding work and during installation of ventilation shafts, fire insulation and water pipes. Altogether 4 static and 4 personal dust samplers and a tyndallometer were used. Mass concentration of total dust and fine dust was determined. For stationary sampling the asbestos content in the fine dust was measured by infrared spectrography. Fibre counting was done by phase contrast and by scanning electron microscopy. Further on fibre sizing and fibre analysis was done in the scanning electron microscope using energy dispersive x-ray spectroscopy (EDX). In addition to the dust measurements occupational anamneses of craftsmen were performed together with an extended medical examination. A cohort of about 400 craftsmen handling asbestos cement products for eight years or more was examined. For the occupational anamneses standardized questionnaires on chronic bronchitis (9) and a special standardized inquiry form concerning the handling of asbestos cement products, other asbestos containing materials and man made mineral fibres were used.

### Results and Discussion

#### Dust measurements

Most of the dust measurements were performed during roofing operation with corrugated asbestos cement sheets containing chrysotile only. Tyndallometer measurements showed high concentrations of fine dust only during the use of the grinding machine. Cutting with this machine is commonly done on sheet corners which overlap on the roof. Once it is usually performed on a stack of sheets in the open air at fixed location (type 1). Otherwise cutting is done directly on the roof (type 2).



For type 1 building sites cutting time is about 3 to 13 % of the total working time with a mean value of 5,7 %.

The probability density function of fine dust mass concentration measured from stationary as well as from personal sampling shows lognormal behaviour (fig. 1).

Measured values of fine dust range from 0.1 to 1.35 mg/m<sup>3</sup> for stationary and from 0.13 to 7.6 mg/m<sup>3</sup> for personal sampling. Median values were 0.34 mg/m<sup>3</sup> for stationary and 1.0 mg/m<sup>3</sup> for personal sampling. Mean value and confidence interval of fine dust estimated from the measured values for a lognormal probability density function (7) are listed in table 1. The chrysotile content of the fine dust determined by infrared spectroscopy amounts to about 10 %.

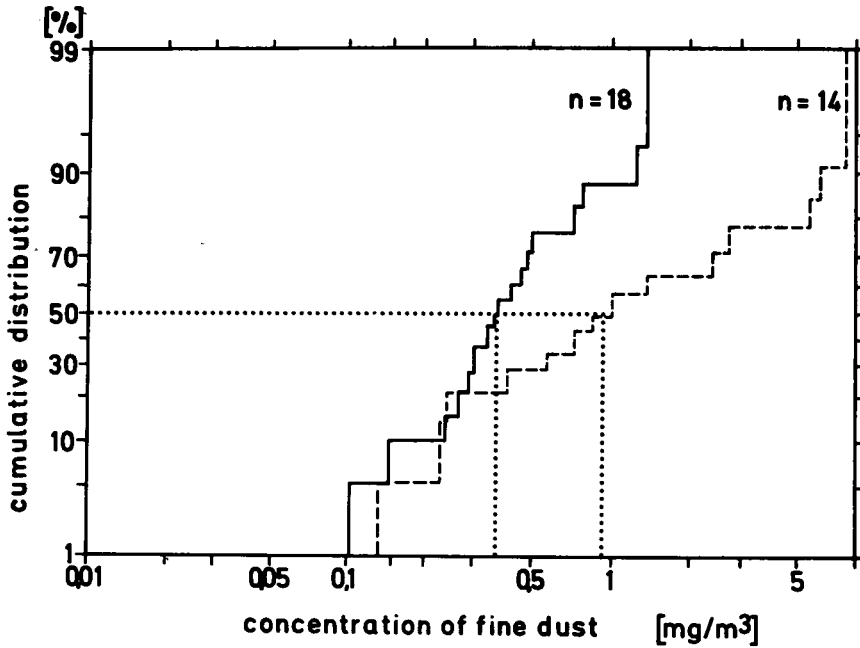
Though there is a different frequency and type of exposure for cutters and bystanders for both types of building sites, the mean exposure to chrysotile fine dust is about the same. By personal dust sampling, it is estimated to

$$0.11 \text{ mg/m}^3,$$

summarizing exposure as well for cutters as for bystanders. This is about half of the mean value of asbestos fine dust exposure of cutting craftsmen on type 1-building sites.

The mean value of fibre concentration during a daily working shift calculated from fibre concentration during cutting processes and from the cutting time only reaches  $1.2 \cdot 10^6$  fibres/m<sup>3</sup> with length  $L > 5 \mu\text{m}$ . As before by personal dust sampling the mean daily fibre concentration to which craftsmen roofing with corrugated sheets are exposed is estimated to be half this value for both types of building sites ( $0.6 \cdot 10^6$  fibres/m<sup>3</sup>).

By stationary dust sampling at cutting locations with the scanning electron microscope median values of  $20 \cdot 10^6$  fibres/m<sup>3</sup> of all fibres  $L > 0,3 \mu\text{m}$  and  $1.4 \cdot 10^6$  fibres/m<sup>3</sup>  $L > 5 \mu\text{m}$  are observed. In fig. 2 and 3 fibre length and diameter distributions of similar working places are plotted.



**Fig. 1:** Cumulative distribution function of fine dust mass concentration at open air cutting locations on type 1-building sites during roofing with corrugated asbestos cement sheets.

————— static sampling.  
 ----- personal sampling.

**Table 1:** Estimated mean value and confidence interval of the fine dust mass concentration measured on type 1 - and type 2 - building sites for static and personal sampling.

roofings with corrugated asbestos cement sheets	sampling	n	Fine dust [mg/m <sup>3</sup> ]	
			estimated mean value	confidence interval
cutting in the stack (type 1)	stationary	19	0,51	0,37 - 0,79
	personal	14	2,2	1,3 - 8,9
cutting on the roof (type 2)	stationary	3	0,5	0,2 - 35
	personal	8	1,82	0,85 - 9,81

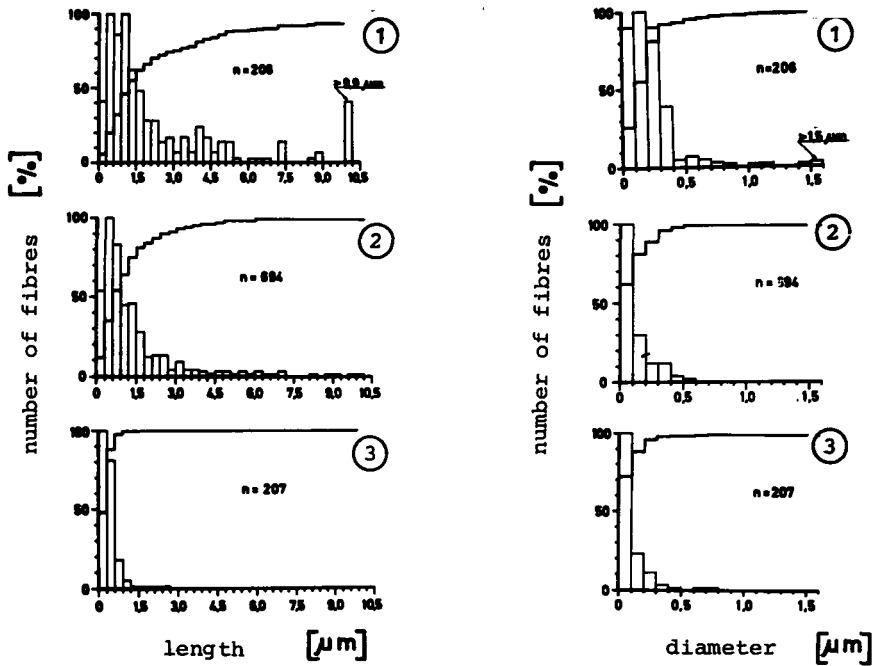


Fig. 2: Length distribution of chrysotile fibres in airborne asbestos cement dust emitted during cutting processes with the grinding machine.  
1. Personal sampling  
2. Stationary sampling at the cutting location  
3. Stationary sampling about 30 m distant from the cutting location.

Fig. 3: Diameter distribution of chrysotile fibres in airborne asbestos cement dust emitted during cutting processes with the grinding machine (compare to subscript of figure 2).

Sampling of airborne dust on nuclepore filters has been performed.

1. personally on a roofer using the grinding machine
2. stationary at the cutting location and
3. stationary about 30 m distant from the cutting location.

The fraction of fibres  $L > 5 \mu\text{m}$  amounts to

- 1.) 14 %          2.) 4 %          and 3.) 0 %.

For the roofer using the grinding machine a fibre length and diameter distribution with median values of  $1.3 \mu\text{m}$  length and  $0.2 \mu\text{m}$  diameter is measured. In sample 2 and 3 shorter and thinner fibres are observed. Even if only stationary sampling at cutting locations is evaluated fibre length and diameter distributions show wide variations between different samples. The fraction of fibres  $L > 5 \mu\text{m}$  compared to all fibres  $L \geq 0.3 \mu\text{m}$  ranges between  $< 4 \%$  and  $31 \%$ .

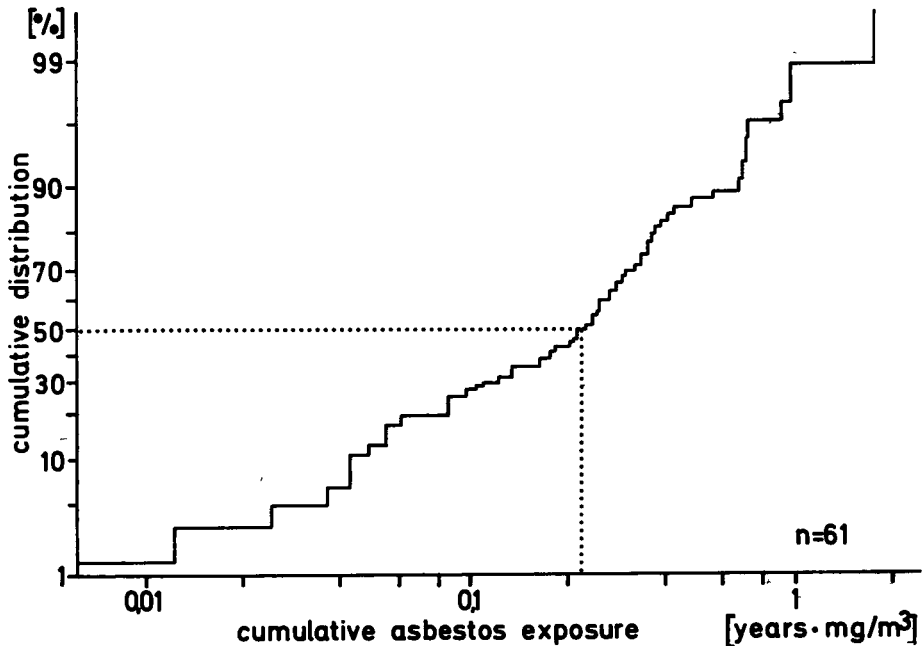
Analysis as well of chrysotile and crocidolite as of chrysotile and amosite emitted simultaneously is performed with EDX in the scanning electron microscope. Identification turns out to be difficult for thin fibres but to be sure for fibres  $L > 5 \mu\text{m}$  (7). No distinct differences arise from length and diameter distributions of chrysotile and crocidolite fibres emitted from asbestos cement products. Amosite fibres from airborne asbestos cement dust in the median are longer and thicker than chrysotile fibres.

### Occupational history

Results of an evaluation of occupational histories of 61 roofers employed by 33 roofing companies are presented in table 2. Since some of them were engaged in more than one position, 93 positions were analysed in all. The frequency of roofings with corrugated sheets of asbestos cement sidings and of roofings with asbestos cement shingles where the grinding machine is not used are presented in table 2.

**Table 2: Occupational histories of 61 roof coverers**  
 Frequency of handling asbestos cement products.

product	handling frequency [days / year]	
	median	$\bar{X} \pm s$
corrugated sheets	34	39,2 ± 31,2
shingles	30	39,4 ± 39,8
front plates	25	30,3 ± 29,2



**Fig. 4:** Cumulative distribution function of the cumulative exposure of chrysotile fine dust which is calculated for the use of corrugated asbestos cement sheets. A mean daily chrysotile fine dust concentration of 0.11 mg/m<sup>3</sup> is assumed when roofing with corrugated asbestos cement sheets is done. The mean duration of asbestos exposure amounts to 16 years.

Roofing with corrugated asbestos cement sheets was carried out up to 125 days per year. More than half of the roofers handle these sheets more than 34 days a year. All roofers work with other asbestos containing products such as shingles or siding also.

Similar to other studies (2) the dust exposure is characterized by the cumulative asbestos exposure. From the years of duration of employment and from the number of days per year when roofing with corrugated sheets is performed, the cumulative asbestos exposure of single craftsmen can be calculated. Taking into account an average chrysotile fine dust concentration of  $0.11 \text{ mg/m}^3$  we obtain the cumulative dose values presented in figure 4.

The probability density function is nearly lognormal. The median of the cumulative asbestos fine dust exposure amounts to  $0.21 \text{ years} \cdot \text{mg/m}^3$  and the mean value is  $0.29 \pm 0.29 \text{ years} \cdot \text{mg/m}^3$ . This corresponds to an exposure of 3 years duration with an average asbestos fine dust mass concentration equal to a limit value of  $0.1 \text{ mg/m}^3$ . Since the mean duration of using corrugated sheets amounts to 16 years for the roofers, the mean exposure per year can be roughly estimated to be 20 % of this limit value.

### Conclusions

Results of measurements of fine dust mass concentration during roofing with corrugated asbestos cement sheets show nearly log-normal behaviour and differ considerably for personal and stationary dust sampling.

The mean fine dust mass concentration for roofers cutting corners of asbestos cement sheets with the grinding machine amounts to about  $2 \text{ mg/m}^3$  asbestos cement fine dust. The mean fibre concentration is estimated to about  $1.2 \cdot 10^6 \text{ fibres/m}^3$   $L > 5 \text{ } \mu\text{m}$ .

Even if only chrysotile containing asbestos cement products are used, fibre length and diameter distributions show wide variations between different samples. The fraction of fibres  $L > 5 \text{ } \mu\text{m}$

compared to all fibres  $L \geq 0,3 \mu\text{m}$  ranges between  $< 4\%$  and  $31\%$ . No distinct differences are seen between length and diameter distributions of chrysotile and crocidolite fibres emitted simultaneously. Amosite fibres in the median are longer and thicker than chrysotile fibres.

From the standardized occupational histories of 61 out of 400 roofers the frequency of handling asbestos products and of using the grinding machine was estimated for present as well as for past working places. From dust measurements and anamnesis cumulative doses of  $0.3 \text{ years} \cdot \text{mg}/\text{m}^3$  asbestos fine dust were calculated.

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Staub-Reinhalt. Luft, No. 38, 402-409



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Contract : 264.77.6 ENV F

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Title of project : Cytotoxic effects of asbestos fibres in relation with their physico-chemical properties.

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#### OBJECTIVE OF THE RESEARCH

The study of the toxicity of fibrous minerals necessitates the precise control of the physico-chemical properties of the particles used. Moreover, such a study must take into account the minerals evolution in the biological medium as well as their sorptive properties regarding toxic substances such as benzopyrene or SO<sub>2</sub>.

For the biological experimentations and for collecting up the determining factors of toxicity, the minerals are chosen according to criteria of morphology, chemical composition and structure.

Effects of samples on red blood cells (haemolysis) and alveolar macrophages (enzyme release) are compared. The reactivity is interpreted in terms of physico-chemical parameters and of modification of the surface of the minerals studied.

Consequently, the research is comprised of two successive stages : the mineralogical study and the biological study.

#### MINERALOGICAL RESEARCH

##### Materials and methods

a) Samples : The following are the batches of particles prepared and characterized :

- particles with a fibrous facies and a different chemical composition : chrysotile, amosite, crocidolite, nemalite (fibrous brucite), glass fibres

- 6 - particles having the same chemical composition and different structure :  
chrysotile and forsterite, quartz and glass fibres  
- asbestos fibres of the amphibole group and of different chemistry :  
amosite and crocidolite.

The asbestos fibres are crushed to a nominal length < 20 µm, the glass fibres < 20 µm, the quartz to a diameter < 5 µm.

b) Preparation of the leached minerals : The dissolution of asbestos by different biologically interesting acids is followed by photoelectron spectroscopy. Moreover, for a comparison with the crude samples, amosite, chrysotile and crocidolite undergo a strong leaching by oxalic acid 0.1 N.

c) Physico-chemical characterization : All the following analyses are applied to the crude and leached minerals :

- Granulometric analysis, homogeneity at the particle level
- Chemical analysis of the major and trace elements
- X-ray examination of the crystallinity and minority phases
- Measurement of the surface area and the infra-porosity.

d) Doping of the minerals : Adsorption of SO<sub>2</sub> and benzo 3,4 pyrene on the asbestos samples has been carried out. The crude or leached minerals undergo a sweep of weakly concentrated SO<sub>2</sub> in dry or wet medium ; the SO<sub>2</sub> adsorption is followed and recorded by flame photometer detection ; in addition, the desorption is obtained, by sweeping by gas current free of SO<sub>2</sub> ; this makes it possible to find out (among others) the quantities adsorbed from studying the adsorption and desorption curves.

Samples were prepared in the same way, in wet medium for biological experimentation only.

For adsorption of benzopyrene, the sample is mixed in a benzopyrene solution in benzene (10<sup>-3</sup> g/ml) for 48 hours. The amounts absorbed are measured by gaseous chromatography. Benzopyrene adsorbed does not correspond to a total saturation of fibers.

## RESULTS

a) Leached minerals : The cinetic study of the behaviour of the main elements of these minerals during leaching gives the following order of stability :

amosite > crocidolite > chrysotile

The magnesium leaching of chrysotile is progressive and over 85 %. Crocidolite and amosite are more stable and the Fe and Mg leaching rates quickly reach their level : respectively 11,5 % and 9 % for amosite, 20 % and 18,5 % for crocidolite.

After leaching, the minerals are washed and lyophilized.

The amosite and crocidolite keep their crystalline structure after strong leaching ; the surface area goes up from 13 and 26 m<sup>2</sup>/g for crocidolite and from 12 to 19 m<sup>2</sup>/g for amosite. On the other hand chrysotile loses its crystalline structure ; the almost complete magnesium leaching increases the surface area considerably and this may reach 300 m<sup>2</sup>/g compared to 26 m<sup>2</sup>/g in the crude state.

b) Doping of asbestos by SO<sub>2</sub> and benzo 3-4 pyrene

	Chrysotile		Amosite		Crocidolite	
	crude	leached	crude	leached	crude	leached
SO <sub>2</sub> adsorbed (mg/g) dry medium	4,2	1,3	0,2	0,05	0,2	0,05
SO <sub>2</sub> adsorbed (mg/g) wet medium	5,7	0,7	1,4	0,3	1,6	0,4
Benzopyrene (mg/g)	0,8	1,6	2,0	2,0	1,4	1,6

In a dry or wet medium, the crude chrysotile adsorbs more SO<sub>2</sub> than the leached chrysotile. The increase in the leached chrysotile's surface area does not, therefore, bring about a higher sorptive capacity : the disappearance of the brucitic layer is directly connected to the sorptive properties.

The amounts adsorbed and the nature of the phenomena differ between benzopyrene and SO<sub>2</sub> ; results on chrysotile may be explained by internal surfaces not easily accessible to benzopyrene.

As far as adsorption of SO<sub>2</sub> and benzopyrene is concerned, the behaviour of amosite and crocidolite appears similar ; SO<sub>2</sub> in a wet medium is much more reactive with amphiboles surfaces ; leaching decreases reactivity of the fibers with SO<sub>2</sub>.

BIOLOGICAL RESEARCH

This research had two aims : firstly, to determine the in vitro effects of asbestos fibres and reference samples on red blood cells (RBC) and alveolar macrophages (AM) ; secondly, to develop a method for culturing pleural mesothelial cells.

Material and methods

a/ Haemolysis. Human RBC and particles were prepared as described elsewhere (1). RBC were used at a final concentration of 0.5 % and incubated under shaking with the particles (37°C). Every minute, the haemolysis was stopped by addition of glutaraldehyde. After centrifugation, the degree of haemolysis was obtained by determination of the optical density at 540 nm. To compare the haemolytic activity of the particles, the following parameter were retained : initial velocity ( $v_i$ ), dose to obtain 100 % haemolysis.  $v_i$  was obtained by fitting the measured haemolysis with theoretical curves.

b/ In vitro release of enzymes from rabbit alveolar macrophages. The experiments were performed as described in ref 1, except that alveolar washings were made under reduced pressure. After culturing with the particles for 2, 4, 6, 8, 18, 20 and 23 hrs, the enzymatic activities were determined both in the adherent cells and in the medium. In addition a control culture without particles was included. There was a duplicate for each experiment, three experiments were carried out for each particle. The enzymes were assessed as follows : lactate dehydrogenase (LDH) by determination of the rate of oxidation of reduced nicotinamide adenine dinucleotide,  $\beta$  galactosidase ( $\beta$  Gal) by the method of Conchie et al (2).

c/ Pleural mesothelial cells (PMC). PMC from the rat parietal pleura have been cultured in various culture media and on different substratum. 5 media differing in their content in amino acids and nucleotides were assessed (Eagle, Ham F10, NCTC 109). Four kinds of substratum were used (Falcon, Linbro, Coston, Corning).

## Results

a/ Haemolysis. The rate of haemolysis was very different according to the type of asbestos fibres used. The vi varied from 0.2 to 200 %/min/0.5 mg and 100 % haemolysis was obtained at a concentration varying from 0.4 to 4 mg/ml. The two amphiboles fibres had a similar effect characterized by a low haemolytic activity. Chrysotile fibres as opposed to amphiboles were highly haemolytic. After leaching, the activities were modified : amphiboles were highly haemolytic and the activity of chrysotile was reduced as compared to the untreated fibres and their activity was close to that obtained with quartz.

There was no significant modification of the haemolysis after SO<sub>2</sub> sorption either with the untreated or with the leached fibres.

b/ In vitro enzyme release from rabbit AM. Chrysotile fibres caused a selective release of  $\beta$  Gal (20 % after 20 hrs of incubation with 50  $\mu$ g/ml) ; at the contrary, amphiboles released both  $\beta$  Gal and LDH, but the rate of release was very low (5 % after 20 hrs of incubation with 100  $\mu$ g/ml). After leaching, chrysotile fibres induced a release of the two enzymes (20 % LDH in the same conditions as above, a similar effect was obtained with quartz. Leached amphiboles induced a higher release of  $\beta$  Gal than LDH.

The adsorption of SO<sub>2</sub> or BP did not significantly modified the reactivity of the fibres.

Nemalite was cytotoxic (LDH and  $\beta$  Gal released) and glass fibres had a reactivity close to that obtained with the untreated amphiboles fibres.

c/ Mesothelial cells. A culture system has been developed for long term maintenance of rat PMC. The cells were obtained by scraping the parietal pleura and cultured on Falcon flasks in NCTC 109 supplemented with 10 % fetal calf serum. The explants formed a confluent monolayer of polygonal cells, within 10-15 days. Subcultures were made in the same medium. The mean population doubling time was approximately 30 hrs. Electron microscopic studies have demonstrated that these cells can engage in phagocytosis of chrysotile fibres.

## Conclusions

In vitro studies are useful for determining the toxicity of asbestos fibres. An important observation is that reactivity is modified by leaching. This implies that, since asbestos fibres are not stable in biological media, their reactivity in the lungs may be modified with time. This fact might explain why chrysotile and amphiboles, which have different cytotoxicities have the same effects after a certain residence time in humans or in animals.

As was found previously with chrysotile (1), adsorption of SO<sub>2</sub> does not greatly modify the effect of asbestos fibres on AM. Although slight increase was usually observed, the difference between unleached and leached samples was maintained and the effect of leaching was predominant. SO<sub>2</sub> therefore does not protect the cell. It is interesting to note that the leached samples adsorbed less SO<sub>2</sub> than the unleached samples ; the adsorption of SO<sub>2</sub> is thus not related to the specific surface area. The decrease in the sorptive properties of the leached samples in relation to unleached ones indicates that cations are important in the binding of SO<sub>2</sub> to the fibres.

The results obtained with BP-treated samples were similar to those obtained with untreated fibres, except with leached chrysotile. The delay observed in the release of enzymes may have been due to a delay in the phagocytosis of BP-treated fibres.

(1) Jaurand et al. Environ. Res., 1978, 17, 216.

(2) Conchie et al. Biochem. J., 1959, 71, 318.

### Publications related to this grant

Jaurand M.C., Bignon J., Magne L., Renier A., Lafuma J. : Interaction des fibres avec les globules rouges et les macrophages alvéolaires in vitro. Rev. Fr. Mal. Resp., 1979, 7, 717-722.

Jaurand M.C., Kaplan H., Thiollet J., Pinchon M.C., Bernaudin J.F., Bignon J. : Phagocytosis of chrysotile fibers by pleural mesothelial cells in culture. Amer. J. Pathol., 1979, 94, 529-532.

Jaurand M.C., Magne L., Bignon J. : Significance of lysosomal enzyme release induced asbestos. In The in vitro effects of mineral dusts. Brown R.C., Chamberlain M., Davies R., Gormley I.P. eds, Academic Press, 1980, pp. 83-87.

Jaurand M.C., Magne L., Bignon J. : Effects of well-defined fibres on red blood cells and alveolar macrophages. In Biological effects of mineral fibres, vol 1, IARC Scientific Publications n° 30, 1980, pp. 441-450.

Jaurand M.C., Bernaudin J.F., Renier A., Kaplan H. Bignon J. : Rat pleural mesothelial cells in culture. In Vitro (in press)

Jaurand M.C., Magne L., Boulmier J.L., Bignon J. : In vitro reactivity with alveolar macrophages and red blood cells of asbestos fibres treated with oxalic acid sulfur dioxide and benzo-3-4-pyrene. Submitted to Exptl. Lung Res.

Contractor : Tumour Center and Institute of Oncology, Bologna,  
Italy

Contract No. ENV/347 I

Project leader : Prof. Dr. Cesare Maltoni

Title of project : Comparative evaluation of the oncogenic risk  
of various types of asbestos of different origin  
and of various other fibrous or otherwise corpusco-  
lated materials

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#### Objective of the research

The purpose of the present research-project is to study the carcinogenic potential of different solid materials, including different types of asbestos, caolin, talc, crystalline and amorphous silica, alumina, different types of natural zeolites, and many others, with the aim of establishing a grading of relative risk.

All the compounds are administered by intraperitoneal injections and several of them also by subcutaneous and intrapleural injections.

#### Materials and methods

The origin of each tested compound is known and registered, and the available quantity of each sample is enough to make possible its physical-chemical characterisation, if necessary.

Sprague-Dawley rats have been employed (groups of 20 males and 20 females are utilized for each compound and for each route). The plan of the experiments, is presented in tables 1-3. The animals are controlled and weighed every two weeks during the whole experiment. The animals are kept alive until spontaneous death.

A complete autopsy is performed on every animal. Histological specimens include: tissues at the site of the injections (with or without lesions), organs and/or apparatuses affected by mesotheliomas, any other organ and/or apparatus with gross changes, and always the thymus, mediastinal lymph nodes, lung, diaphragm, liver, spleen and kidneys.

The experiments were started on January 27, 1981.

#### Results

The results up to the 13th week, are shown in tables 1-3. Only two out of 1,400 animals died, which means that the treatment, by whatever route, is well tolerated.

#### Additional comments.

Other corpuscolated compounds will be included in the study in the coming weeks.

The end of the biophase of the started experiments is foreseen for Autumn 1983, and the final report of the whole experiment will be ready by the end of that year.

TABLE 1

Experiment H22101: Response by intraperitoneal and/or intrapleural and/or subcutaneous injection to Aberton, at the dose of 25 mg in water (1 cc), *una tantum*. Results after 13 weeks.

Exp. No.	GROUP	COMPOUND	ROUTE	TREATMENTS	ANIMALS (Sprague-Dawley rats, 8 weeks old at start)				TUMORS					
					Sex	No. at start	No. survivors	% survivors	At the site of injection		In other sites			
									No.	%	Summary frequency	Other type		
H22101	I	Cresidolite	Intraperitoneal injection	25 mg/ 1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	II	Cresidolite	Intra-pleural injection	25 mg/ 1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	III	Cresidolite	Subcutaneous injection	25 mg/ 1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	IV	Chrysothile (Canada)	Intraperitoneal injection	25 mg/ 1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	V	Chrysothile (Canada)	Intra-pleural injection	25 mg/ 1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	VI	Chrysothile (Canada)	Subcutaneous injection	25 mg/ 1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	VII	Chrysothile (Rhodesia)	Intraperitoneal injection	25 mg/ 1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	VIII	Chrysothile (California)	Intraperitoneal injection	25 mg/ 1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	IX	Amosite	Intraperitoneal injection	25 mg/ 1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	X	Anthrophyllite	Intraperitoneal injection	25 mg/ 1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	XI	None (Control)	Intraperitoneal injection	1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	XII	None (Control)	Subcutaneous injection	1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	XIII	None (Control)	Intra-pleural injection	1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
TOTAL					M	260	260	100.0						
					F	260	260	100.0						
					M and F	520	520	100.0						



TABLE 2

Experiment 822102: Response by intraperitoneal and subcutaneous injection to various inorganic particles, at the dose of 25 mg in water (1 cc), una tantum.  
Results after 13 weeks.

EXP. NO.	GROUP	COMPOUND	DOSE	TREATMENT	ANIMALS (Sprague-Dawley rats, 6 weeks old at start)									
					Sex	No. at start	No. survivors	% survivors	At the site of injection		TUMORS			
									No.	%	In other sites		Benign	Other types
822102	I	Kaolin	Intrape- ritoneal injection	25 mg/ 1 cc H <sub>2</sub> O una tantum	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	II	Kaolin	Subcuta- neous injection	25 mg/ 1 cc H <sub>2</sub> O una tantum	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	III	Talc	Intrape- ritoneal injection	25 mg/ 1 cc H <sub>2</sub> O una tantum	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	IV	Talc	Subcuta- neous injection	25 mg/ 1 cc H <sub>2</sub> O una tantum	M	20	18	90.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	V	Crystal- line silica	Intrape- ritoneal injection	25 mg/ 1 cc H <sub>2</sub> O una tantum	M	20	20	100.0	0	-	0	-	0	-
F					20	20	100.0	0	-	0	-	0	-	
M and F					40	40	100.0	0	-	0	-	0	-	
VI	Crystal- line silica	Subcuta- neous injection	25 mg/ 1 cc H <sub>2</sub> O una tantum	M	20	20	100.0	0	-	0	-	0	-	
				F	20	20	100.0	0	-	0	-	0	-	
				M and F	40	40	100.0	0	-	0	-	0	-	
VII	Amorphous silica	Intrape- ritoneal injection	25 mg/ 1 cc H <sub>2</sub> O una tantum	M	20	20	100.0	0	-	0	-	0	-	
				F	20	20	100.0	0	-	0	-	0	-	
				M and F	40	40	100.0	0	-	0	-	0	-	
VIII	Amorphous silica	Subcuta- neous injection	25 mg/ 1 cc H <sub>2</sub> O una tantum	M	20	20	100.0	0	-	0	-	0	-	
				F	20	20	100.0	0	-	0	-	0	-	
				M and F	40	40	100.0	0	-	0	-	0	-	
IX	Alumina	Intrape- ritoneal injection	25 mg/ 1 cc H <sub>2</sub> O una tantum	M	20	20	100.0	0	-	0	-	0	-	
				F	20	20	100.0	0	-	0	-	0	-	
				M and F	40	40	100.0	0	-	0	-	0	-	
X	Alumina	Subcuta- neous injection	25 mg/ 1 cc H <sub>2</sub> O una tantum	M	20	20	100.0	0	-	0	-	0	-	
				F	20	20	100.0	0	-	0	-	0	-	
				M and F	40	40	100.0	0	-	0	-	0	-	
XI	None (Control)	Intrape- ritoneal injection	1 cc H <sub>2</sub> O una tantum	M	20	20	100.0	0	-	0	-	0	-	
				F	20	20	100.0	0	-	0	-	0	-	
				M and F	40	40	100.0	0	-	0	-	0	-	
XII	None (Control)	Subcuta- neous injection	1 cc H <sub>2</sub> O una tantum	M	20	20	100.0	0	-	0	-	0	-	
				F	20	20	100.0	0	-	0	-	0	-	
				M and F	40	40	100.0	0	-	0	-	0	-	
TOTAL					M	240	240	100.0						
					F	240	240	100.0						
					M and F	480	480	100.0						

TABLE 3

Experiment 3E2103: Exposure by intraperitoneal and/or intrapleural and/or subcutaneous injection to iscolite, at the dose of 25 mg in water (1 cc), *una tantum*. Results after 13 weeks.

EXPERIMENT	GROUPS	COMPOUND	DOSE	TREATMENTS	ANIMALS (Sprague-Dawley rats, 6 weeks old at start)									
					Sex	No. at start	No. surviving	% surviving	TUBERCLES					
									At the site of injection		In other sites			
No.	%	No.	%	No.	%	No.	%							
3E2103	I	Erioste	Intrape-ritoneal injection	25 mg/ 1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	II	Erioste	Intrapleural injection	25 mg/ 1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	III	Erioste	Subcutaneous injection	25 mg/ 1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	IV	Bordenite	Intrape-ritoneal injection	25 mg/ 1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	V	Bordenite	Subcutaneous injection	25 mg/ 1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	VI	Phillipite	Intrape-ritoneal injection	25 mg/ 1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	VII	Phillipite	Subcutaneous injection	25 mg/ 1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	VIII	None (Control)	Intrape-ritoneal injection	1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	IX	None (Control)	Subcutaneous injection	1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
	X	None (Control)	Intrapleural injection	1 cc H <sub>2</sub> O <i>una tantum</i>	M	20	20	100.0	0	-	0	-	0	-
					F	20	20	100.0	0	-	0	-	0	-
					M and F	40	40	100.0	0	-	0	-	0	-
TOTAL					M	200	200	100.0						
					F	200	200	100.0						
					M and F	400	400	100.0						

Contractor : Environmental and Medical Sciences Division, AERE  
Harwell, Oxon OX11 0RA, UK.

Contract No. 277-77-10 ENV UK

Project leader : A. Morgan

Title of project : Deposition and clearance of inhaled asbestos fibres

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#### Objective of the research

The original objectives of this programme were to study (a) the effect of fibre dimensions on the regional deposition of inhaled fibres in experimental animals and (b) the effect of fibre dimensions on the clearance of fibres deposited in the alveolar region of the lung.

Much work has been carried out on the deposition of inhaled asbestiform minerals in the rat and their subsequent clearance which has been summarised by Morgan (1979). More recently, this work has been extended to cover the deposition of thicker man-made mineral fibres (Morgan *et al.*, 1980).

Studies of the deposition and clearance of fibres inhaled by rodents has been complemented by studies of the characteristics of amphibole fibres encountered in human lung samples taken at autopsy (Morgan and Holmes, 1980). In particular, a study has been made (Morgan and Holmes, in press) of the concentrations and characteristics of amphibole fibres in the lungs of UK gas-mask factory workers exposed to crocidolite during the second World War. Detailed topographic studies of the distribution of amphibole fibres in sagittal slices of human lung are also being performed.

#### Materials and Methods

Methods developed for the measurement of concentrations and dimensions of amphibole fibres in lung, based on the hypochlorite digestion technique and optical microscopy, were described in the previous progress report.

#### Results

##### 1. Amphibole fibres in the lungs of UK gas-mask factory workers

The concentrations and dimensions of uncoated and coated amphibole fibres in the lungs of some workers at the Leyland, Nottingham and Blackburn gas-mask factories who died with mesothelial tumours have been measured with the light microscope. Also included in the study were lungs of three miners from Wittenoom, Australia, from where the crocidolite used in military respirators was reputed to have originated.

A comparison of fibre densities (fibres  $\text{mm}^{-2}$ ) on filters measured by optical and electron microscopy showed that about 15% of the fibres present were detected with the OM and that this corresponds to the proportion of

fibres exceeding 0.2  $\mu\text{m}$  in diameter.

The lengths of both uncoated and coated fibres were distributed log-normally. For the two largest groups of gas-mask factory workers the count median lengths (CMLs) of uncoated and coated fibres were very similar, being about 12 and 26  $\mu\text{m}$  respectively. The CMLs of uncoated fibres in the Wittenoom miners were only about 3  $\mu\text{m}$ , probably due to the impairment of macrophage-mediated clearance by the very large number of long fibres present. The concentrations of crocidolite fibres in the lungs of the gas-mask factory workers ranged from 0.07 to  $216 \times 10^6/\text{g}$  dry weight. In two of the Wittenoom miners the concentrations approached  $10^9/\text{g}$  dry weight.

As shown in Fig. 1 the fibre concentration in the lungs of the largest (Nottingham) group, are distributed log-normally with a median value of about  $4 \times 10^6/\text{g}$  dry weight. Concentrations of various types of asbestos fibres in the lungs of 22 workers from the same factory have also been measured by Jones *et al.* (1981) with the EM. They found that crocidolite fibres predominated in the majority of cases and, as can be seen in Fig. 1, their crocidolite fibre concentrations are also distributed approximately log-normally. As would be expected, because of the greater resolution of the electron microscope, the curve is displaced by almost an order of magnitude and the median value is about  $40 \times 10^6/\text{g}$  dry weight.

The third line in Fig. 1 represents the concentrations of amphibole fibres in the lungs of 100 patients who died with pleural mesothelioma reported by Whitwell *et al.* (1977). These workers measured fibres greater than 6  $\mu\text{m}$  in length with the optical microscope using a Fuchs-Rosenthal counting chamber. Over 90% of these cases had industrial exposure to asbestos in, for example, shipyards, asbestos factories and insulation. That the concentrations of amphibole fibres in this series are also distributed log-normally is shown in Fig. 1. The median value is  $0.6 \times 10^6/\text{g}$  dry weight or about 7 times lower than the median for the Nottingham gas-mask factory workers measured with the OM. However, in an intercomparison between Whitwell's laboratory and our own, involving lung tissue from 37 patients, although the results were well correlated ( $r = 0.92$ ) we detected over 5 times as many fibres exceeding 5  $\mu\text{m}$  in length. This indicates that finer fibres can be detected with the membrane filter technique than the counting chamber. Taking this consideration into account, and also the fact that Whitwell *et al.* did not include fibres less than 6  $\mu\text{m}$  in length in their assessment, it appears that the distribution of fibre concentrations in the Nottingham gas-mask factory workers and in Whitwell series are quite similar with comparable median values.

It is worthwhile commenting on the factors which result in a log-normal distribution pattern of amphibole fibre concentrations in the lungs of the Nottingham gas-mask factory workers and possibly in other occupationally exposed populations. The main factors which determine the shape of the curve are a) the dose-response relationship for mesothelioma induction and b) the distribution of dose in the population. It seems certain that people with high concentrations of crocidolite fibre in lung are at greater risk of dying with mesothelial tumours than those with low. For example, Jones et al. showed that, with the Nottingham workers, there is a positive correlation between duration of exposure and incidence of mesothelial tumours and Hobbs et al. (1981) have reported a similar relationship for the Wittenoom miners. We consider that, except at very high dose levels, when clearance is impaired, the residual fibre concentration in lung provides the most accurate index of integrated exposure but such information can only be obtained post-mortem. If it were possible to measure post-mortem the fibre concentrations in the lungs of all the Nottingham gas-mask factory workers, irrespective of cause of death, it should be possible to derive the dose-response relationship for the induction of mesothelial tumours by crocidolite asbestos. One complicating factor is that in heavily exposed subjects (certainly those with fibre concentrations exceeding  $10^8$ /g dry weight) there will be a competing risk of mortality from asbestosis.

Extrapolation of the lines in Fig. 1 indicates that there may be a small, but significant, risk of developing mesothelial tumours even when the concentration of crocidolite fibres in the lung is below the level normally considered to result from occupational exposure. In the study reported by Whitwell et al. there was a considerable degree of overlap in amphibole fibre concentrations in the lungs of the control series and 100 cases of mesothelioma. This is to be expected if the probability of developing a mesothelioma at low fibre concentrations is small. In deciding whether mesothelial tumours are spontaneous, or caused by asbestos exposure, it is clearly important to have available methods which permit the accurate determination of relatively low concentrations of amphibole fibres in the lung.

## 2. Topographic distribution of uncoated and coated amphibole fibres in the human lung

Work described briefly in the previous progress report on the topographic distribution of uncoated and coated amphibole fibres in sagittal sections of human lung has been extended in the period under review. A slice 0.5 in thick was cut from the periphery of a sagittal section of lung from a worker with asbestosis. A slice was cut into blocks 0.5 in long. The positions of these blocks relative to the remainder of the section are shown in Fig. 2. Part of

each block was digested with sodium hypochlorite and the remainder dried to give the wet/dry ratio. The concentrations and length distributions of both uncoated and coated optically visible amphibole fibres were measured in each block. A number of interesting results emerged as follows:-

- a) The regions of the lung most distant from the hilum (eg sections A1, A12, B1, B10 etc) have much lower fibre concentrations than other peripheral samples, presumably due to the poor ventilation of these regions.
- b) In the pleural region adjacent to the chest wall (sections A1 to A12) there was a distinct periodicity in the concentrations of both uncoated and coated fibres corresponding to the intercostal spacing. Lower concentrations were found in the region corresponding to the intercostal space. A similar effect has been demonstrated using non-fibrous radioactive particles and autoradiography (Barnes, 1971).
- c) Overall, there was a positive correlation ( $r = 0.82$ ) between the concentrations of uncoated and coated fibres.
- d) There was also a positive correlation ( $r = 0.83$ ) between the total fibre concentration and the concentration of non-fibrous dust in the various blocks measured on an arbitrary scale of 1 to 6.

### Conclusions

As pointed out in the discussion of the results obtained on gas-mask factory workers it is possible, in principle, to determine the probability of a given fibre concentration in lung inducing a mesothelial tumour. To do this, it is necessary to identify an occupationally exposed population in which there is a reasonably high incidence of mesothelial tumours. Such populations would include, for example, the UK gas-mask factory workers exposed to crocidolite or the Devonport dockyard workers. Having identified such a population it would then be necessary to analyse lung tissue for all those dying with a mesothelial tumour and also from a representative selection of workers dying of other causes. Investigations into the feasibility of such a study are proceeding.

Finally, with regard to the topographic distribution of fibres in human lung, sagittal sections of lungs from Finnish anthophyllite fibres have been obtained. It is proposed to extend the present study using this material, as virtually all the anthophyllite fibres can be detected by OM.

### Acknowledgement

This work is supported in part by the Asbestosis Research Council.

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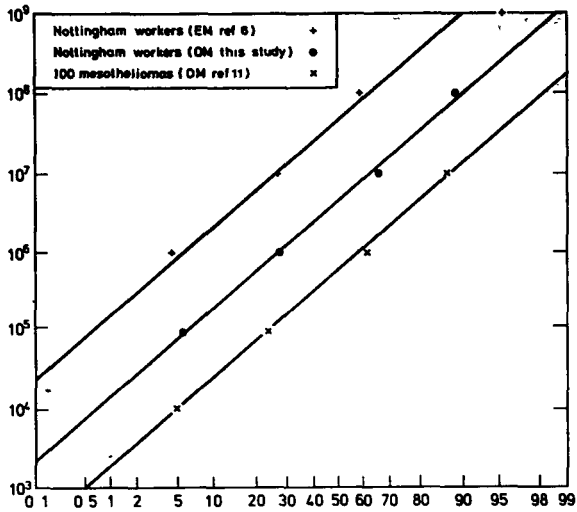


Fig. 1 Distributions of fibre concentrations obtained from the Nottingham gas-mask factory workers. The upper line (+) represents results obtained by Jones *et al.* (1981) for Nottingham workers using the electron microscope. The lower line (x) represents the concentrations found in 100 cases of mesothelioma reported by Whitwell *et al.* (1977).

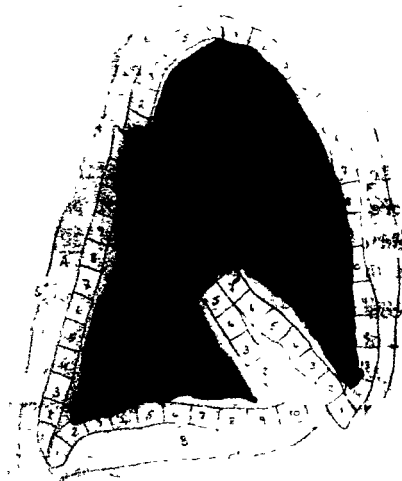


Fig. 2 Photograph of sagittal slice of human lung after removal of peripheral region for analysis.



Contractor : International Research and Development Co Ltd, Newcastle  
Contract No. : 281-77-8 ENV UK  
Project Leader : P.E. Harley, D.E. Gibbs  
Title of Project : CONTINUATION STUDY OF IMPROVED TECHNIQUES FOR THE  
DETECTION, QUANTIFICATION AND IDENTIFICATION OF  
ASBESTOS AND OTHER FIBROUS MATERIALS FOR THE USE  
IN THE FIELD OF ENVIRONMENTAL STUDIES

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#### OBJECTIVES OF THE PROJECT

The objectives of the project are described in detail elsewhere<sup>1</sup>, but may be broadly summarised as:-

- (a) The continuation of studies performed under CEC/IRD Contract No. 281-77-8 ENV UK in order to exploit the phenomenon of the magnetic alignment of asbestos fibres. This preceding contract culminated in the successful manufacture of a novel microscope system for the evaluation of environmental asbestos fibre levels.
- (b) A study of existing and potential techniques and systems which might be applied to the objective quantification of airborne asbestos dusts.
- (c) A study of potential systems for the direct evaluation of fibrous dust concentrations in the environment. To be of value, such systems are required to sample the dust laden air and provide immediate concentration data, thus overcoming the cumbersome nature of the existing membrane filter technique.

#### IMAGE ANALYSING EQUIPMENT AS APPLIED TO ASBESTOS DUST CONCENTRATION DETERMINATION

In the mid-term report<sup>2</sup>, it was explained that the existing methods of asbestos dust quantification based on the membrane filter and optical microscope, were limited by subjective factors. It was also indicated that recent developments had made automatic fibre level determination possible, thereby obviating the subjective element.

A study has been performed to investigate the suitability of the commercial systems available, in order to analyse asbestos dust samples collected on membrane filters in the conventional manner.

The study was conducted as follows:

- (a) All known manufacturers of image analysis hardware were contacted with a view to obtaining specifications for their equipment.
- (b) The replies from the responding companies were studied to ascertain the general capabilities of automatic image analysis, as applied to asbestos fibre counting.
- (c) Of the companies approached, one, in particular, had a system which was used much more extensively, in order to gain an insight into the implications of automatic image analysis as applied to the quantification of asbestos dusts. This system (the Joyce Loebel Magiscan 1) was chosen because:-
  - (i) The company agreed to the useage.
  - (ii) This is the only system, amongst those surveyed, which supports an operational software package dedicated to the analysis of environmental asbestos samples.

The final technical report for this project includes the amassed data relating to image analyser systems investigated, together with details of experiments relating to asbestos fibre counts performed manually and automatically, using samples created for the purpose in the in-house dust chamber facility and "real" samples acquired whilst performing routine environmental monitoring work.

The image analyser was used in conjunction with other work in the project, notably the analysis of the orientation distribution of magnetically aligned fibres.

The overall conclusion is that image analysis technology, whilst still in its infancy as regards fibrous aerosol applications, is already capable of adopting the role of an objective evaluation system.

### CONTINUOUS MONITOR

The mid term report<sup>2</sup> delineated the philosophy behind the development of an instrument for the continuous inspection of an environment for fibrous dust particles.

Liaison with professional hygienists in the asbestos industry, together with other bodies, revealed that there is a well defined need for an instrument. A target specification has been formulated, based on these discussions, and, on an in-depth study of the technology available.

Several schemes are proposed by which means, a realistic continuous monitor could be developed and one of these approaches is explored in depth.

Conclusions reached, are that a monitoring instrument capable of detecting fibrous particles of respirable size at concentrations in air below the legislated minima, and with a suitable size, cost and portability, is a feasible entity.

### MAGNETIC ALIGNMENT

The phenomena whereby asbestos fibres can be forced to align either with, or normal to, a magnetic field<sup>3</sup>, formed the cornerstone of this and the preceding project. Particular areas which have been studied and which are described in detail in the technical report are as follows:-

- (a) Empirical studies have been performed to determine the optimum means by which asbestos fibres as supported on membrane filters, can be aligned in a manner compatible with viewing in an optical microscope.
- (b) Using the methods of alignment derived in (a) above, studies have been performed to characterise the asbestiform grouping of respirable fibres as determined by characteristic patterns of alignment. The experimental work in this area has been performed with the help of an image analyser and specially commissioned software<sup>4</sup>.

- (c) Using aligned samples, experiments have been performed to determine whether the alignment of fibres can be used to enhance the performance of an image analysis system.
- (d) The behaviour of asbestos fibres in aqueous suspension, and under the influence of dynamic magnetic fields has been observed in a specially constructed microscope assembly incorporating a large water cooled electro-magnet.
- (e) The use of a magnetic field to align fibrous particulates of non-asbestiform species has been investigated. Of particular interest are carbon fibres, which have been found to align readily, and blown glass fibres, which are only influenced to a very small degree by the field. Methods of modifying the alignment chemistry to improve alignment of glass has been investigated to a certain degree, but with no concrete results.

#### STUDIES IN SUPPORT OF THE RAPID FIBRE COUNTER

The Vickers M88 Rapid Fibre Counter, the direct derivative of the preceding project, has been used extensively as both a means of measurement in other project phases (particularly alignment technique optimisation) and in order to characterise its performance in dealing with real environmental samples of asbestos dusts.

Samples obtained during surveys performed by IRD's Environmental Services Group, have been subjected to evaluation by multifarious means, including evaluation on the M88, manual counting by a number of different operators, and image analysis. Most samples were derived from demolition operations involving the removal of asbestos from old buildings, though some originated in a friction materials factory.

Another means of investigating the performance envelope of the M88 involved the double exposure of filters to asbestos dusts, and to blown glass fibre particles, both from controlled dust chamber sources. Exploitation of the refusal of certain glass fibres to magnetically align was used to introduce background "noise". Glass fibres are, however, easily counted by the manual operator, thus allowing "signal to noise" studies to be performed.

## CONCLUSIONS

As a result of the diverse nature of the project activities, detailed conclusive summaries are relegated to the technical report.

The two most important general areas, which have been studied are those relating to automatic image analysis, and the prospect of a continuous monitor. The latter, in particular, is ripe for further development.

## REFERENCES

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**TOPIC 15 : AIR QUALITY**  
**Effects of air pollutants on plants**

**Contractor:** Universität Hohenheim  
Institut für Landeskultur und Pflanzen-  
ökologie, P.O.B. 700562, D-7 Stuttgart 70

**Contract n<sup>o</sup>:** ENV 423 - 80 D

**Project leader:** Prof. Dr. Uwe Arndt, Dr. Willfried Nobel

**Title of the project:** Air pollution effects to wild plants in  
rural ecosystems

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#### Objective of the research

About air pollution effects to forest-, horticultural and agricultural plants there exist numerous results of researches. The sensitivity of wild plants is nearly completely unknown. But exactly wild plants and their communities reflect the natural conditions of the habitat, and enables conclusions to stress, stress capacity, and stability of ecosystems. Consequently the knowledge of the different characteristics of resistance of wild plants to air pollutants as SO<sub>2</sub> and HF, which are most important in Middle Europe, is of special interest.

#### Materials and methods

In the present research project, supported by the CEC for one year, 12 characteristic grass species were chosen, all species of typical grassland (*Molino-Arrhenatheretea*) in South-West Germany. The seeds were purchased on the market respectively from the botanic garden of the university and were cultivated in single pots in the green house. Three weeks after sowing the plants were cut and three weeks furthermore they were fumigated for three weeks with SO<sub>2</sub> in concentrations of 1 mg/m<sup>3</sup> = 0,4 ppm. The SO<sub>2</sub>-fumigation system consists of a SO<sub>2</sub>-dosage system (Fa. Wösthoff, Bochum), a SO<sub>2</sub>-measuring instrument (Fa. Beckman, Munich) and two plastic chambers with 30 l, each hold 5 pots with experimental plants.

After fumigation time the grasses were cut and from each pot 30 leaf blades were selected arbitrarily. From these leaves the total length and the length of the chlorotic or necrotic leaf parts were measured with a ruler.



## Results

The growth of the leaf length was different comparing the unfumigated control and the fumigated plants:

### I. Control greater than fumigated:

*Alopecurus pratensis*, *Cynosurus cristatus*, *Deschampsia cespitosa*, *Festuca rubra*, *Holcus lanatus*, *Lolium perenne*,  
*Phleum pratense*

### II. Control equal as fumigated:

*Festuca pratensis*

### III. Control smaller than fumigated:

*Arrhenatherum elatius*, *Dactylis glomerata*, *Poa pratensis*,  
*Trisetum flavescens*

A significant decrease of the leaf growth showed however only *Festuca rubra* and *Phleum pratense*.

The leaf damages, recorded as the length of chlorotic and necrotic parts, were converted in percent of the total leaf length and coordinated to three degrees of damage:

### I. Small damage (untill 25 % of leaf length):

*Dactylis glomerata*, *Deschampsia cespitosa*, *Lolium perenne*

### II. Moderate damage (untill 75 % of leaf length):

*Arrhenatherum elatius*, *Cynosurus cristatus*, *Festuca rubra*,  
*Holcus lanatus*

### III. Severe damage (over 75 % of leaf length):

*Alopecurus pratensis*, *Festuca pratensis*, *Phleum pratense*,  
*Poa pratensis*, *Trisetum flavescens*

The control plants showed comparatively only very small chlorotic or necrotic parts, only *Trisetum flavescens* showed moderate damage. With regards to the feeding value it is of interest, that grasses with severe damage all together are attributed to the more valuable species. Stress by  $\text{SO}_2$  consequently could result in a succession of grass species to less valuable forage grasses.

## Conclusions and additorial comments

12 characteristic grass species of typical grassland (*Molinio-Arrhenatheretea*) in South-West Germany were fumigated with  $\text{SO}_2$  ( $1 \text{ mg/m}^3 = 0,4 \text{ ppm}$ ). Severe damage (length of the chlorotic or necrotic leaf parts) showed *Alopecurus pratensis*, *Festuca pratense*

sis, *Phleum pratense*, *Poa pratensis* and *Trisetum flavescens*, which all together are attributed to the more valuable forage grasses.

The experiments will be repeated in fumigation chambers in the field with  $\text{SO}_2$   $0.6 \text{ mg/m}^3 = 0.2 \text{ ppm}$  and  $0.14 \text{ mg/m}^3 = 0.05 \text{ ppm}$ . To investigate the effects of HF-stress to wild plants mapping vegetation has been started around two habitats influenced by brickworks. Investigating the effects on plant communities under controlled conditions fumigation experiments will be carried out in open top chambers. The projected investigations are objects of a further research project applied for the CEC as a continuation or an extension of the present project.

Contractor : Université d'Aix-Marseille III

Contract n° ENV-341-80-F

Project leader : Deveze Louis, Professeur

Title of project : Transport de polluants phytotoxiques par les embruns

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#### OBJECTIVE OF THE RESEARCH

Previous studies have shown that littoral vegetation on the French Mediterranean Coast undergo a particular sort of degradation in direct account with her more or less high exposition to sprays coming from sea.

#### Symptoms of declinement observed :

##### - on plane-trees (Platanus acerifolia)

The part of the foliage turned towards the sea, offers marginal necrosis respecting the nervures. The progress of damages is made by the face turned towards the sea, first attacked to the face turned towards the land and is translated by a withering and a premature fall of leaves and by the progressive dead of the branches.

##### - on Alep pines (Pinus halepensis)

Necrosis set out to the pine-needle from the basis, with the same direction like in the case of plane-trees. The fall of dried pine-needles carries along the dead of the tree.

#### Among the bushes exposed to the marine sprays

Similar necrosis and high recession of vegetation are established. It is the case among the most prevalant species :

- privet (Ligustrum vulgare)
- laurel (laurus nobilis)
- holm-oak (Quercus ilex).

The analysis of the marine sprays made at the level of vegetation has shown the presence of important quantities of oil hydrocarbons and anionic tensides as also of large quantities of fluorides.

Identical symptoms from the ones observed before, have been reproduced in laboratory, by spraying on young Alep pines cultivated in full mould of an artificial spray having a same composition as collected marine sprays :

NaCl 20 g/l ; NaF 0,1 % ; LAS\* 0,1 % ; Fuel oil 10%

The association of these four main constituents has given the same symptoms than precedently.

On the other hand, a spray containing only NaCl or condensate water does not conclude on the apparition of phytotoxicity symptoms.

Particularly, others sorts of experimentations on cultivations of vegetal tissues have for object to examine the action of different pollutants in the marine sprays.

The researches made within the plane of a contract have for object to strike the balance as detailed as possible about the chemical composition of the marine sprays, in order to specify the extent of this sort of pollution and to establish the consequence of this phenomenon. These actions have been completed by a mapping of the places affected by the declination of the vegetation, along the French Mediterranean Coast.

#### MATERIALS AND METHODS

##### Traping and chemical composition of the marine sprays :

In order to determine the nature of the chemical composition sprays deposited on the vegetation, two sorts of collectors have been placed in position ;

- a system of deposition gauge formed by a support, put in an open ground containing a funnel of 30 cm diameter and a receiving bottle of 10 liters.
- squared plates of glass about 30 x 30 cm exposed vertically on rigid supports in the way of the sea.

Then again, a system of sampling on the superficial sheet of sea water formed with a similar roller described by Harvey has been used to examine the phenomenon of superficial concentration of the tensides and setting apart them in farthermost analysis.

The global grades of tensides, hydrocarbons and fluorides have been determined by spectrophotometry; analysis more detailed about the tensides have been made by HPLC on grafted columns of silica RP 18 with detection by spectrophotometry at 230 nm, after the concentration of liquid-liquid extraction with ethyl acetate.

(\* ) Linear alkylbenzene sulfonate

Damage of tensides and physiological effects of pollutants :

The trials on the degradation tensides have been experimented in laboratory on non sterile sea-water buffered and enriched in phosphate, ammonium chloride and continued in HPLC.

The experimentation on the cultivations of *Psoralea bituminosa* tissues have been studied on Murashige and Skoog medium, in the presence of different pollutants. The growth inhibition has been measured by weighing with regard to check samples cultivated in the same conditions.

RESULTS AND DISCUSSION

The means of measurements are made on the collectors, directed in several places :

- a check site not at all polluted : "Ile Port-Cros", apart from all urban centres
- a site nearlier of the coast : "Ile de Porquerolles"
- the "Rade de Marseille"
- a site very polluted (industrial zone of Lavera, situated between Marseilles and Fos-sur-Mer. (Tables n° 1 and 2).

These measurements show that as privilege places as "Ile Port-Cros", the level of pollution is not negligible.

The study of chromatographycal tensides cuts off at Port-Cros in relation with biodegradability studies made in laboratory, shows that the degradation affects preferentially some isomers, the less branched out and depends on the deposits of phosphate, ammonium and carbon source.

The pollution made by hydrocarbons on vegetation looks like near Marseilles appeared in several places along the Mediterranean Coast.

These conclusions are confirmed by the courbes of inhibition growth of the *Psoralea bituminosa* tissues which show that a same percent of inhibition growth is achieved for more important concentrations of hydrocarbons than fluoride or tensides. Fluoride and tensides have a weakness concentration in the marine sprays. Among the constituents of fuel oil, a parafinic hydrocarbon like hexadecan have important activity. Aromatic hydrocarbons as naphtalen and phenonthren have a higher activity.

TABLE N° 1

Check samples made in Marseilles				
Mean values of principal elements (mg)				
(10500 mg of Na)				
Boron B	70	Fluorine F	140	
Magnesium Mg	1400	Chlorine Cl	17500	
Calcium C	10500			
Others elements (same conditions)				
Silicon Si	504	Copper Cu	51,1	
Phosphorus P	17,5	Zinc Zn	91	
Sulphur S	1890	Arsenic As	4,2	
Potassium K	350	Bromine Br	9,1	
Vanadium Va	9,8	Strontium Sr	59,5	
Chromium Cr	11,9	Cadmium Cd	2,1	
Iron Fe	51,1	Barium Ba	3,5	

TABLE N° 2

Mean values between anionic tensides (LAS) and chlorides and between hydrocarbons (HC) and chlorides in several places of the coast				
	Port-Cros	Porquerolles	Marseilles	Lavera
$\frac{\text{LAS}}{\text{Cl}^-} \times 10^3$	0,98	2,42	3,48	3,88
$\frac{\text{HC}}{\text{Cl}^-} \times 10^3$	20,2	41	43,8	N.D.

### CONCLUSIONS

The process of the formation of marine sprays leads to send in the atmosphere, important quantities of different pollutants, in places far away from industrial and urban source of pollution.

This particular sort of contamination in the atmosphere is translated in visible damages of the vegetation.

The tables n° 1 and 2 show the presence of sprays in different chemicals, well-known for their toxicity, especially sal-heavy metals.

It will be better to no rid of their contribution to unfavourable effects for the organisms.

### PUBLICATIONS AND ORAL COMMUNICATIONS

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Transport de composants phytotoxiques par les embruns marins.  
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Colloquy on the pollution, Martigues, (M.E.C.V.), February 11th 1981.

Contractor: C.N.R.S., France

Contract n° ENV/342 F

Project leader: O. Queiroz, Directeur de Recherche, Phytotron, CNRS  
91190 - Gif-sur-Yvette, France

Title of project: Non visible effects of plant pollution by SO<sub>2</sub>:  
primary sites for SO<sub>2</sub> action, enzymic mechanisms and  
physiological cost (development, productivity, life  
length). Biochemical methods for early detection of  
these effects

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#### Objective of the research

The working hypothesis considers that development and survival of plants in permanently polluted areas should be dealt with as a problem of adaptation i.e. by combining two complementary approaches: (a) modelisation, under controlled environment, of the underlying biochemical and biophysical mechanisms (b) comparison with behaviour in field conditions.

(a) The model evolved in this laboratory is based on preceding results showing that extended changes in enzyme capacity (enzymic potentiality of the tissue) and in metabolic activity of bean leaves occur within a few days under non-necrotic pollution by SO<sub>2</sub> with doses lower than those required to produce ultrastructural changes. Therefore it is considered that the primary effect of SO<sub>2</sub> intervenes at the metabolic level. The present research seeks for:

- 1) the primary metabolic "target" of pollution;
- 2) the kinetics of enzymic and metabolic readjustments during pollution;
- 3) evaluation of the physiological cost of this modified metabolism.

(b) Application to field conditions is studied in collaboration with J. Bonte, I.N.R.A., Station d'Etude de la Pollution Atmosphérique, at Montardon. Bean plants grown in field under permanent non-necrotic pollution are analysed at the Phytotron, Gif-sur-Yvette for comparison with the biochemical responses predicted by the model.



## Materials and methods

Experiments on pollution were carried out with *Phaseolus vulgaris* var. *Menil* in specially designed chambers affording strict control of photoperiod, temperature, humidity, nutrition and concentration of SO<sub>2</sub>. Plants 3 weeks old were separated into 2 batches of 6-8 plants, one for control the other for continuous pollution with 0.06, 0.10 or 0.15 ppm SO<sub>2</sub>. Samples of leaves of different ages were analysed after 0, 6, 12, 16 and 21 days of pollution.

Enzyme measurements. Enzyme capacity was measured as total extractable activity for PEP carboxylase (PEPC), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), glutamate dehydrogenase (GDH), glucose-6-P dehydrogenase (G6PDH), NADP-malic enzyme (ME), aspartate aminotransferase (AAT) and peroxydases (POD). Specific reactions were measured by spectrophotometry.

Separation of isozymes. The isozymic pattern of malic enzyme and isocitrate dehydrogenase is analysed by electrophoresis on polyacrylamide gels.

Proteins. Soluble proteins were measured by the Folin method.

Organic acids. After extraction by alcohol, organic acids were analysed by silica gel column chromatography and contents measured titrimetrically or by spectrophotometry utilising specific enzyme reactions. Identification was also achieved by co-chromatography.

Amino acids. Separation, identification and measurement of content in the leaves were achieved with an automatic ion-exchange analyser.

Polyamines. Separation, identification and content were obtained automatic micro-analysis.

Total sulphur content. Measurement is carried out with a LECO analyser at the I.N.R.A. Station, Montardon.

Photosynthesis and respiration during 24h cycles are monitored with an infra-red CO<sub>2</sub> analyser.

## Results

### A - Experiments under controlled conditions

#### I. The onset of the metabolic response to pollution

The kinetics of the increase in enzyme capacity were shown to depend on the concentration of SO<sub>2</sub> in the air and on the stage of development of the leaf.

(a) Effect of the concentration of SO<sub>2</sub>. Changes in enzyme capacity (shown in Fig. 1 in % of the control) are triggered earlier, develop a larger amplitude and decrease earlier under 0.15 ppm SO<sub>2</sub> than under 0.10 and 0.06. Hence it appears that increase in SO<sub>2</sub> concentration, within non-necrotic limits, accelerates and enlarges the total process.

Total sulphur content in the leaf is higher after a 3-week pollution by 0.15 ppm than by 0.10. It should be emphasized that there is good quantitative and temporal correlation between increase in enzyme capacity and increase in total sulphur content. This latter stabilizes after about two weeks at a level depending on the SO<sub>2</sub> content in the air.

(b) Effects of leaf age. The kinetics of the changes in enzyme capacity under a given level of pollution depend on the age of the leaf as shown in Fig. 1, i.e. on the metabolic level of the tissues at the beginning of the pollution. Analysis of the metabolism of non polluted leaves (control plants) show a progressive decrease of enzyme capacity with the age. At the beginning of the experiment the plants bear 3 three-lobed leaves labelled F1 (first, adult leaf), F2 (in exponential growth phase) and F3 (in primordium phase): in contrast with F1 and F2 wholly or partly grown in SO<sub>2</sub>-free air prior to pollution, F3 develops under pollution. Results show that the younger the leaf at the beginning of pollution the longer the lag period and the smaller the amplitude of the change in enzyme capacity. Accumulation kinetics for total sulphur content are in good agreement with timing of the enzymic response.

In order to ascertain that these differences between the leaves depend on their age and not on their position on the plant, the first three-lobed leaf was subjected to 7 days of pollution by 0.1 ppm SO<sub>2</sub> beginning at different stages of its development. Measurements of changes in the capacity of malic enzyme and isocitrate dehydrogenase confirmed the kinetics observed for the corresponding F1, F2 or F3 leaves as described above.

## II. Mechanisms underlying the changes in enzyme capacity

Several hypothesis are considered to account for the increase in enzyme capacity in polluted leaves: de novo synthesis of the existing enzyme forms or of isozymes, changes in the synthesis/degradation balance or activation of existing enzymes. Preliminary results suggest that there would be no modification of the isozymic pattern at least in the case of ME and IDH in polluted plants and probably no significant de novo synthesis of enzymes. In contrast, small but significant modification of the  $K_m$  of IDH was measured during pollution: the affinity of the enzyme for its substrate appears to increase in the polluted leaves. Research is in progress.

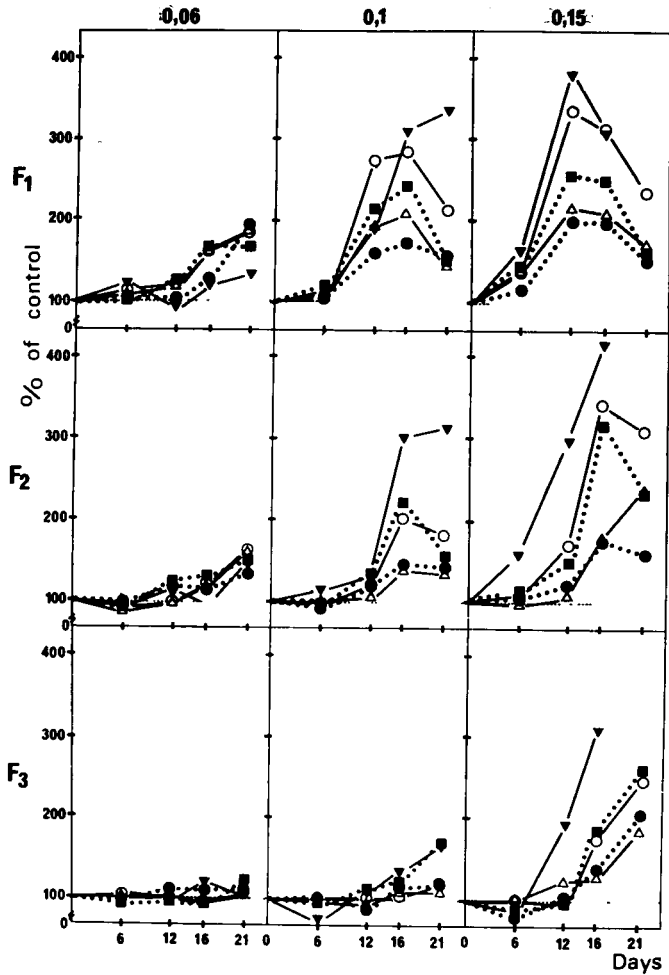


Fig 1. Variation of enzyme capacity during 21 days of pollution by 0.06, 0.1 or 0.15 ppm SO<sub>2</sub> in leaves of different ages (F<sub>1</sub>, F<sub>2</sub>, F<sub>3</sub>, see text). Results in % of those in non treated control plants.

POD ▼—▼ ; IDH ○—○ ; ME ■...■ ; AAT ●....● ; GDH △—△

### III. Physiological consequences of the metabolic readjustment

(a) CO<sub>2</sub> exchanges were continuously recorded during 24h cycles at different moments of the experiments. Increases up to 26% of net photosynthesis and up to 80% of dark respiration were observed for polluted plants during the changes in enzyme capacity.

(b) A pH-stabilizing mechanism could be involved in the metabolic response with the purpose of counteracting acidification processes which may occur during SO<sub>2</sub> fixation. Consistent with this hypothesis are the observed variations in the level of polyamines (putrescine and spermidine) in agreement with those of their precursor arginine and opposite to those of organic acids, mainly malate (Fig 2).

(c) Earlier senescence could result from the acceleration of metabolism: this assumption is supported by the prolonged increase in peroxydase capacity and in the level of serine in the treated leaves.

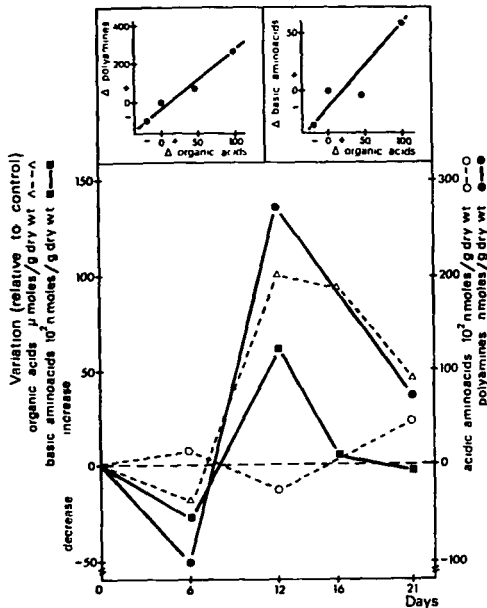


Fig 2. Difference in the content of organic acids, basic and acidic amino acids and polyamines between polluted and control plants as a function of the number of days of pollution. Inserts: correlation between the variation of content in organic acids and content in polyamines and basic amino acids.

### B. Experiments under field conditions

Bean plants are included in field experiments carried on by J. Bonte at the I.N.R.A. Station, Montardon. Plants are subjected to non-necrotic pollution during the whole growth cycle; leaves are sampled at different phases of plant development, lyophilized and sent to Gif-sur-Yvette for biochemical analysis. Changes in the capacity of IDH, ME, POD, PEPC and in level of malate were detected that suggest an acceleration of the metabolism consistent with the model. Earlier senescence has also been observed, together with a decrease in productivity (fruits, -15%; seeds, -23%; aborted seeds, +35%).

### Conclusions

Permanent subnecrotic pollution of bean plants by SO<sub>2</sub> does not act on a specific metabolic target: the primary response consists of an increase in the capacity of a large number of enzymes. The hypothesis of an acceleration of the general metabolism is also supported by changes in metabolite levels and in net photosynthesis and respiration. This effect is relatively slower in younger than in older leaves: the higher the metabolic activity of the tissues at the onset of pollution, the smaller the amplitude of the metabolic readjustment required to respond to pollution. These data, and variation in total sulphur content, suggest that the increase in metabolizing capacity of the cell would tend to stabilize sulphur content and internal pH (through increase in polyamines). The physiological cost of this adaptive process would imply earlier senescence and lower final productivity. Adequacy of this model to behaviour under field conditions is under current research.

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Pierre M., Queiroz O.- Enzymic and metabolic changes in bean leaves during continuous pollution by subnecrotic levels of SO<sub>2</sub>. Environ. Pollut. (Series A) 25, 41-51 (1981)

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Contractor: Research Institute for Plant Protection,  
Binnenhaven 12, Wageningen, the Netherlands

Contract no: 330-79-8 ENV N

Project leader: Dr. A.C. Posthumus

Co-workers: O. Cleij, G.W.H. Laurens, M.S. Schrijver and  
Drs. A.E.G. Tonneijck

Title of project: Research on the influence of different air pollutants,  
separately and in combinations, on agricultural,  
horticultural and forestry crops

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### Objectives of the research

In continuation of the research during the first phase of the Second Environmental Research Programme of the EEC (contract no 297-77-8 ENV N) new fumigations were carried out to study the effects of several air pollutants under controlled conditions. In general the same plant species were used. The evaluation of foliar injury, the establishment of concentration-response relationships and no-adverse-effect levels and the determination of possible growth retardations were the main objectives.

Attempting to relate the results of laboratory studies to the field situation has often proved to be difficult. As a result increased attention has been directed towards examining plant responses following simultaneous exposures to two or more pollutants. Moreover, preliminary experiments were performed in practice using open-top chambers or by exposing plants to polluted ambient air.

Results should be of value for the effect monitoring network with plants as is used in the Netherlands and for the practice of agriculture and nature management. For this reason effects of air pollutants on some economically important crops were studied.

### Materials and methods

#### Fumigations with PAN, O<sub>3</sub> and PAN + O<sub>3</sub>

Plants of small stinging nettle were fumigated under controlled conditions. Experiments with different O<sub>3</sub>-concentrations were carried out during three hours both in the morning and in the afternoon. In a similar way plants were treated with 125 or 250 µg/m<sup>3</sup> PAN, 450 µg/m<sup>3</sup> O<sub>3</sub> or the mixtures with the same concentrations.

To determine the no-adverse-effect level for PAN plants were fumigated during varying exposure periods with concentrations ranging from 100 till 1000  $\mu\text{g}/\text{m}^3$ . After one week the presence of at least one plant showing specific symptoms was taken as a criterion for the occurrence of PAN-injury.

#### Fumigations with $\text{SO}_2$ , $\text{O}_3$ and $\text{SO}_2 + \text{O}_3$

Young plants of big plantain, small stinging nettle and tobacco 'Bel W3' were fumigated for two weeks with  $\text{SO}_2$ ,  $\text{O}_3$  and  $\text{SO}_2 + \text{O}_3$  in fumigation greenhouses. Using a fixed  $\text{O}_3$ -concentration of 75  $\mu\text{g}/\text{m}^3$   $\text{SO}_2$ -concentrations were 100, 200 and 300  $\mu\text{g}/\text{m}^3$ . To compare these results with those under more practical conditions the same species were treated in open-top chambers with unfiltered ambient air or with air to which 200  $\mu\text{g}/\text{m}^3$   $\text{SO}_2$  was added.

Big plantain was exposed for four weeks to  $\text{SO}_2$ ,  $\text{O}_3$  and their mixtures. Average concentrations were successively: 440  $\mu\text{g}/\text{m}^3$   $\text{SO}_2$  and 64  $\mu\text{g}/\text{m}^3$   $\text{O}_3$ , 290  $\mu\text{g}/\text{m}^3$   $\text{SO}_2$  and 70  $\mu\text{g}/\text{m}^3$   $\text{O}_3$ , and 160  $\mu\text{g}/\text{m}^3$   $\text{SO}_2$  and 44  $\mu\text{g}/\text{m}^3$   $\text{O}_3$ .

To study the effects of these air pollutants on more economically important crops perennial ryegrass was fumigated for four weeks with 160  $\mu\text{g}/\text{m}^3$   $\text{SO}_2$ , 70  $\mu\text{g}/\text{m}^3$   $\text{O}_3$  or the combination. Because of its sensitivity big plantain was also used in these experiments.

#### Fumigations with $\text{SO}_2 + \text{NO}_2$ , $\text{NO}_2 + \text{O}_3$ and $\text{SO}_2 + \text{NO}_2 + \text{O}_3$

Perennial ryegrass was treated with the combinations  $\text{SO}_2 + \text{NO}_2$ ,  $\text{NO}_2 + \text{O}_3$  and  $\text{SO}_2 + \text{NO}_2 + \text{O}_3$ . Four experiments, each of three weeks, were carried out with different concentrations: 60 - 110  $\mu\text{g}/\text{m}^3$   $\text{SO}_2$ , 40 - 60  $\mu\text{g}/\text{m}^3$   $\text{O}_3$  and 50 - 90  $\mu\text{g}/\text{m}^3$   $\text{NO}_2$ . Plants of tomato, lettuce, endive and radish were also fumigated in some of these experiments.

#### Influence of HF-polluted ambient air

Based on results from artificial fumigations the influence of HF on enzyme activities was studied in leaves of field exposed gladioli. Corms of two varieties, the sensitive variety Snowprincess and the resistant variety Flowersong, were put in standard soil in plastic containers and grown outdoors. After eight weeks of growth two containers per variety were brought to each of six experimental fields at different distances of two HF-sources. Following an exposure period of two weeks leaf tips were harvested to determine the activities of enolase and peroxidase. Lined filter papers were

exposed during the same period. In this way 10 experiments were performed from late May till the middle of October 1979.

## Results

### Fumigations with PAN, O<sub>3</sub> and PAN + O<sub>3</sub>

As had already been found for PAN, O<sub>3</sub> also can injure the leaves of small stinging nettle significantly. For both morning and afternoon exposures a threshold concentration of ca 450 µg/m<sup>3</sup> O<sub>3</sub> could be established, above which injury augmented with increasing concentration. In contrast with results of PAN-fumigations O<sub>3</sub> did not influence dry matter weights of the different organs.

Fumigations with the combination PAN + O<sub>3</sub> caused a clear antagonistic response regarding foliar injury, when plants were treated in the morning with 250 µg/m<sup>3</sup> PAN and 450 µg/m<sup>3</sup> O<sub>3</sub>. In all other treatments synergism never occurred.

The no-adverse-effect level for foliar injury of small stinging nettle by PAN can be given as follows:

$$\log c \text{ (ppb)} = -0.64 \log t \text{ (hours)} + 1.79$$
$$r = -0.94 \quad P \leq 0.01$$

For co-ordinates above and to the right side of the diagram visible injury due to PAN is to be expected (see Figure 1).

### Fumigations with SO<sub>2</sub>, O<sub>3</sub> and SO<sub>2</sub> + O<sub>3</sub>

In the fumigations of two weeks in the fumigation greenhouses 100 µg/m<sup>3</sup> SO<sub>2</sub> appeared too low to cause a synergistic response in combination with 75 µg/m<sup>3</sup> O<sub>3</sub>. It was concluded that 200 µg/m<sup>3</sup> SO<sub>2</sub> was the lower limit in this type of experiments. As a result slight synergistic responses were found for tobacco 'Bel W3' and small stinging nettle. However, only big plantain responded to all combination treatments with decreases in dry matter production. Addition of 200 µg/m<sup>3</sup> SO<sub>2</sub> to unfiltered ambient air in open-top chambers caused much higher levels of foliar injury for all three species. Tobacco 'Bel W3' clearly showed symptoms specific for O<sub>3</sub>, while plants treated with unfiltered ambient air without SO<sub>2</sub> were hardly affected.

In the first two long-term experiments with big plantain no synergistic responses regarding leaf injury occurred. In the last one with the lowest concentrations foliar injury due to the pollutant mixture was higher than



after the  $O_3$ -treatment, while  $150 \mu\text{g}/\text{m}^3$   $\text{SO}_2$  did not cause visible symptoms. In general the combination reduced production of dry matter and leaf area more than  $O_3$ . However, no relationship between these parameters and levels of leaf injury could be found.

The experiments with perennial ryegrass showed that this species did not respond with a reduction in dry matter production, while big plantain did. However, a slight discoloration of the leaf tips of this grass was visible after exposures of four weeks to all treatments.

#### Fumigations with $\text{SO}_2 + \text{NO}_2$ , $\text{NO}_2 + \text{O}_3$ and $\text{SO}_2 + \text{NO}_2 + \text{O}_3$

In none of the experiments with these combinations perennial ryegrass showed visible symptoms or growth reduction. For the other species the combination  $\text{NO}_2 + \text{O}_3$  and  $\text{SO}_2 + \text{NO}_2 + \text{O}_3$  resulted in significant numbers of injured leaves. Only in one treatment  $\text{SO}_2 + \text{NO}_2$  was harmful. In these experiments the presence of  $\text{O}_3$  was decisive, the more so as numbers of injured leaves depended on the  $\text{O}_3$ -concentrations.

#### Influence of HF-polluted ambient air

Only plants exposed at two of the six fields differed significantly in fluoride accumulation, when results of the ten exposure periods were combined. The same was true for the fluoride accumulation in limed papers. As a result, differences were found in the length of the necrotic leaf tips for the sensitive gladiolus variety Snowprincess. Yet, activities of enolase and peroxidase in leaves of both varieties were not influenced.

#### General conclusions

1. Small stinging nettle, the indicator plant for PAN, can also be injured by  $\text{O}_3$ .
2. When PAN is present in really phytotoxic concentrations,  $\text{O}_3$  clearly reduces the effect of PAN.
3. Comparison of the experimentally determined no-adverse-effect level for foliar injury of small stinging nettle by PAN and the measured concentrations in the Netherlands shows that PAN-injury in the field is not to be expected.
4. Addition of  $200 \mu\text{g}/\text{m}^3$   $\text{SO}_2$  to filtered air with  $75 \mu\text{g}/\text{m}^3$   $\text{O}_3$  in greenhouses may result in synergistic responses regarding foliar injury of tobacco Bel W3 and small stinging nettle. However, addition of the same

SO<sub>2</sub>-concentration to unfiltered ambient air in open-top chambers is much more harmful.

5. Perennial ryegrass appears to be rather insensitive to the treatments as far as growth is concerned, while other plant species react very clearly.
6. Results of the treatments with the combinations SO<sub>2</sub> + NO<sub>2</sub>, NO<sub>2</sub> + O<sub>3</sub> and SO<sub>2</sub> + NO<sub>2</sub> + O<sub>3</sub> reveal that the presence of O<sub>3</sub> is decisive.
7. Although artificial fumigations with HF reduce enolase activities in leaves of monocotyledonous ornamentals consistently, no such influence may be found when gladioli are exposed for two weeks in areas with HF-polluted ambient air.

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- Mooi, J. en A.E.G. Tonneijck: Invloed van SO<sub>2</sub>, O<sub>3</sub> en SO<sub>2</sub> + O<sub>3</sub> op planten. Proc. SO<sub>2</sub>-symposium, Wageningen, Pudoc (1980): 119.
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- Posthumus, A.C.: Monitoring of levels and effects of air-borne pollutants on vegetation. Use of biological indicators and other methods; national and international programmes. Proc. ECE-Symposium on the effects of air-borne pollution on vegetation, Warsaw (1980): 296-311.
- Tonneijck, A.E.G.: Influence of HF on enzyme activities in monocotyledonous ornamentals. Proc. ECE-Symposium on the effects of air-borne pollution on vegetation, Warsaw (1980): 316.

#### Oral communications

- Posthumus, A.C.: Plants as bio-indicators for atmospheric pollution. European Environmental Summerschool E 4, Jülich, 1 June 1979.
- Posthumus, A.C.: Monitoring of levels and effects of air-borne pollutants on vegetation. Use of biological indicators and other methods; national and international programmes. ECE-Symposium on the effects of air-borne pollution on vegetation, Warsaw, 22 August 1979.
- Posthumus, A.C.: Biological indicators of air pollution. 32nd School in Agricultural Science, University of Nottingham, Sutton Bonington, 1 September 1980.
- Posthumus, A.C.: Some problems concerning the exposure-response relationships of the influence of air pollutants on plants. RIV/ECE International workshop on air pollution deposition, its calculation and effects on flora, fauna and materials, Bilthoven, 4 November 1980.

Tonneijck, A.E.G.: Influence of HF on enzyme activities in monocotyledonous ornamentals. ECE-Symposium on the effects of air-borne pollution on vegetation, Warsaw, 22 August 1979.

Tonneijck, A.E.G.: Some effects of air pollutants on plants, separately and in combinations. 1st Meeting of Contact Group "Air pollution effects on plants" of the Commission of the European Communities, Wageningen, 12 September 1980.

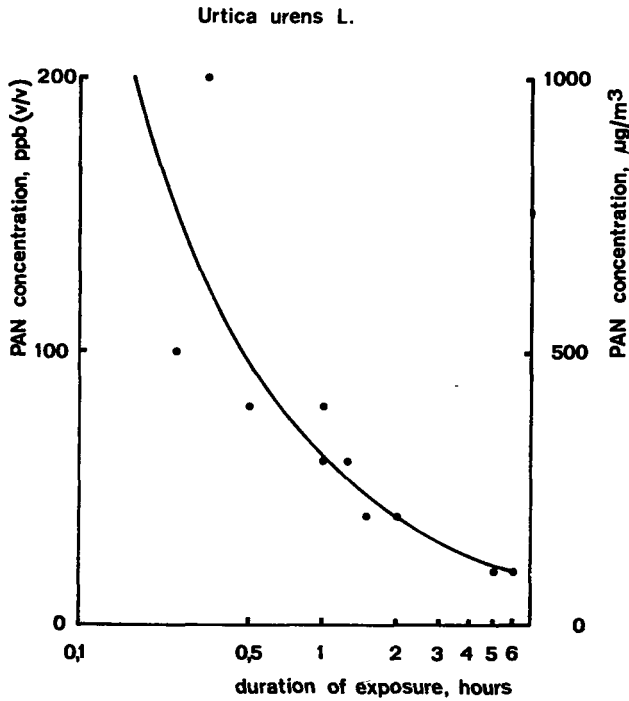


Figure 1: no-adverse-effect level for foliar injury of small stinging nettle by PAN

**Contractor:** NATURAL ENVIRONMENT RESEARCH COUNCIL through its  
INSTITUTE OF TERRESTRIAL ECOLOGY (ITE)

**Contract No:** 280-77-1. ENV UK, SUPPLEMENTARY AGREEMENT NO 1

**Project Leader:** J.N.R. JEFFERS

**Title of Project:** EFFECTS OF AIR POLLUTION BY SULPHUR AND ITS DERIVATIVES  
ON PLANTS

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#### OBJECTIVES OF RESEARCH

To describe the loads of atmospheric pollutants in a semi-rural area of Scotland and assess their effects on plant growth, especially trees, enabling effects of changing amounts of pollutants to be predicted. Specifically:

- A. To measure the diurnally and seasonally changing concentrations of SO<sub>2</sub>, NO<sub>x</sub> and O<sub>3</sub> above the canopy of an intensively studied forest of Scots pine (*Pinus sylvestris*).
- B. To describe chemical properties of precipitation (rain) in northern Britain including the characterisation of inputs to the intensively studied forest.
- C. To develop techniques for measuring fluxes of SO<sub>2</sub> to forests.
- D. Using data from A-C,
  - i to describe the ways in which forests influence the pathways and inputs of pollutant sulphur to soil;
  - ii to describe direct effects of polluted atmospheres on epicuticular waxes of pine needles; and
  - iii to initiate studies of the direct effects of gaseous pollutants on growth of pines.

#### MATERIALS AND METHODS

1. Sites: The intensively studied pine forest (Devilla Forest) with its associated field laboratory is located in central Scotland, mean winter concentrations of SO<sub>2</sub> being 30-40 µg m<sup>-3</sup>. Other observations were made from ITE's laboratories near Edinburgh and Aberdeen; a site for new studies of effects of gaseous pollutants on tree growth is being developed in Glasgow.
2. Instruments:
  - i Concentrations of gaseous pollutants were measured at Devilla at 10-minute intervals using automatic analysers.
  - ii Assessments of crown leaching were made with equipment recommended by Lakhani and Miller (1978).
  - iii Open-top chambers, 1.2 m diameter x 1.5 m high, have been designed for exposing single (young) pine trees to filtered or unfiltered atmospheres, and possibly different mixtures of filtered and unfiltered air.

## RESULTS

### A. Concentrations of SO<sub>2</sub>, NO<sub>x</sub> and O<sub>3</sub> above the canopy of Scots pine at Devilla.

Continuous measurements for more than 3 years show clearly that concentrations of SO<sub>2</sub>, NO, NO<sub>2</sub> and O<sub>3</sub> vary diurnally and seasonally. The patterns of SO<sub>2</sub> and NO<sub>x</sub> are similar, concentrations being smaller in summer than in winter; concentrations of O<sub>3</sub> were maximal in spring. Detailed analyses have shown:

- i The occurrence of oxides of nitrogen has not been appreciated fully. The average ratio of NO<sub>2</sub>/SO<sub>2</sub> at Devilla is 1.3. Thus, in terms of concentrations, oxides of nitrogen are important. At other rural sites, NO<sub>x</sub>/SO<sub>2</sub> ratios average 0.8, in contrast to a ratio of 0.5 for known emissions. The difference is probably attributable to the more efficient removal of SO<sub>2</sub> than that of NO and NO<sub>2</sub>.
- ii The frequency of large gas concentrations predicted from an assumed log-normal distribution differs from those based on extreme value statistics (Fig 1b). These differences are important because it is thought that occasional large concentrations are more important than mean concentrations when considering effects on plants. The occurrence of smaller concentrations of SO<sub>2</sub> and NO<sub>x</sub> conform to a log-normal frequency distribution (Fig 1a).

### B. Nature of precipitation

The north-west to south-east trends of increasing acidity and of sulphate concentrations, previously identified, have been confirmed by further data from 18 sites in northern Britain (Fig 2a). Additionally, other ions have been measured. Nitrate and pollutant sulphate are both strongly correlated with acidity (H<sup>+</sup> ion) (Fig 3), nitrate accounting for c 30% of total acidity, the balance being associated with 'excess' (pollutants) sulphur. When concentrations are multiplied by amounts of rain it seems that amounts of SO<sub>4</sub><sup>2-</sup>, NO<sub>3</sub><sup>-</sup> and H<sup>+</sup> reaching ground are similar in the north-west and south-east (Fig 2b). The proportion of sulphate attributable to dry deposition of SO<sub>2</sub> on gauge surfaces was greater in the south-east than in the north-west.

### C. Flux measurements

A fluxatron based on a rapid response sulphur analyser has been developed and is now ready for field testing: it detects SO<sub>2</sub> at ambient concentrations down to 4 µg SO<sub>2</sub> m<sup>3</sup> (1.5 ppbv). Field measurements will be made at Devilla in 1981.

### D. Interrelations of air pollutants and trees

- i Effects of Scots pine on precipitation reaching soil. At Devilla forest S, Ca and Mg concentrations increased in precipitation on passing through the canopy, with gross precipitation < throughfall stemflow, pH decreasing in the same sequence (pH 4.2, 3.7, 3.3). Their lime potentials (pH - ½p (Ca, Mg)) showed that stemflow had more capacity for acidifying soil than gross precipitation and

throughfall. Dry deposition, wet deposition and crown leachates, accounted for 46%, 32% and 22% of the  $37 \text{ kg SO}_4\text{-S ha}^{-1} \text{ yr}^{-1}$  reaching the forest floor during the first year. Distribution of S (and H ions) on soil depended upon the 'pathway' followed. Amounts deposited round stems, by stemflow, were more than 100 times greater per unit area than those where gross precipitation fell on soil without being intercepted by the canopy. Thus there is a mosaic of inputs to soil with large localised inputs around stembases.

- ii Leaf surface studies on Scots pine. From a scanning electron microscopy study of structural changes of epicuticular wax with time erosion of surfaces exposed to polluted air was found to be more rapid than of those exposed to clean air (Fig 4a). Changes also occurred in cuticular transpiration which was greater from older needles and from needles from the polluted site - cuticular transpiration is inversely related to cuticular resistance (Fig 4b) Estimated dry matter production at the polluted site decreased by 5% when water supply was limited. Contact angles between water droplets and needle surfaces decreased where wax had been eroded, so allowing water to wet the needle surface more readily.
- iii Gas effects on growth of Scots pine. To provide realistic field data about the responses of trees to ambient gas concentrations, a field experiment, using young pines (3-yr old) grown in open-top chambers provided with filtered or unfiltered air, is being established in a polluted area in the west of Scotland (Glasgow). Development work to ensure uniform gas concentrations, adequate air turbulence and a low aerodynamic resistance ( $r_a$ ) is now well advanced as is the construction of air filtering systems.

#### CONCLUSIONS AND SYNTHESIS

This integrated research programme, based in Scotland, and forming part of the EEC programme, has now passed the main descriptive phase. Utilising knowledge of air pollution properties ( $\text{SO}_2$ ,  $\text{NO}_x$  and  $\text{O}_3$  acid rain), the foremost research objective is now to understand plant responses using one species, Scots pine. Gas monitoring has shown that very large and potentially damaging concentrations of mixtures of pollutants can occur in an area having small mean concentrations. We wonder how often damage has been attributed to  $\text{SO}_2$  not knowing that comparable concentrations of  $\text{NO}_x$  may have also been present. The importance of  $\text{NO}_x$  as a gas pollutant has been demonstrated clearly, and it has been shown that nitrate in rainfall (in addition to sulphate) makes a substantial contribution to the acidity of precipitation. In studying the ways in which forests influence the pathways and inputs to soil, a potentially important indirect effect has been identified, namely the capacity of pine to greatly intensify local concentrations of pollutants entering the soil, especially where dry deposits of pollutants accumulating on foliage are large. Studies of direct effects on needles have shown that pollutants may influence plant growth by modifying the protective (epicuticular) wax. Having shown convincingly that atmospheres are likely to contain mixtures of pollutants, ITE is directing its effort at an understanding of whole-plant responses to ambient mixtures of pollutants in conditions as close as practicable to those occurring in forests: open-top chambers, backed

up by 'process' studies in controlled conditions, are being used.

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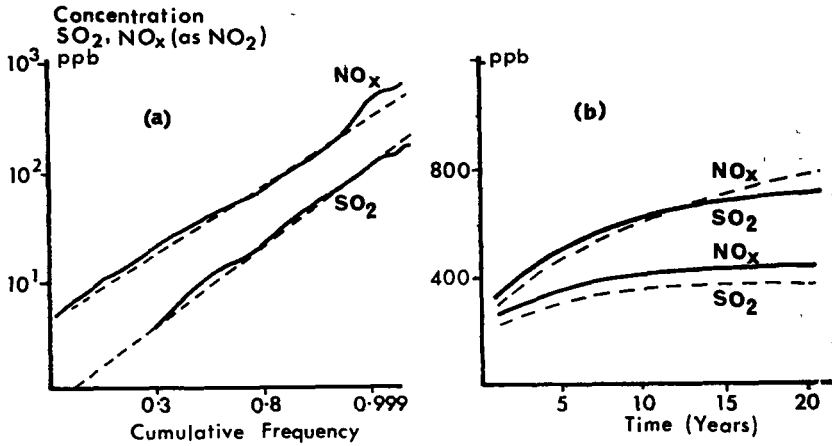
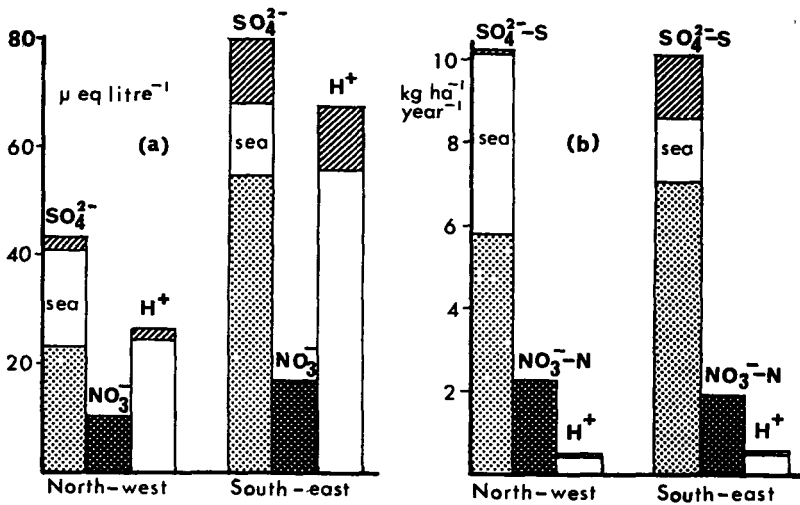


Figure 1. (a) Log-normal distributions of NO<sub>x</sub> and SO<sub>2</sub> concentration data. Actual (—) and theoretical fitted (---) distributions.

(b) Predicted maximum concentrations (10-minute sampling) based on log-normal distributions (—) and extreme value statistics (---).



▨ Dry deposition on gauge attributed to SO<sub>2</sub>

Figure 2. (a) Geographical differences in rainfall quality (ion concentrations, μ eq litre<sup>-1</sup>).

(b) Geographical differences in total inputs in rain (kg ha<sup>-1</sup> yr<sup>-1</sup>).



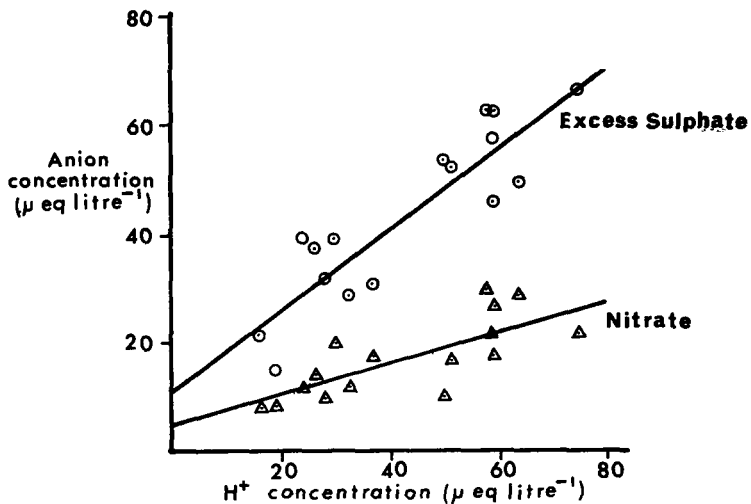


Figure 3. Relationship between concentrations of nitrate (Δ), 'excess' sulphate (○) and acidity in rainfall collected, 1980 (ITE monitoring network).

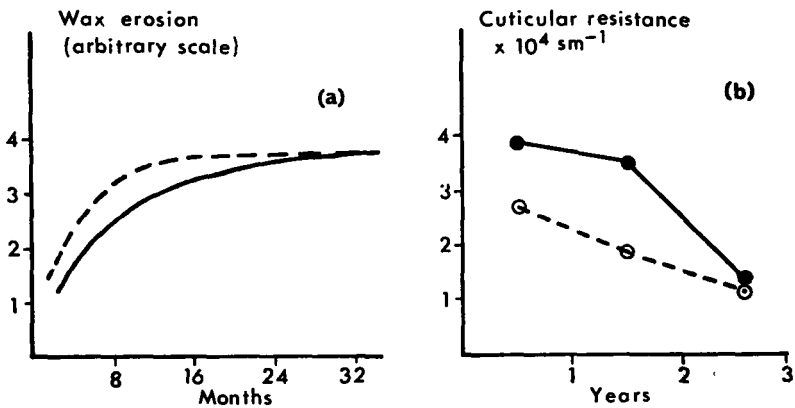


Figure 4. Erosion of epicuticular wax structure (based on an arbitrary scale) and changes in cuticular resistance of ageing needles of Scots pine sampled from polluted (---) and unpolluted (—) environments.

Contractor : Imperial College of Science and Technology

Contract n° : ENV-358 UK

Project leader : Dr. J.N.B. Bell

Title of project : The impact of ozone on vegetation in south-east England

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#### Objective of the research

This research forms part of a four-year study with the primary objective of assessing the impact of ozone on vegetation in an area to the west of London.

#### Materials and methods

A total of 45 sites were established within a semi-circular study area, of radius 80 km, to the west of, and centred in, London. At all of these sites, the amount of phytotoxic ozone was determined using five replicate plants of the indicator *Nicotiana tabacum* cv. Bel-W3 grown in self-watering containers. The mean concentration of nitrogen dioxide was determined with diffusion tubes, using a technique recently developed by AERE, Harwell for use indoors. The growth of commercial cultivars of *Phleum pratense*, *Lolium perenne*, *Trifolium repens*, *Trifolium pratense* and *Raphanus sativus* (2 cultivars) was determined at 20 of the 45 sites. Five replicate plants of each cultivar were grown in self-watering containers at each of these sites. Each site was visited at weekly intervals during a 20-week period from mid-May to late September 1980, in order to assess the amount of injury on the *Nicotiana* leaves and to measure the size of the other plants. Every four weeks, the plants and diffusion tubes were replaced; the plants in the growth study were then dried and weighed. Relationships between plant size and dry weight were established for each cultivar so that growth rates could be calculated from field data.

Experiments to compare the growth of a number of plant species and cultivars in ambient air with that in charcoal-filtered air were carried out at two sites in open-top chambers. The plants were grown from seed in the chambers in a fertilized peat/sand mix in individual pots supplied with water by subirrigation. The concentration of ambient ozone was recorded continuously using a Dasibi monitor.

## Results

Ambient concentrations of ozone during the summer of 1980 were abnormally low in the U.K. At Ascot, for example, the percentage of days during the period 1 May-30 September on which the maximum hourly mean concentration exceeded 0.08 ppm was 1.3% (i.e. 2 days). For comparison, the equivalent values for south-east England over the period 1971-1978 ranged from 6.1% (1977) to 29.3% (1976).

Nevertheless, significant reductions of plant growth in ambient air, compared with that in filtered air, were observed in a number of species. The results of the various experiments carried out in open-top chambers are summarised below (reductions in growth are expressed as a percentage of the filtered-air value).

(a) Forage crops - chambers at 2 sites.

Percentage of days when maximum hourly mean  $[O_3]$  > 0.06 ppm = 6% (Leatherhead) and 7% (Ascot).

Significant ( $P < 0.05$ ) reductions in above-ground dry weight at both sites of *Trifolium repens* cv. Grasslands Huia (7.5% at Leatherhead; 12% at Ascot) and *Trifolium pratense* cv. Hungaropoly (6.5% at Leatherhead; 9% at Ascot).

No significant effects at either site on above-ground dry weight of *Lolium perenne* cv. S24, *Lolium multiflorum* cv. RvP, *Dactylis glomerata* cv. Roskilde and *Phleum pratense* cv. Erecta.

(b) Vegetables

Percentage of days when maximum hourly mean  $[O_3]$  > 0.06 ppm = 11%.

Significant ( $P < 0.05$ ) reductions in leaf dry weight of *Spinacia oleracea* cvs. Sigmaleaf (20%) and Long Standing Round (21%).

No significant effects on dry weights of *Phaseolus vulgaris* cv. Provider and *Brassica oleracea* cvs. Calabrese and Early Purple Sprouting.

(c) Native (wild) species

Percentage of days when maximum hourly mean  $[O_3]$  > 0.06 ppm = 5.5%.

Significant ( $P < 0.05$ ) reduction in above-ground dry weight of *Reseda lutea* (35%).

No significant effects on dry weights of *Papaver rhoeas*, *Agropyron repens*, *Anagallis arvensis*, *Avena Fatua*, *Crepis capillaris*, *Chenopodium album*, *Chenopodium rubrum*, *Festuca rubra*, *Digitalis purpurea*, *Sanguisorba minor*, *Lathyrus aphaca*, *Chenopodium bonus-henricus* and *Lotus uliginosa*.

(d) Cereals (vegetative growth only)

Percentage of days when maximum hourly mean  $[O_3]$  > 0.06 ppm = 13%.

Significant ( $P < 0.05$ ) reductions in relative growth rates of *Hordeum vulgare* cvs. Mavis Mink (8%), Wing (8%), Georgie (8%), Hassan (11%) and Sundance (12%).

No significant effects on relative growth rates of *Hordeum vulgare* cvs. Abacus and Magnum, *Avena sativa* cvs. Printa and Maris Oberon, *Secale cereale* cv. Rheidol and *Triticum aestivum* cv. Timmo.

The average amount of leaf injury to *Nicotiana tabacum* plants over the 20-week period at each study site was significantly positively related to the distance of the site from London ( $r = 0.438$ ;  $P < 0.01$ ), i.e. there was less injury in, and close to, London. This result may be caused by the presence in urban areas of higher concentrations of nitric oxide (NO), which destroys ozone via the reaction  $\text{NO} + \text{O}_3 \rightleftharpoons \text{NO}_2 + \text{O}_2$ .

There is no simple method of measuring ambient concentrations of NO; however, the concentrations of nitrogen dioxide ( $\text{NO}_2$ ) at the study sites should give a measure of the relative levels of NO present. The mean concentration of nitrogen dioxide ( $C_{\text{NO}_2}$  ppm) at each site did decrease with increasing distance from London (X km) according to the equation  $\ln C_{\text{NO}_2} = 2.80 - 0.0148X$  ( $r = 0.628$ ;  $P < 0.001$ ). The concentrations found at certain sites which lay within 250 m of a major road were consistently higher than those at neighbouring sites. The nitrogen dioxide concentration was negatively correlated with the amount of ozone-induced leaf injury ( $r = -0.381$ ;  $P < 0.05$ ). The concentrations of  $\text{NO}_2$  recorded were comparable to those obtained using physico-chemical monitors. This technique provided a satisfactory method of determining long-term mean  $\text{NO}_2$  concentrations in the field.

It was not possible to elucidate any relationship between the amount of ozone-induced leaf injury on *Nicotiana tabacum* and the growth rates of the other plant species studied in the field when variation through time was considered. This was basically because the ambient concentration of ozone depends on several meteorological variables (temperature, sunshine, etc.), which themselves affect plant growth rates.

The relationships between growth rates, ozone-induced leaf injury on *Nicotiana*, and other variables at the study sites were examined using a multiple regression technique. The relative growth rates of both clover species (*Trifolium repens* and *Trifolium pratense*) were significantly ( $P < 0.05$ ) lower at sites with high amounts of *Nicotiana* leaf injury; in contrast, the relative growth rates of both grass species (*Lolium perenne* and *Dactylis glomerata*) were not significantly related to the amount of

*Nicotiana* injury. These findings are consistent with the results of the open-top chamber experiments, in which the growth of the clover species, but not the grass species, was significantly reduced in ambient air. The results of controlled fumigation studies also indicate that the *Trifolium* species are more sensitive to ozone than *Lolium* and *Dactylis*.

The relative growth rate of neither *Raphanus sativus* cultivar was related to the amount of *Nicotiana* injury. However, when the data were expressed as the difference in relative growth rate between Cherry Belle (an ozone-sensitive cultivar) and Scarlet Globe (an ozone-resistant cultivar), a significant relationship with the amount of *Nicotiana* injury was found. At sites with little injury, the growth rates of the two cultivars were similar; at sites with high amounts of *Nicotiana* injury, the growth rate of the ozone-sensitive cultivar was consistently lower than that of the ozone-resistant cultivar.

#### Conclusions

The ambient concentrations of ozone in south-east England during the summer of 1980 were sufficient to adversely affect the growth of several different plant species. These particular species are known to be relatively sensitive to ozone. However, the concentrations of ozone during 1980 were abnormally low, and a more marked impact on plant growth might be observed in more normal summers.

High concentrations of ozone were not confined to urban areas; in contrast, the amount of ozone, as determined using indicator plants, was lower at urban sites than at more rural sites. Thus, adverse effects of ambient ozone on the growth of sensitive plant species might be expected to have occurred over a considerable proportion of the 10,000 km<sup>2</sup> study area.



**TOPIC 15 : AIR QUALITY**  
**Health effects**

**Contractor :** Biochemisches Institut für Umweltcarcinogene  
Sieker Landstraße 19, D-2070 Ahrensburg, F.R.G.  
**Contract n°:** 138-76-10 ENV D

**Project leader :** Prof.Dr.G.Grimmer

**Title of project :** Investigation on carcinogenic impact of  
used lubricating oil from vehicles

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**Objective of the research :** The objective of the proposed investigation is to estimate the carcinogenic risk resulting from handling with used lubricating oil and the identification of the most effective substances, responsible for the carcinogenic impact of used oil. It, furthermore, occurred to be necessary to investigate a series of used oil samples collected from gasoline and DIESEL fuel driven passenger cars and trucks.

**Introduction :** The estimation of the carcinogenic potency of a lubricating oil sample from chemical analytical data presumes the identification of carcinogenic classes of compounds occurring in the oil as well as information on their contribution to the biological effect of the total sample. A comparison of the tumorigenic effect of single fractions (which may contain one or more defined classes of compounds) with that of an unseparated sample of lubricating oil using a carcinogen specific test model (e.g. skin painting) allows to establish a balance of the biological effect and to recognize the most effective class of compounds (analysis of the balance of impact = ABI). ABI have been performed in case of e.g. vehicle exhaust and PAH containing more than 3 rings have been shown to be the most effective fraction in this matrix (1). Due to these results lubricating oil was separated into PAH-containing and PAH-free fractions. Since 2- and 3-ring PAH do not exhibit significant carcinogenic activities (2), the PAH-fraction has been separated into PAH containing 4 - 7 rings and into PAH containing less than 4 rings.

**1. Identification of carcinogenic constituents of used lubricating oil :**

To determine the biological active part of PAH or single compounds as e.g. benzo(a)pyrene occurring in used lubricating



oil, it is separated into PAH-containing and PAH-free fractions. The biological effect of these fractions then is compared with that of the original lubricating oil. Painting resp. dropping onto the skin of mice was used as carcinogen specific test.

1.1 Material : For the animal experiments an oil was used which had been aged on the chassis dynamometer under standard conditions simulating city traffic using a test vehicle with gasoline engine (1500 cm<sup>3</sup>, 55 kW/5800 min<sup>-1</sup>; 232 mg BaP/kg oil). Fuel with lead content of 0.4 g/l type CEC European Reference Fuel RF-01-T 74 was used.

1.2 Separation into fractions : Lubricating oil was dissolved in cyclohexane and the PAH extracted with nitromethane (v/v, 6 times) from this solution. Separation of the PAH according to the number of rings was performed by chromatography of the residue of the nitromethane phase on Sephadex LH 20 (elution with propanol-2). One half of the fractions 1 - 3 was recombined yielding a reconstituted oil sample. The fractions contained the following classes of compounds : (1) paraffins and alkenes, free of PAH (91.6 m-%); (2) PAH with 2 and 3 rings (incl. fluoranthene)(7.3 m-%); (3) PAH with more than 3 rings (from chrysene)(1.14 %).

1.3 Results of the animal experiments : Table 1 shows the results of the experiments with used lubricating oil and fractions thereof (dropping onto the skin of femal CFLP mice). The fraction containing PAH with more than 3 rings exhibits the highest tumorigenic activity. It accounts for only 1.14 % by weight but for about 70 % of the biological impact of the oil. Simultaneously tested benzo(a)pyrene (in three dosage) showed after a probit analysis that the BaP content of the oil sample explains about 18 % of the total carcinogenic activity(3).

The result of ABI characterizes PAH containing more than 3 rings to be the most relevant class of compounds in used lubricating oil. According to this significant biological effect it seems possible to calculate the biological impact of oil samples from the mass concentrations of PAH.

## 2. Chemical investigation of fresh and used engine lubricating oil in respect to the PAH-content.

2.1 Method : The enrichment of PAH from mineral oil products has been published elsewhere (4, 5). Condition of GC: Instrument Perkin Elmer, type Sigma 2, CPSil 5 column 0.4 mm x 52 m with 0.72  $\mu$ m coating; 2 ml He/min carrier gas flow; injection bloc temp.: 270°C. To check the responses of the FID, a reference solution containing 14 PAH standards was used quantitatively. The PAH isolated from the sample were dissolved in toluene and injected splitless at 110°C oven temp.. After 5 min. the split was opened and the column heated up to 270°C using a temperature-program of 3°C/min.

The quantitative evaluation of the electronically registered FID-signals was performed by comparing the peak areas with that of the internal standard (picene), added initially.

Instrument : Spectra Physics type SP 4100-02.

MS conditions : MAT VARIAN type 112S was used which was coupled to a gas chromatograph Perkin-Elmer type F 22.

## 2.2 Results

2.2.1 Profile of the PAH from lubricating oil - Inventory by GCGC/MS (fresh oil) : PAH, sulphur- and oxygen-containing polycyclic aromatic compounds (S-PAC; O-PAC) which occur in concentrations higher than 0.02 mg/kg in a lubricating oil sample were identified by comparison with reference substances (34) or, if reference compounds were lacking, characterized by mass spectrometry (58 compounds). Glass capillary gas chromatography (=GCGC) combined with mass spectrometry (=MS) was used for this inventory. Mass spectra of typical individuals from several classes (S-PAC, O-PAC and methyl derivatives) are discussed in detail (6). The profile of polycyclic aromatic compounds (=PAC) occurring in mineral oils is significantly different from that obtained by incomplete combustion or pyrolysis of organic materials.

2.2.2 Investigation of fresh commercially available lubricating oils : To get a survey of the range of PAC-concentration in different fresh lubricating oils, 20 commercial samples have been investigated for 12 selected PAC. The results are

summarized in Table 2.

2.2.3 Investigation of fresh re-refined oils: Re-refined oils contain the same PAC as commercially available lubricating oils. The results of an investigation of 8 re-refined samples are included in Table 2.

2.2.4 Investigation of used lubricating oil from passenger cars with gasoline powered engines : Oil samples from vehicles with different mileage (3300 - 110300 km) were investigated. They were driven 1000 to 6000 km with the same oil. The range of the PAH concentration is included in Table 2.

The samples investigated do not exhibit any relation between PAH concentration of the lubricating oil and the driven distance.

2.2.5 Investigation of used lubricating oil from passenger cars with DIESEL powered engines : Table 2 shows the results taken from used lubricating oils from DIESEL driven passenger cars. An increase to about 0.1 - 1 mg BaP/1000 km per litre was observed in case of DIESEL-driven vehicles, whereas an increase of about 5 - 10 mg BaP/1000 km per litre could be detected in case of gasoline powered cars.

2.2.6 Investigation of used lubricating oil from trucks with DIESEL engines : Oil samples were taken after various driving intervals (10 000; 20 000; 30 000 km) from trucks (192 and 265 PS, respectively). Even after 30 000 km no increase of the PAH concentration could be observed. The BaP concentration of about 0.2 mg/kg in used oil differs insignificantly from that in fresh oil. This could be observed in 5 different trucks.

2.2.7 Investigation of used lubricating oil from buses with DIESEL engines : Oil samples from 4 different buses (200 PS = 147 kW) after 10 000, 20 000 and 30 000 km were taken. In no case an increase of the PAH concentration related to the mileage could be detected.

### 3. Conclusion and Recommendation

By painting onto the skin of mice, the used lubricating oil of gasoline powered vehicles produce carcinomas.

Therefore there is a carcinogenic risk resulting from handling with used lubricating oil owing to the content of PAH with more than 3 rings.

**Tab. 1: Comparison of the Carcinogenic Impact of Used Lubricating Oil and Fractions Thereof Applied to the Skin of Mice. (65 CFLP mice female/group, 2 x 0.1 ml test material per week, 104 weeks total, solvent : acetone + cyclohexane 3 + 1)**

Proportion to total oil (%)	test material	individual dose (mg)	Incidence Papillom/Carcinoma	Animal bearing local tumors (%)
100 %	used lubricating oil sample	0.625 1.875 5.625	3 8 14	0 9 29
91.6 %	PAH-free fraction	0.5725 1.7175 5.1525	0 0 1	1 2 0
7.3 %	PAH-containing fraction (with 2 and 3 rings)	0.0456 0.1369 0.4106	2 1 4	2 1 2
1.1 %	PAH-containing fraction (more than 3 rings)	0.0069 0.0206 0.0619	0 7 20	0 2 13
100 %	reconstituted oil from all fractions	0.625 1.875 5.625	1 4 16	2 2 19
	Benzo(a)pyrene	0.003846 0.007692 0.015385	6 6 6	20 44 54
	Solvent (acetone + cyclohexane)		0	1

Concentration of benzo(a)pyrene in used oil : 232 µg BaP/g oil.

Tab. 2: Ranges of PAH-concentration of Fresh and Used Lubricating Oils (Passenger car, Truck)  
(mg/kg)

Oil	fresh		re-refined		used		used	
	distance (km)	Number of samples	8	20	1000 - 6000 gasoline	574 - 6112 Diesel	10 cars	20 000 Diesel
Fluoranthene	0.068 - 2.750	20	5.1 - 68.8	15.6 - 99.0	1.3 - 17.3	0.44 - 1.24		
Pyrene	0.062 - 6.530		0.9 - 11.5	32.7 - 184.0	1.4 - 27.3	0.93 - 2.50		
Benzonaphthothiophene	0.097 - 9.430		1.8 - 4.8	- - -	1.0 - 1.4	1.49 - 1.50		
Chrysene + Triphenylene	0.207 - 13.400		8.1 - 29.5	11.0 - 74.0	7.5 - 12.3	2.00 - 2.60		
Benzofluoranthenes	0.027 - 0.320		3.7 - 7.5	5.7 - 43.4	1.8 - 7.8	0.46 - 0.58		
Benzo(e)pyrene	0.054 - 0.583		0.61 - 3.90	6.4 - 43.5	1.3 - 5.7	0.34 - 0.54		
Benzo(a)pyrene	0.008 - 0.181		0.19 - 0.95	5.2 - 35.1	0.7 - 6.6	0.20 - 0.30		
Perylene	0.007 - 0.127		0.05 - 0.13	1.9 - 10.0	0.6 - 0.8	0.12 - 0.21		
Indeno(1,2,3-cd)pyrene	0.001 - 0.009		0.06 - 0.48	2.1 - 12.5	0.8 - 4.5	0.13 - 0.13		
Benzo(ghi)perylene	0.019 - 0.139		0.11 - 1.43	4.4 - 69.8	2.1 - 8.8	0.36 - 0.35		
Anthanthrene	0.008 - 0.060		0.02 - 0.06	1.6 - 10.8	0.5 - 2.3	0.04 - 0.03		
Coronene	0.002 - 0.016		0.02 - 0.65	2.8 - 23.5	0.1 - 3.4	0.10 - 0.11		

Benzonaphthothiophene = Benzo(b)naphtho(2,1-d)thiophene  
 Benzofluoranthenes = mixture of BbF + BbJ + BkF

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## SUMMARY

The results of the investigation on the carcinogenic impact of used lubricating oil from cars shows :

1. Used lubricating oil from gasoline driven engines exhibits a dose-dependent carcinogenic effect in the skin dropping test model with mice.
2. The carcinogenic effect in this model predominantly results from the content of PAH with more than 3 rings (70 %). A small effect comes from PAH with less than 4 rings.
3. Benzo(a)pyrene counts for only 18 % of the total activity in used oils.
4. Fresh lubricating oil contains about 0.01 mg benzo(a)-pyrene/kg, used oil from gasoline driven vehicles about 1000 fold this amount.
5. The PAH concentration in used lubricating oils from DIESEL driven vehicles (passenger cars, trucks, buses) is not increased even after high mileages.

This investigation was performed by :

Chemical studies : Prof.Dr.G. Grimmer, Prof. Dr.J.Jacob, K.-W. Naujack, G. Dettbarn, Biochemisches Institut für Umweltcarcinogene, Ahrensburg.

Animal studies : Dr. H.Brune, Dr. R. Deutsch-Wenzel, Beratungsforum für Präventivmedizin und Umwelttoxikologie, Hamburg.

Mathematical-statistical evaluation: Prof. Dr.J. Misfeld, Institut für Mathematik, TH Hannover.

Histology : Prof. Dr. U. Mohr, Med. Hochschule Hannover, Institut für exp. Pathologie.

**Contractor** : Gesellschaft zur Förderung der Lufthygiene und  
Silikoseforschung e.V., an der Universität  
Düsseldorf

**Contract No.** : 289-76-10 ENVD, Project 1

**Project leaders** : Prof.Dr.med. H.-W. Schlipkötter  
Prof.Dr.med. F. Pott  
Dr.rer.nat. Tomingas

**Title of project:** Sampling of suspended particulate matter, analysis  
of polycyclic aromatic hydrocarbons (PAH), frac-  
tionating, and testing for carcinogenicity.

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#### Objective of Research

1. The relative proportion of PAH in suspended particulate matter.
2. The carcinogenicity of the total extract, the PAH fraction, the propanol fraction, and the recombination of the three fractions in the subcutaneous (s.c.) test with mice.

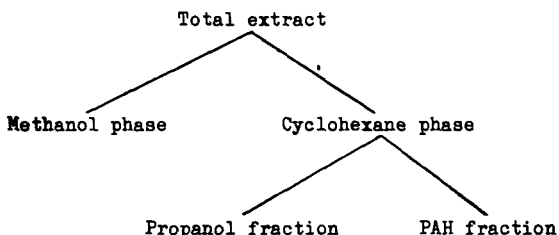
#### Materials and Methods

In winter 1978/79 suspended airborne particulate matter was collected for 3.3 months on filters in three areas: an urban industry district (Duisburg-Neuenkamp), an urban residential district (Düsseldorf), and a rural district (Krahm, Bergisches Land).

The sampling of airborne particulate matter was carried out with a folded glass fibre filter (Dräger), surface area:  $15\text{-m}^2$ , air flow rate:  $1700\text{ m}^3/\text{h}$ , collection of particles  $> 0.3\text{ }\mu\text{m}$  in size with an efficiency of 99.98 %.

As the BaP content in the atmosphere at the three sampling stations, varies to a large extent, different filter areas were needed for the animal experiment; in contrast to Krahm only a considerably smaller filter area of Duisburg was taken for the experiment.

The filters were extracted with methanol, liquid-liquid partition of the extract between water and cyclohexane, fractionating of the cyclohexane phase on an alumina column according to the following scheme:



In the PAH fraction 8 PAH were determined by direct measurement of the fluorescence intensity on the thin-layer plate. Figure 1 shows the relative proportions of the 7 other PAH to benzo(a)pyrene (BaP): PAH profiles.

The components of the propanol fraction are especially more polar cyclic hydrocarbons containing functional groups such as phenolic OH-groups, aldehyde or ketone groups, carboxyl groups and N-heterocyclic compounds.

Animal experiments: The total extract, PAH fraction, and propanol fraction of each sampling station, moreover the recombination of the methanol phase, PAH fraction, and propanol fraction of Duisburg were dissolved or suspended in tricaprylin. 4 doses of each substance mixture (with one exception) were prepared. The dose levels (0.11 µg, 0.33 µg, 1.0 µg, 3.0 µg) refer to the BaP content or to the BaP amount which would be in the fraction as if the PAH had not been separated. In order to achieve equal BaP levels the extracts of the three districts had to be concentrated differently; concerning the rural area more extract in volume was consequently necessary to obtain the same BaP level as in the extracts of the urban districts.



A single subcutaneous injection of 0.5 ml was administered to female NMRI mice; Histoacryl<sup>®</sup> was used to seal the point of perforation. Each animal group treated with one dose contains 60 mice, one group only 56 mice; it amounts altogether with the control groups to 2516 mice. In two groups, which obtained the highest dose of a substance mixture, in about 30 % of the animals a necrose of the epithelium occurred upon the location of the injected substances; consequently the applied substances were eliminated at least partly. The tumour rates which will occur in these groups will have to be interpreted with caution. Tumours and tumourous tissue found at the application site will be analysed histologically.

#### Results

Fig.1 shows the relative proportion of 7 PAH to BaP (= PAH profiles) being contained in the suspended airborne particles of the three sampling stations.

Table 1 shows the tumour rates 9, 12, 15, and 18 months respectively after the beginning of the experiment. Till now only those tumours are diagnosed histopathologically which occurred during the first 12 months of the experiment. The further data are based on macroscopic findings.

Fig. 2 shows the tumour incidence at the application site in per cent with 95 % level of significance 18 months after the s.c. application of total extract, PAH fraction, and propanol fraction respectively obtained from the three sampling stations. The columns refer to the average tumour incidence shown in table 1.

#### Discussion

The PAH profiles analysed from three sampling stations in West Germany during winter 1978/79 showed on the whole a great similarity among one another and also in comparison with those of former years (1,2). It seems that the widely varying PAH profiles of the emissions found in the atmosphere become relatively uniform when found in suspended particles. Apart from that the long collection period of more than three months causes a levelling of fluctuations in the PAH profile. Recent results confirm the similarity of PAH profiles even when 135 PAH were detected (3).

The total extracts of airborne particulate matter collected in the three areas in winter 1978/79 induced approximately the same tumour rate as the PAH fractions which were isolated thereof. These results are contrary to former findings with extracts of airborne particles collected in winter 1975/76; in these experiments the tumour rates caused by the total extracts of urban areas significantly exceed those being induced by the PAH fractions; the total extract of the rural area, however, caused a significantly lower tumour rate than the corresponding PAH fraction (4).

The PAH fractions of the three sampling stations were obtained from different air volumes so that they contained the same BaP content. Thus the PAH fractions of Duisburg and Krahm led to nearly the same tumour rate. The PAH fraction of Düsseldorf was significantly less effective; but the highest dose applied was about 10 % lower than in the PAH fractions of Duisburg and Krahm. In former experiments four PAH fractions containing the same BaP level showed the same carcinogenic effect (4).

The results show definitely that the airborne particles of both cities as well as those of the rural area contain besides the PAH further carcinogenic substances which can be summarized with the term "polar substances". This confirms former findings (4).

#### References

1. Tomingas, R., F. Pott and G. Voltmer (1978): Profiles of Polycyclic Aromatic Hydrocarbons in Suspended Particles of Different Cities in the Western Federal Republic of Germany. Zbl.Bakt.Hyg., I.Abt.Orig.B 166, 322-331.
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3. König, J., W. Funcke, E. Balfanz, B. Grosch, T. Romanowski und F. Pott (1981): Vergleichende Untersuchung von atmosphärischen Schwebstoffproben aus 5 Städten der Bundesrepublik Deutschland auf ihren Gehalt an 135 polyzyklischen aromatischen Kohlenwasserstoffen. Staub - Reinhalt. Luft (in press).
4. Pott, F., R. Tomingas, A. Brockhaus, and F. Huth (1980): Studies on the Tumorigenicity of Extracts and their Fractions of Airborne Particulates with the Subcutaneous Test in the Mouse. Zbl.Bakt., I.Abt. Orig.B 170, 17-34.

Table 1: Examination of the carcinogenicity of extracts and fractions of suspended particles from Duisburg-Neuenkamp, Düsseldorf and Krahm, sampling period winter 1978/79, using the subcutaneous test on mice (60 mice per group), results 9, 12, 15, and 18 months respectively after injection (till 12 months the s.c. tumours are confirmed histo-pathologically).

Declaration of extract or fraction	Dose * (µg)	mice with s.c. tumours (%) after			
		9 mon.	12 mon.	15 mon.	18 mon.
Tricaprylin (carrier)	-	-	-	-	-
Extract of unused filter	0.11	-	-	1.67	3.33
" " " "	3.00	-	-	-	-
Total extract, Dui.	0.11	11.67	11.67	13.33	15.00
" " " "	0.33	11.67	13.33	16.67	20.00
" " " "	1.00	11.67	18.33	21.67	26.67
" " " "	3.00	15.00	26.67	35.00	36.67
PAH fraction, Dui.	0.11	6.67	13.33	16.67	20.00
" " "	0.33	6.67	15.00	15.00	15.00
" " "	1.00	18.33	26.67	28.33	28.33
" " "	3.00	11.67	20.00	26.67	30.00
Propanol fraction, Dui.	0.11	6.67	6.67	8.33	8.33
" " "	0.33	5.00	6.67	8.33	10.00
" " "	1.00	1.67	1.67	3.33	6.67
" " "	3.00	3.33	3.33	3.33	5.00
Recombination of methanol, PAH and propanol fraction, Dui.	0.11	6.67	6.67	10.00	11.67
" " "	0.33	5.00	6.67	8.33	10.00
" " "	1.00	5.00	8.33	8.33	11.67
Total extract, Düss.	0.11	3.33	6.67	6.67	6.67
" " "	0.33	8.33	13.33	13.33	15.00
" " "	1.00	6.67	11.67	16.67	21.67
" " " **	3.00	8.93	14.29	19.64	21.43
PAH fraction, Düss.	0.11	3.33	5.00	6.67	8.33
" " "	0.33	1.67	1.67	5.00	5.00
" " "	1.00	11.67	18.33	25.00	30.00
" " "	2.65	1.67	1.67	6.67	11.67
Propanol fraction, Düss.	0.11	3.33	5.00	5.00	5.00
" " "	0.33	10.00	10.00	10.00	10.00
" " "	1.00	6.67	10.00	13.33	13.33
" " "	3.00	11.67	18.33	18.33	20.00

\*) BaP dose per animal or BaP amount which would be in the fraction if the PAH had not been eliminated.

\*\*\*) Only 56 mice in this group.

Table 1 continued:

Declaration of extract or fraction	Dose * (µg)	mice with s.c. tumours (%) after			
		9 mon.	12 mon.	15 mon.	18 mon.
Total extract, Krahm	0.11	5.00	6.67	10.00	13.33
" "	0.33	5.00	8.33	13.33	16.67
" "	1.00	3.33	5.00	10.00	15.00
" "	3.00	1.67	5.00	11.67	20.00
PAH fraction, Krahm	0.11	5.00	6.67	8.33	10.00
" "	0.33	13.33	16.67	16.67	18.33
" "	1.00	20.00	26.67	31.67	35.00
" "	3.00	11.67	13.33	15.00	15.00
Propanol fraction, Krahm	0.11	6.67	11.67	11.67	11.67
" "	0.33	5.00	8.33	10.00	11.67
" "	1.00	3.33	5.00	5.00	5.00
" "	3.00	13.33	20.00	21.67	25.00

-----  
 \*) BaP dose per animal or BaP amount which would be in the fraction if the PAH had not been eliminated.

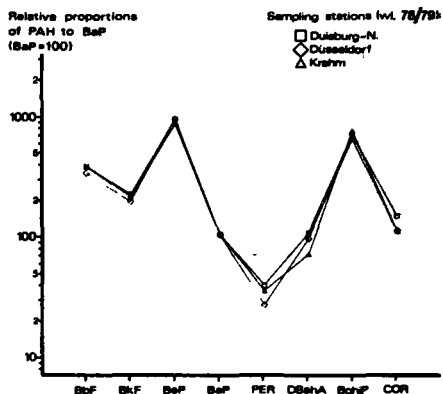


Figure 1: PAH in suspended particles of 3 sampling stations; relative proportions of 7 PAH to BaP (BaP = 100); sampling period: winter 1978/79.

Abbreviations: Benzo(b)fluoranthene (BbF), Benzo(k)fluoranthene (BkF), Benzo(e)pyrene (BeP), Benzo(a)pyrene (BaP), Perylene (PER), Dibenz(a,h)anthracene (DBahA), Benzo(ghi)-perylene (BghiP), Coronene (COR).

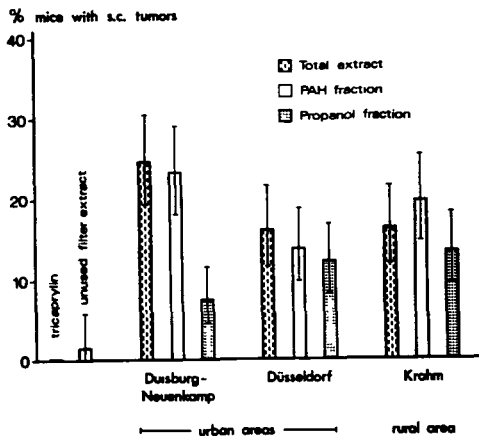


Figure 2: Tumour incidence at the application site in per cent with 95 % level of significance 18 months after s.c. injection of extracts derived from airborne particles. Total extract, PAH, fraction and propanol fraction obtained from 3 sampling stations; sampling period: winter 1978/79. The columns refer to the average tumour incidence shown in table 1. (The highest dose of the PAH fraction of Düsseldorf remained about 10 % under those of the other fractions; cf. discussion.)

**CONTRACTOR** : *Association pour le Développement de la Recherche sur le Cancer (ADRC), 94800, Villejuif, France*

**CONTRAT** : ENV -375-F Project 1

**PROJECT LEADER** : *Ivan CHOUROULINKOV*

**TITLE OF PROJECT** : *Effects of the physiological liquids on the activity of BaP and Diesel Particulate extracts.*

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**OBJECTIFS OF THE RESEARCH :**

Almost all experiments with pure or complexe chemical carcinogens are performed using strong organic solvent. This manner do not represent the physiological conditions of intoxication with, for example, air pollutants. The bioavailability of the toxic compounds and the role, eventually protective of the physiological liquids (serum, lung secretion) are not well known. Consequently the health effect may be surestimated or underestimated.

Our first objectif was to study the effect of the serum on the in vitro cellular uptake of benzo(a)pyrene (BaP) and on the cytotoxicity and SCEs with Diesel Particulate extracts (DPE).

**MATERIALS AND METHODS :**

- **Compounds**

- . Tritium-labelled Benzo(a)pyrene (BaP) (Amersham)
- . Normal purified BaP (Sigma)
- . Tritium-labelled Benzo(e)pyrene (BeP) (CEA-Saclay)
- . Dichloromethane Diesel Particulate extracts (DPE)

- **Cells**

- . Syrian Hamster embryo primary culture cells for BaP uptake and SCEs induction with BaP
- . V79 cell line for cytotoxicity and SCEs induction with DPE

- **Mesure of BaP uptake** - using liquid scintillation counting (Intertechnique 5L-32 couter).

- SCEs test. In V79 cells :  $1.0 \times 10^6$  cells were plated and incubated for 16 h in 10 mm dishes with 10 ml Eagle's MEM with 10 % inactivated FBS in humidified incubator at 37°C in presence of 5 % CO<sub>2</sub>. The medium is then replaced with serum free MEM medium containing the studied compound. After 1 h treatment, the BrdU (5 µg/ml) with complete medium is added to the cells for 7 hours to cover one S-phase. After that, the BrdU was replaced by complete medium for another 18 h. Then 6 µg/ml of Colcemid was added for 2 h. The cells then was collected for preparation.

With the Syrian hamster embryo cells (secondary culture), the technic was the same. The number of the seeded cells was  $2.5 \times 10^6$ /dish, the treatment 17-18 h and the BrdU was kept for 9 h.

- Cytotoxicity - Was determined by the cloning efficiency of the V79 cloned cells.

#### RESULTS :

##### 1 - Serum and BaP uptake and BaP SCEs induction

A quantitative study on the in vitro uptake of benzo(a)pyrene and benzo(e)pyrene by Syrian hamster embryo cells and the induction of SCEs has been carried out. The amounts of benzo(a)pyrene and benzo(e)pyrene taken up by the cells decreases when the concentration of serum in the culture medium increases and it appears that serum prevents benzo(a)-pyrene or benzo(e)pyrene uptake. We observed no significant differences in this respect between the two hydrocarbons ; chromatographic results show however that benzo(a)pyrene is metabolized by these cells, but that benzo(e)pyrene is not metabolized under the conditions used.

Our results taken together suggest that serum seems to shield cells from benzo(a)pyrene and benzo(e)pyrene uptake and decreases the induction of sister chromatid exchanges.

2 - Induction of SCEs with DPE in serum

Table 1 - SCEs incidence in V79 cells treated with DPE in serum and acetone solution.

Dose µg/ml	SCEs m ± SE	
	DPE in acetone	DPE in serum
0	2.80 ± 0.38	2.63 ± 0.24
1.75	4.73 ± 0.38	4.67 ± 0.45
3.5	4.70 ± 0.31	4.10 ± 0.45
7.0	6.80 ± 0.61	4.63 ± 0.33

The serum seems "inactivate" some but not all active compounds revealed in SCEs system.

3 - Cytotoxicity in V79 cells of DPE

Table 2 - Cytotoxicity (%) in V79 cells induced with DPE in acetone or in serum.

Dose µg/ml	DPE in acetone	DPE in serum
0	0.	0.
3.5	17.7	0.
7.0	37.4	0.
14.0	89.7	0.
28.0	100.0	19.0

The DPE in serum do not have the same cytotoxicity than in acetone.



Table 3 - Cloning-efficiency of V79 cells in presence of DPE in acetone (14 µg/ml) as fonction of serum (FCS) quantity in the culture medium.

Dose µg/ml	% FCS in MEM	Cloning efficiency %
14.0	0	0.
14.0	1	1.4 ± 0.2
14.0	2	30.6 ± 2.9
14.0	3	59.0 ± 2.7
14.0	4	67.2 ± 4.6
14.0	5	70.2 ± 4.6
0.	0	43.8 ± 5.1
0.	5	77.0 ± 4.7

Even small quantity of the serum can protect the cells from the toxic effect of DPE.

This preliminary results indicate clearly a protective role of serum. We can not discuss the mecanism now. Further studies are necessary using other methods and other material, for example particulate from Diesel exhaust or from air pollution.

**RESULTS :**

- . Progress Report - February 1981
- . The uptake and Release of BaP and BeP in vitro by Syrian Hamster embryo cells as function of serum concentration.  
H. COULOMB, Z. GU, S. AUDU and I. CHOUROULINKOV.  
Carcinogenesis, in press.

CONTRACTOR : *Association pour le Développement de la Recherche sur Le Cancer (ADRC), 94800, Villejuif, France*

CONTRAT : N° ENV-375-F Project 2

PROJECT LEADER : *Ivan CHOUROULINKOV*

TITLE OF PROJECT : *Biological effects of air pollutant extracts in short term test systems.*

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OBJECTIFS :

It has been well established that air pollution can present health risks for man. Experimentally, the presence of mutagens and carcinogens has been demonstrated. Nevertheless for the real or else exact evaluation of the health risks, we need a regular controls of the air pollution's quantity and quality.

Our objectifs were then : (a) to measure monthly the air pollution in a well defined city-point ; (b) to evaluate the mutagenic and carcinogenic activities of the air pollution extracts ; (c) to improve different short term test systems ; and (d) to try to qualify the air in the studied point.

MATERIAL AND METHODS :

Collection and extraction of the pollutants. The pollutants were collected on the teflon filters. To avoid chemical modifications, these filters were renewed daily. Each filter was weighed before and after collection. The "Cold" technic was used for extraction of organic pollutants. The teflon with dust are plunged successively in 2 acetone baths, 3 ml of acetone for 1 mg of dust and for 20 minutes each with shaking. The acetone then was filtered and evaporated (rotavapor apparatus). For the bioassays the final extracts was dissolved in acetone.

Bioassays : Five different short term tests were used for this study.

- Test d'Ames : *Salmonella thiphimurium*, strains 98 NR<sup>+</sup> and NR<sup>-</sup>, with or without metabolic activation.

- SCE's test in V79 cell line.

- Induction of forward gene mutation (induction of 6-thioguanine resistant) in V79 cell line according to the method of Abbondandolo et al. (1977).

- Cell transformation with Syrian Hamster embryo cells (technic of Berwald and Sachs (1965), Di-Paolo (1969) and Pienta (1979)).

- Short term skin tests (sebaceous glands and hyperplasia tests) for carcinogenicity in mouse. The destruction of the sebaceous glands and the increasing of the epidermal thickness indicate carcinogenic and promoting activities (Lazar and Chouroulinkov - 1974).

*RESULTS :*

Results from cell transformation and induction of 6-Tg<sup>r</sup> essays were negative. But these do not mean a lack of mutagens and of carcinogens in the extracts. They only indicate that these systems are not easy to use for systematic and rapid evaluation. They are too laborious.

The results from the other tests are summarized in table 1.

Summary of the results from the studies with the  
air pollutants

Month	Dust $\mu\text{g}/\text{m}^3$	Organic extract %	Activity in Ames test	Activity in SCE's test	Activity in : Seb.Gl. test	Hyper. test
Jan.	90.0	67.5	++++	+	-	++++
Feb.	80.5	32.7	++++	-		
Ma.	106.0	32.0	+++	+		
Av.	106.5	6.8	+	+		
May.	63.8	33.4	+	++		
June	41.4	96.0	+	+++		

- Quantity : They are very big variations in the quantity of the dust and of the extractible fraction. These variations can be explained by climatic conditions and by human activities, which also interfere with the quality of the extracts.

- Biological effects - In Ames test all extracts are active. They are more active in 98 NR<sup>+</sup> and in presence of S9. The extracts from the first three months were more active than others. In SCEs test the results were inverse, extracts from the first three months were less active. These results indicate the presence of different compounds, some mutagens active in Ames test, and other active in SCEs test.

- In short term skin test - We have only the results from January gland test but highly active in hyperplasia test like phorbol ester (TPA) a well known promoter. This result indicates the presence of promoter(s) in the extract but does not exclude the presence of initiators.

In conclusion, the combined use of different short term tests may provide useful information on the mutagenic and carcinogenic activities of the air pollution, and eventually on the mode of action in lung cancer induction : initiation and/or promotion.

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- . Action biologique d'extraits organiques des particules atmosphériques urbaines : Effet cytotoxiques et mutagènes. B. FESTY, F. COVIAUX, M.Y. COURTOIS et I. CHOUROULINKOV. Pollution Atmosphérique 85, 50-57, 1980.
- . Action biologique d'extraits organiques des particules atmosphériques urbaines : Effets cancérogènes. I. CHOUROULINKOV, Y. LE MOULLEC et B. FESTY. Pollution Atmosphérique 85, 58-61, 1980.
- . Genotoxicité des particules atmosphériques urbaines en suspension. Y. COURTOIS, I. CHOUROULINKOV, B. FESTY. Congrès Pollution Atmosphérique, Oct. 1980, Buenos-Aires.

Contractor : INSERM - Pollution atmosphérique  
Vigoulet-Auzil (Toulouse)  
Contract no. : ENV/426 F  
Project leader : Prof. P. BOURBON  
(collaborators : G. BOMPART, P. LEVY)  
Title of project : Effects of NO, NO<sub>2</sub>, NO + CO, NO + NO<sub>2</sub> on plasma and  
lungs.

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Previous experiments carried out, in vitro, indicate that :

- a) Fixation of CO on human blood or rabbit blood in presence of NO in similar concentrations is diminished by the formation of methemoglobin and nitrite methemoglobin. NO is oxidised in nitrate. Furthermore, it appears that oxidation of nitrite in nitrate is realised by red-blood cells. Spectrography study of blood treated by NO indicates presence of methemoglobin, nitrite methemoglobin.
- b) Later are studied in vivo effects on hemoglobin of CO, NO, NO + CO, NO + NO<sub>2</sub> after exposures of rabbits. No competitive effect on hemoglobin between CO and NO was observed. The level of nitrates in serum is related to the level of (NO)<sub>x</sub> after six hours of exposure. This increase is observed beyond 20 vpm of NO.

Results : see table I

On account of these results we have studied the time history of NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> in washing liquid and in the lung tissue of guinea pigs exposed during 6 hours to 20 vpm/NO<sub>2</sub>.

After exposition, eight guinea pigs are shared in four groups of two. The first group is killed at the end of the exposure (time "0") and the others after 24, 48 and 72 hours. For every guinea pig are carried out :

1. Exsanguination by heart puncture
2. Washing of lungs by pulmonary artery with a liquor NaCl (9‰)

T A B L E I

For every experience : 8' Results : Arithmetic mean : °	Hb gr%	mlCO/gr Hb		Meth. : %		NO <sub>3</sub> : mg/l	
		before Intox.	After Intox.	before Intox.	after Intox.	before Intox.	after Intox.
Atmosphere1:500 vpm CO	12.8+1.0	0.009±0.000	0.32+0.02				
Atmosphere2:500vpmCO+80vpmNO	13.2+0.7	0.009+0.001	0.33+0.04	0.52+0.09	0.60+0.08	14.8+1.6	46.1+6.8
Atmosphere3: 80 vpm NO	14.3+0.7			0.56+0.13	0.66+0.19	23.1+6.6	52.4+6.2
Atmosphere4:80 v pm CO+80vpmNO	10.1+0.6	0.013+0.001	0.18+0.02	0.48+0.14	0.57+0.16	14.0+8.6	35.8+0.6
Atmosphere5: 80 vpm CO	9.9+1.6	0.012+0.002	0.20+0.03	0.43+0.20	0.60+0.13		
Atmosphere6 : 40 vpmNO+40vpmNO <sub>2</sub>	10.3+0.9			0.51+0.21	0.60+0.27	14.5+4.8	45.3+4.5
Atmosphere7 :20 vpm NO	10.4+0.5			0.17+0.14	0.19+0.14	11.1+2.9	18.1+2.2
Atmosphere8 : 10. vpm NO	11.6+2.0			0.31+0.23	0.32+0.13	16.0+5.4	14.9+3.5

T A B L E I I

EVALUATION OF NITRATES AND NITRITES IN WASHING SOLUTION OF LUNGS, IN GRINDED LUNGS AND PLASMA OF GUINEA PIGS AFTER 6 HOURS EXPOSURE TO NO<sub>2</sub> = 13 VPM, NO = 10 VPM

	NON EXPOSES	EXPOSES				
		0 h.	24 h.	48 h.	72 h.	
in washing solution of lungs	NO <sub>3</sub> <sup>-</sup>	1.3 ± 0.4	3.4 ± 1.6 ( p < 0.01)	3.6 ± 1.8 ( p < 0.01)	2.5 ± 0.5 ( p < 0.01)	2.4 ± 1.8 ( N.S.)
	NO <sub>2</sub> <sup>-</sup>	0.0	0.0	0.0	0.0	0.0
/ug in grinded lungs	NO <sub>3</sub> <sup>-</sup>	0.7 ± 0.5	3.8 ± 2.9 ( p < 0.01)	3.4 ± 2.5 ( p < 0.01)	2.8 ± 0.7 ( p < 0.001)	1.5 ± 0.9 ( N.S.)
	NO <sub>2</sub> <sup>-</sup>	0.0	0.0	0.0	0.0	0.0
mg/l in plasma	NO <sub>3</sub> <sup>-</sup>	1.8 ± 0.8	4.6 ± 1.4 ( p < 0.001)	2.9 ± 0.6 ( p < 0.01)	2.3 ± 0.5 ( N.S.)	2.0 ± 0.2 ( N.S.)
	NO <sub>2</sub> <sup>-</sup>	0.0	0.0	0.0	0.0	0.0

T A B L E II I

EVALUATION OF NITRATES AND NITRITES IN WASHING SOLUTION OF LUNGS, IN GRINDED LUNGS, AND PLASMA OF GUINEA PIGS AFTER 6 HOURS EXPOSURE TO 20 VPM NO<sub>2</sub>

NO <sub>3</sub> <sup>-</sup> - NO <sub>2</sub> <sup>-</sup>	NON EXPOSES	EXPOSES			
		0 h.	24 h.	48 h.	72 h.
in washing solution of lungs	NO <sub>3</sub> <sup>-</sup>	3.9 ± 1.5	4.5 + 1.4 (N.S.)	4.0 + 1.6 (N.S.)	3.9 + 1.3 (N.S.)
	NO <sub>2</sub> <sup>-</sup>	0.0	0.0	0.0	0.0
µg in grinded lungs	NO <sub>3</sub> <sup>-</sup>	7.9 + 1.2 (p < 0.001)	2.9 + 0.5 (N.S.)	2.8 + 0.5 (N.S.)	2.4 + 0.6 (N.S.)
	NO <sub>2</sub> <sup>-</sup>	0.0	0.0	0.0	0.0
mg/l in plasma	NO <sub>3</sub> <sup>-</sup>	5.4 + 0.7 (p < 0.001)	2.2 + 0.3 (N.S.)	2.5 + 0.4 (N.S.)	2.6 + 0.4 (N.S.)
	NO <sub>2</sub> <sup>-</sup>	0.0	0.0	0.0	0.0



3. Lungs are washed by perfusion of trachea with 20 ml liquor Na Cl (9‰)
4. Lungs are grinded in 5 or 10 ml Na Cl (9‰), centrifuged and treated by trichloroacetic acid and centrifuged. Nitrites, nitrates reduced in  $\text{NO}_2$  by Cd, are measured by Griess Saltzman method.

Results : see tables II and III

Conclusion

- a)  $(\text{NO})_x$  are oxidised in the plasma in nitrates.
- b) After exposition to  $\text{NO}_2$ ,  $\text{O}_2$ ,  $\text{NO}_2 + \text{NO}$  during 48 hours,  $\text{NO}_3^-$  are present in the lung but in greater quantities in the second instance.

Publications

- Etude in vitro de l'action de l'oxyde nitrique sur la courbe de dissociation de l'oxyhémoglobine.

Pierre BOURBON, Guy BOMPART et Pierre LEVY  
Note présentée par M. René TRUHAUT  
C.R. Acad. Sc. Paris - T. 285 - 3 oct. 1977

- Contribution à l'étude in vitro des effets de l'oxyde nitrique et de l'oxyde de carbone sur le sang d'origine humaine et de lapin.

G. BOMPART et coll.  
Toxicological European Research - Vol. I - no. 5 - Sept. 1978

- Effets in vivo de l'oxyde nitrique, de l'oxyde de carbone et de leur association sur le sang de lapin.

P. BOURBON, G. BOMPART, P. LEVY  
Toxicological European Research - Vol. II - no. 6 - Nov. 1979

Contractor : Laboratorio di Genetica  
Università di Pisa

Contract no. : ENV/388/I

Project Leader : Prof. N. LOPRIENO

Title of project : Studies on the genotoxic activity of particulate  
and of extracts of Diesel exhaust material by means  
of in vitro and in vivo mutagenicity system

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#### Objective of the research

Several studies have demonstrated the presence of mutagenic chemical organic compounds on the particulate produced in Diesel exhaust: the potential carcinogenic activity of such chemicals for human exposure has been suspected on the basis of mutagenic in vitro studies.

The aim of the present research is to develop as many information as possible on the potential mutagenic activity of solid particulate collected from Diesel exhaust which can be inhaled by human population and on the mutagenic activity of extracts in vitro and in vivo on different microbial and mammalian systems.

#### Materials and methods

Salmonella TA strains have been employed, together with S.pombe, S.cerevisiae and HeLa human cells as a preliminary set of biological materials for screening Diesel particulate extracts with DCM.

In vivo experiments with mice and Salmonella in a combined host mediated methodology has been preliminarily performed.

#### Results and discussion

As the mutagenic activity of solid particulate previously defined by us has been shown to be influenced by the size of the particles, the sonication has been applied to the particulate before the mutagenic assay. In these conditions it has been found that the mutagenic specific activity of the particulate dissolved in water is a function of the time (minutes)

of sonication (14 revertants/min) in the case of TA 98 strain.

The OXY fraction obtained from the DCM-treated Diesel exhaust solid material has been found mutagenic in Salmonella and in S.pombe and convertogenic in S.cerevisiae. The fraction was too toxic to human cells in culture and could not be evaluated for the induction of UDS.

Other studies in progress.

Publication

N. LOPRIENO et al. : In vitro mutagenicity analyses on Diesel particulate extracts by different genetic systems.  
International Symposium on Health Effects of Diesel Engine Emissions.  
EPA, 75-92, 1981.

Contractor : Laboratoire de Toxicologie de l'Environnement

Université de Liège - Prof. D. RONDIA

Contract : 162-77-1 ENV. B

Project Leader : F. DE WIEST

Title of project : Experimental Study of the Factors Influencing the  
Availability and Biological Effects of Polynuclear  
Aromatic Hydrocarbons Adsorbed onto Soot Particles

---

#### Objective of the research.

Our first research demonstrated the physico-chemical properties of carbon blacks and combustion soots<sup>(1)</sup> vs adsorbed polycyclic hydrocarbons (PAH) and the elution kinetics of PAH from these respirable particles by blood plasma solutions<sup>(2)</sup>. It showed that : a) adsorption and elution of the PAH are independent from the oxidized surface state of the carbon, taken as a model for aged soots; b) elution of PAH is proportional to time of contact but independent, in percentage eluted, from the PAH concentration per unit carbon surface; c) speed of elution is inversely proportional to the number of benzene rings in the PAH but slower for natural soots than for artificially enriched carbon black particles of slightly larger diameter.

The present research aimed at the checking of a hypothesis formulated a few years ago<sup>(3)</sup>: the existence in the respirable fraction of combustion soots, of photoderivatives of benzo(a)pyrene (BaP) possibly in a free radical form, which genotoxic activity was much higher than that of BaP itself, or its metabolites.

The developments of mutagenicity testing on bacteria (Ames test) made possible the reproducible measurement of a genotoxic activity of PAH derivatives in minutes amounts and the search for a correlation between nature of the PAH, extent of its photodegradation, and degree of biological activity.

#### Materials and methods.

The carbon blacks and soots used in this experiment are described elsewhere<sup>(1,2,4)</sup>, the knowledge of their surface properties being indispensable in the study of PAH adsorption on particles and elution from these

particles by organic or physiologic solvents. The conditions of PAH - spiked particles irradiation have also been studied, so as to approach natural illumination conditions in our country (cold winters with heavy PAH emissions and alternating cloudy and bright sun episodes). The irradiation process<sup>(5)</sup> takes advantage for a property of the soots i.e. the independence of their affinity of OH groups (thus water) and for PAH, as well as the very low water-solubility of the PAH. The demonstration of this fact allowed us to simulate atmospheric photodegradation by the irradiation of water suspensions of PAH-spiked carbon blacks with known surface state.

Particles coated with  $\pm 2$  monomolecular layers of PAH are dispersed in water and irradiated by UV light (310 to 400 nm) at room temperature. An actinometric method was devised to measure the energy reaching the suspension<sup>(6)</sup> in the experiment and in the atmospheric conditions.

The degradation of the PAH was estimated by spectrophotometric and spectrofluorimetric techniques after benzene extraction of PAH from the water suspended soots; it has been extrapolated to atmospheric conditions prevailing in Belgium for the different seasons and has allowed the estimation of "half-lives" of specific PAH in airborne suspended matter. Concentrations of possibly interfering atmospheric pollutants have also been measured by automatic instrumentation (ozone and nitrogen oxides)<sup>(7)</sup>.

The biological properties of the benzene extracts after dissolution in DMSO have been evaluated by submitting them to the Ames test procedure with and without hepatic S 9 activators (from rats pretreated with 1254 Aroclor) on strains TA 98 and TA 100, both possessing the resistance transfer factor and showing good sensibility to polycyclic hydrocarbons; several concentrations have been assayed in order to check the existence of a dose-response relation.

Two pure polycyclics have been submitted to the experiment (benzo(a)anthracene, BaA, and benzo(a)pyrene, BaP) as well as one methylated derivative (7,12-dimethylbenzo(a)anthracene, DMBA); atmospheric soots collected in Liege and experimental soots obtained from natural gas flames have also been used, their extracts being tested in crude form or after

fractionation into broad classes. The chemical identification of the photodegradation products has not been accomplished at this time due to extraneous reasons.

### Results.

#### Photodegradation on Carbon blacks.

Some results of the photodegradation experiments have been published<sup>(5)</sup>, the irradiation conditions being chosen in relation to four typical atmospheric situations :

- A. - summer anticyclonic, clear sky, warm (20-25°C);  
illumination period : 14 hours; UV mean radiant energy 76 J/cm<sup>2</sup>/day
- B. - spring cyclonic, partly clouded sky, fresh (10-15°C); illumination period 10 hours (end March); UV mean radiant energy 39 J/cm<sup>2</sup>/day;
- C. - winter cyclonic, cloudy sky; cold (less than 7°C); illumination period 9 hours (end February); UV mean radiant energy 6.5 J/cm<sup>2</sup>/day;
- D. - winter anticyclonic, clear sky, dry and very cold, East winds;  
illumination period : 7.5 hours; UV mean radiant energy 16 J/cm<sup>2</sup>/day.

They show that photodegradation kinetics are of the type  $\ln Co/Ct = K.t$ , Co and Ct being the initial and final PAH concentration in the carbon black; K is specific for the hydrocarbon and differs from the corresponding constants calculated from methanol-water PAH suspensions without carbon black; it is not temperature dependent between 7 and 24°C; the kinetics are not influenced by the state of the carbon surface (raw or after controlled mild oxydation).

DMBA adsorbed on black particles is extremely sensitive to long wavelength ultraviolet light (table 1); it is completely destroyed in a few hours, its calculated half-life being of two hours only. Independently from the possibility of emission of a hydrocarbon with such steric peculiarity in combustion processes, this justifies its absence in most atmospheric polycyclics analysis.

BaP is also very photosensitive (strong absorption in the near UV region), its half-life being of the order of 4 hours in condition A, 10 hours in condition C and 5 in condition D, thus permitting some narrow range transportation of the emitted polycyclic.

Of the three compounds studied, BaA is the least photosensitive. Its absorption peaks are indeed around 300 nm, which energy in the solar spectrum and in the UV lamp used is very low. Its half-life is 6 hours in condition A, 8 hours in condition B, 18 in condition C and 10 in condition D; long range transport is certainly possible and must be considered even if its carcinogenic potency is much lower than that of BaP.

#### Mutagenicity assays.

The extracts of irradiated spiked carbon blacks (between 2 and 4 mg PAH/g; active surface of the carbon :  $\pm 10 \text{ m}^2/\text{g}$ ), irradiated experimental soots and atmospheric suspended particles have been submitted to the Ames test procedure on strains TA 98 and TA 100 after dissolution in DMSO and sterilisation by filtration on membrane filters. The figures based on pure PAH have been presented at the 5th Intern. Symposium on Polynuclear Aromatic Hydrocarbons<sup>(8)</sup>; the last result concerning the mutagenicity of atmospheric soots is ready for publication<sup>(9)</sup>.

The most interesting findings are those obtained without microsomal activation of the organic extracts, thus clearly showing the existence of a direct mutagenic effect of the compounds formed during the irradiation. They also show that at least some of these compounds are relatively stable as they have resisted the lengthy process of extraction, eventual chromatography on silica column, evaporation to dryness and redissolution in DMSO. We are however unable to infer that the measured activity represents the total of the photodegradation products :

- DMBA, the most photosensible of the compounds tested, yields only a low mutagenic activity without S 9 activation on either TA 98 or TA 100 (table 2); the reactivity of this compound and of the irradiation products is very different from those of true polycyclic hydrocarbons.
- Photoderivatives of BaP on the contrary are extremely mutagenic on TA 98 (table 3), a good indicator of frameshift mutation potency. The mutagenic activity parallels closely the amount of the photodegradation or, more exactly, the amount of BaP destroyed (the bacterial test being conducted either with the whole extract or after chromatographic separation into a hydrocarbon fraction, with an activity similar to the same amount of BaP, and a polar more active fraction).
- Photoderivatives of BaA are also mutagenic but less active than those of BaP and, as opposed to BaP, on TA 100 only, an indicator of base-sub-

stitution mutation (table 4)

Table 1 - Actinometric constants  $\lambda_a$  as determined in the experimental irradiation conditions with HAP adsorbed on carbon black (in  $\text{min}^{-1}$ )

Irradiation condition.	BaA	DMBA	BaP
A	0.0064	0.060	0.030
B	0.0180	0.167	0.086
C	0.0450	0.423	0.211
D	0.0850	0.790	0.397

Table 2 - Mutagenic activity of the organic content of DMBA enriched carbon blacks after UV irradiation.

Strain	$\mu\text{g}$ per plate		Number of revertant colonies par plate	
	DMBA	Photodé- rivatives.	With S 9	Without S 9
TA 98	25.0	0.0	30	n.t.
TA 98	8.0	17.0	99	50
TA 98	15.2	9.8	77	49
TA 100	25.0	0.0	101	n.t.
TA 100	7.0	18.0	137	79
TA 100	13.8	11.2	155	95
TA 100	19.2	5.8	147	-

- DMBA concentration in the carbon black : 2500  $\mu\text{g}/\text{g}$
- UV irradiation : condition B; irradiation times between 0 and 4 hours
- Average of 4 replicates; spontaneous revertants:19 (TA98); 97(TA 100)
- n.t. : negative test.

Mutagenic potency of the irradiated extracts with microsomal activation does not yield many new or interesting results. The different strain sensibility shows however a) that mutagens resulting from irradiation are different from those resulting from metabolic activation and b) that, at least for BaP the potency of photodegradation compounds could be higher than that of metabolic activation; analytical work has to be developed before giving more weight to this conclusion.

Experimental and urban soots.

An experimental soot was obtained by natural gas burning in an air deficient flame. Soot was collected and submitted to the irradiation procedure.



Its extracts, crude and after separation into two fractions have been used in the Ames test.

Table 3 - Mutagenic activity of the organic content of BaP enriched carbon blacks after UV irradiation.

Strain	Positive controls Experiment 1 Pure BaP µg/plate	Organic content after irradiation Experiment 2		Number of revertant colonies per plate.			
		BaP µg/plate	Photode- rivaties µg/plate	With S 9		Without S 9	
				Exp. 1	Exp. 2	Exp. 1	Exp. 2
TA 98	10	9.7	29.5	134	632	n.t.	588
TA 98	20	19.2	20.0	159	226	n.t.	202
TA 98	25	25.3	13.9	150	131	n.t.	110
TA 100	5	4.9	9.0	166	136	n.t.	n.t.
TA 100	10	11.2	7.2	181	228	n.t.	n.t.
TA 100	10	11.2	16.3	181	313	n.t.	n.t.
TA 100	8.1	8.1	19.7	231	571	n.t.	n.t.

- BaP concentrations in the blacks : 3290 µg/g(TA 98) - 2780 µg/g (TA100)
- UV irradiation : condition D; irradiation times between 6 and 24 hours
- Average of 4 replicates; spontaneous revertants: 29(TA 98); 127(TA 100)
- n.t. : negative test.

The results (table 5) are obviously more complex than with pure PAH; they show a clear increase with the irradiation intensity for the total extract whereas the activity the aromatic fraction decreases markedly. Activities on TA 100 strain without S 9 activation show also that a major part of the mutagen activity does not need metabolic activation. They confirm thus the results of the experiments done with pure PAH but reveal in the same time a certain number of synergisms or antagonisms.

Respirable suspended matter collected in the center of the town in winter (214 µg/m<sup>3</sup>, benzene soluble matter 19 µg/m<sup>3</sup>; BaP 50.6 ng/m<sup>3</sup>) has also been extracted, separated into an aliphatic (57%), an aromatic (5.7%) and a polar fraction (37.3%). The crude extract and the fractions have been submitted to the Amest test which results are given in table 6 confirming again the bulk of the experimental results.

Table 4 - Mutagenic activity of the organic content of BaA enriched carbon blacks after UV irradiation.

Strain	ug per plate		Number of His revertant colonies per plate	
	BaA	Photoderivatives.	With S 9	Without S 9
TA 98	25.0	0.0	42	n.t.
TA 98	9.5	15.5	521	n.t.
TA 98	17.8	7.2	240	n.t.
TA 98	20.0	5.0	129	n.t.
TA 98	22.8	2.2	95	n.t.
TA 98	30.3	9.7	291	n.t.
TA 100	25.0	0.0	98	n.t.
TA 100	7.2	17.3	480	211
TA 100	9.2	15.8	428	-
TA 100	12.5	5.5	397	97
TA 100	14.0	4.0	422	145

- BaA concentrations in the carbon blacks : 1800, 2500 and 4000 ug/g
- Ultraviolet irradiation : condition C, irradiation times between 0 and 24 hours
- Average of 4 replicates spontaneous; revertants : 23 (TA98); 102 (TA 100)
- n.t. : negative test.

Table 5 - Mutagenic activity of the organic content of natural gas soot after UV irradiation : number of His revertant colonies per plate in the Ames test.

Irradiation conditions.	Compounds tested.	ug/per plate.	TA 98		TA 100
			With S9	Without S9	Without S9
0	BaP standard	5	230	932	0
0	Total extract	25	592	2095	
	Aromatic fraction		389	723	
	Polar fraction		143	145	
C	Total extract	25	1066	1619	1015
	Aromatic fraction		379	439	302
	Polar fraction		237	79	85
A	Total extract	25	1834	1264	
	Aromatic fraction		226	141	
	Polar fraction		247	n.t.	

- Average of 4 replicates; spontaneous revertants : 20(TA98);145(TA100)
- Raw composition of the soot : total extract 68 mg/g soot; aliphatic fraction : 0%; aromatic fraction : 69.9%; BaP : 2.3%; polar fraction : 19.8%; acid fraction : 10.3%.

Table 6 - Mutagenic activity of the organic content of atmospheric suspended matter collected in Liege (winter 1980) : number of His revertant colonies per plate in the Ames Test.

Compounds tested.	ug/per plate.	TA 98		TA 100	
		With S 9	Without S 9	With S 9	Without S 9
BaP standard	5	180	n.t.	759	n.t.
Total extract	260	464	257	922	523
Aliphatic fraction	148	n.t.	n.t.	n.t.	n.t.
Aromatic fraction	15	190	104	488	343
Polar fraction	97	70	118	282	216

- Average of 4 replicates; spontaneous revertants : 20 (TA 98); 145(TA100)  
 - n.t. : negative test.

#### Conclusions.

Our study has demonstrated the easy degradation, by near ultraviolet light, of some PAH adsorbed on experimental or actual soots and the strong direct mutagenicity of the compounds formed. Clear winter days result in the highest mutagenic activity of atmospheric origin due to the amount of polycyclics present (cold temperature, poor dispersion of the pollutants) and the rather high intensity of the solar UV light.

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**TOPIC 15 : AIR QUALITY**  
**Measurements of air pollutants**

Contractor: Fraunhofer-Institut für Atmosphärische Umweltforschung,  
D-8100 Garmisch-Partenkirchen

Contract n<sup>o</sup> ENV-372-80-D

Project Leader: Dr. Reinhold Reiter

Title of Project: Recording of CO<sub>2</sub> and O<sub>3</sub> simultaneously at 0.7, 1.8, and 2.9 km ASL and of O<sub>3</sub> gradients from 1.0 to 2.9 km by cable car telemetry. Clarification of variations considering the different influence factors.

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## 1. OBJECTIVE OF THE RESEARCH

The purpose of this research is the study of time variations of CO<sub>2</sub> and O<sub>3</sub> in the lower troposphere through simultaneous recordings at different levels (e.g. by cable car telemetry) to elucidate the dependence of the vertical profile of the concentration of both gases on the diurnal and annual variation, on meteorological parameters, on the vertical exchange in both directions; it includes further derivation of anthropogenic sources and photochemical processes (O<sub>3</sub>). Through parameterization of CO<sub>2</sub> and O<sub>3</sub> data obtained by meteorological influence factors, the above objective shall be achieved step by step. Under the first contract which expired on 31.12.80 (see working program and schedule of our proposal) all technical prerequisites for the performance of work should be provided and first results available showing that the approach is properly designed to achieve the goal. This report can only give a few rare examples of results. Nevertheless they show that the proposed schedule has fully been met and that the facilities and methods are appropriate to our purposes.

## 2. MATERIALS AND METHODS

### a. O<sub>3</sub>

The 3 recording stations (small horizontal distance) in the valley (740 m), on the Wank (1780 m) and on the Zugspitze (2964 m) have been equipped with chemoluminescence devices and automatic calibration systems so that the quality of recordings meets the present highest demands. Further, an ozone radiosonde (ECC) was electronically adapted and calibrated in our lab such that it delivers from summer '80 along with our other facilities aboard the Zugspitze cable car currently profiles of ozone from 1.0 to 2.96 km altitude. In all, 520 individual profiles of ozone have been taken in this manner.

### b. CO<sub>2</sub>

At the stations mentioned under 2.a, the CO<sub>2</sub> has been continuously recorded. Innovations during the contract period 1980: Installation of a fully-automatic recording station closely below the Zugspitze peak; this work proved very time-consuming so that the station could be put into operation only from the end of fall 1980. All 3 stations are equipped with automatic calibration systems based on the Keeling CO<sub>2</sub> scale.

### c. Evaluations

All evaluations are made by computer. Parameterization by means of the simultaneously known meteorological conditions is in progress.

## 3. RESULTS

In this brief report only some few results can be discussed. Essentially more information is available which will be combined with data currently obtained after continuation of the program from 1 January 1981.

a. O<sub>3</sub>

Fig.1 shows that in compiling data from 1977 - 80, incl., the earlier published [1] extreme daily variation is found at the valley station where the O<sub>3</sub>-conc. exceeds in the afternoon the values from Wank and Zugspitze during intense insolation (rel. sunshine duration SD >80%). This holds equally for spring and fall. If we have only diffuse light (sunshine duration <1 h), we note in Fig. 1 (right half) that a daily variation does still exist at the valley station but the maximum values do no longer exceed those at the mountain stations. Hence this phenomenon is statistically established and the question arises as to the thickness of this layer with extremely high ozone concentration. The question is answered by using the Zugspitze cable car telemetry for the derivation of vertical ozone profiles.

Fig.2 shows on a typical single day in summer the evaluation of 17 O<sub>3</sub>-profiles. Line A shows the successively detected vertical O<sub>3</sub>-profiles. We observe the nocturnal deficit which extends to an altitude of about 2 km ASL and is filled-up in the course of the day. In the afternoon we find even in the lowest layer an O<sub>3</sub>-conc. higher than that at Zugspitze peak which remained constant throughout the day. The simultaneously measured temperature gradients (line B in Fig.2) reveal from morning till noon a temperature inversion at about 2.2 km alt. which dissolves through vertical exchange in the afternoon. At the end we have an adiabatically mixed layer. Yet, no appreciable O<sub>3</sub>-transport from the valley to the Zugspitze is observed. The inversion in line B is also apparent till early afternoon from the kink in the O<sub>3</sub>-profile (line A). The change of the O<sub>3</sub>-profile suggests photochemical O<sub>3</sub> production in the lower layer between ground and inversion but by no means an O<sub>3</sub>-transport from higher levels to the lower troposphere. Line C gives in addition the vertical profiles of the positive electrical conductivity showing a light kink at 2.2 km alt. with higher values above. This, too, indicates that no drastic transport from Zugspitze level down to the valley took place. Line D in Fig.2 contains the O<sub>3</sub> daily variations, calculated from the vertical O<sub>3</sub>-profile, at the different altitudes (km) from 100 to 100 m. We clearly note how the O<sub>3</sub>-conc. increased currently during the day in a layer thickness to about 2.2 km where the increase dropped however slowly with height. From 2.3 km alt. and above the O<sub>3</sub>-conc. remained constant. It can also be seen that in late afternoon in the valley (1 km) higher concentrations have been reached than at the Zugspitze (3 km). Hence, this case points definitely to an afternoon photochemical production of ozone in the lower troposphere.

Fig.3 shows another type. It is distinguished by the fact that a daily variation of O<sub>3</sub> exists in the near-ground air layer but remained small (see lines A + D), nevertheless this increase prevailed up to 2.2 km. In a mean-level layer (Fig.3, line D) the O<sub>3</sub>-conc. remained constant up to 2.7 km altitude. Above, we find however a steep increase in the O<sub>3</sub>-conc. which leads to essentially higher concentrations as have been measured in the valley (contrast to Fig.2!). This behavior of the O<sub>3</sub>-profile - clearly evident from Fig.3, line A + D - suggests that we have in the present case a subsidence of stratospheric air into the troposphere. This is confirmed by the high Be7 concentration and the temperature profiles in line B. From 14.00, it is obvious that adiabatic warming takes place. If we view in line C the positive electrical conductivity, we recognize by the steep increase of conductivity at higher altitude from 14.00 that extremely pure stratospheric air subsided. But this air could neither through subsidence nor through mixing processes penetrate to the valley floor. From this we may conclude that stratospheric O<sub>3</sub> injections are less important to the O<sub>3</sub> behavior at lower altitudes.

b. CO<sub>2</sub>

The results of CO<sub>2</sub> recordings, see also [2], at 740 and 1780 m are summarized in Fig.4. In graphs a - d we clearly observe the season-dependent daily variation in the valley which is governed by the following components: During exposure to light and higher temperatures net-photosynthesis prevails through CO<sub>2</sub> decomposition and in warm seasons at night, CO<sub>2</sub> production dominates by respiration of plants and decay processes in humus. The small maxima in the winter months in the valley - identified by the behavior of other trace gases - are definitely of anthropogenic origin while the influence of the biomass plays practically no longer a role in these seasons. It clearly follows from graph e in Fig.4 that from spring to fall the intensity of insolation (sunshine duration >80% or <1 h) has indeed a decisive influence on the net photosynthesis by day.

4. CONCLUSIONS

Thus, it can be concluded that the installed facilities are suitable in every respect for studying the subject posed. At the same time it is however beyond doubt that essentially more data must be gathered in order to arrive through an in-depth parameterization at a final solution.

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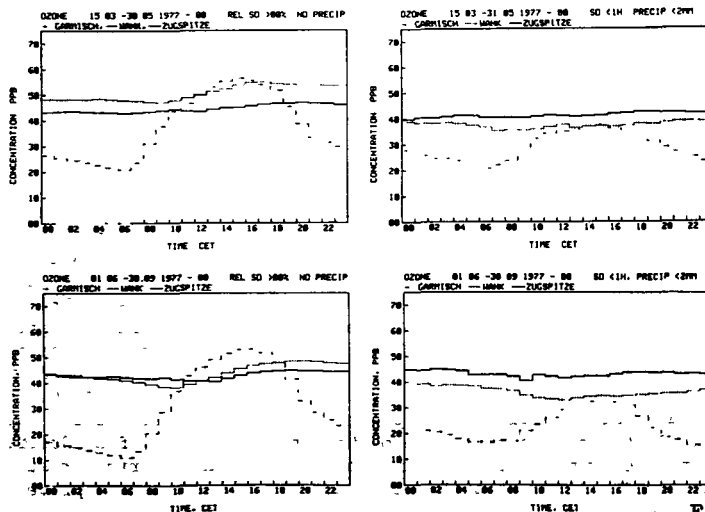


Fig.1



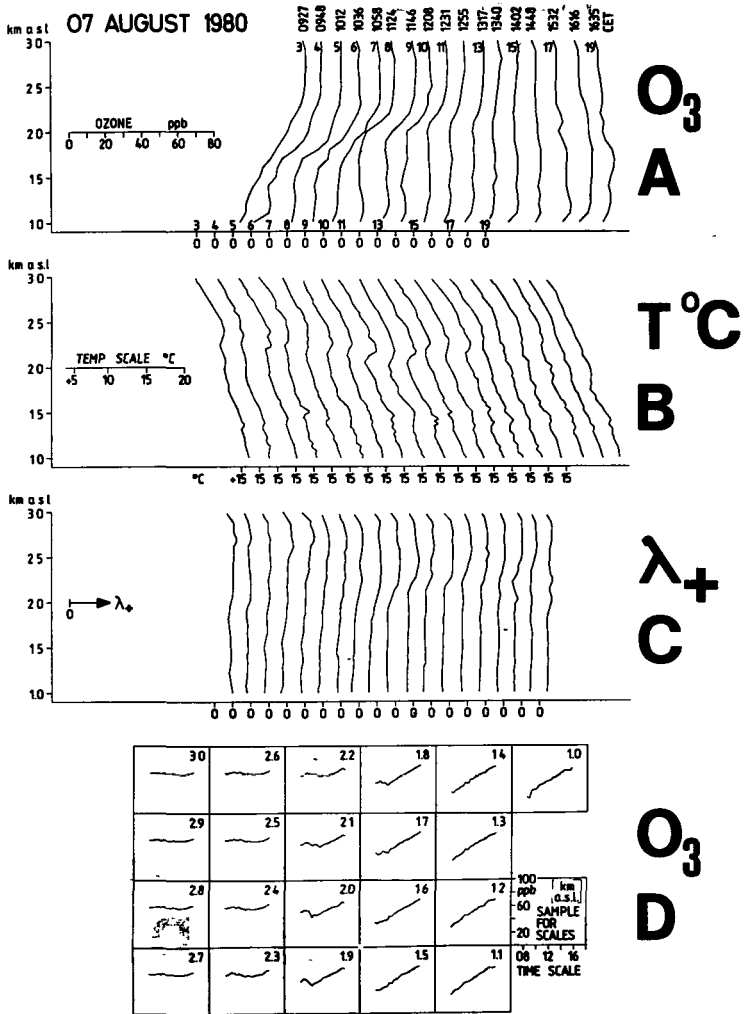
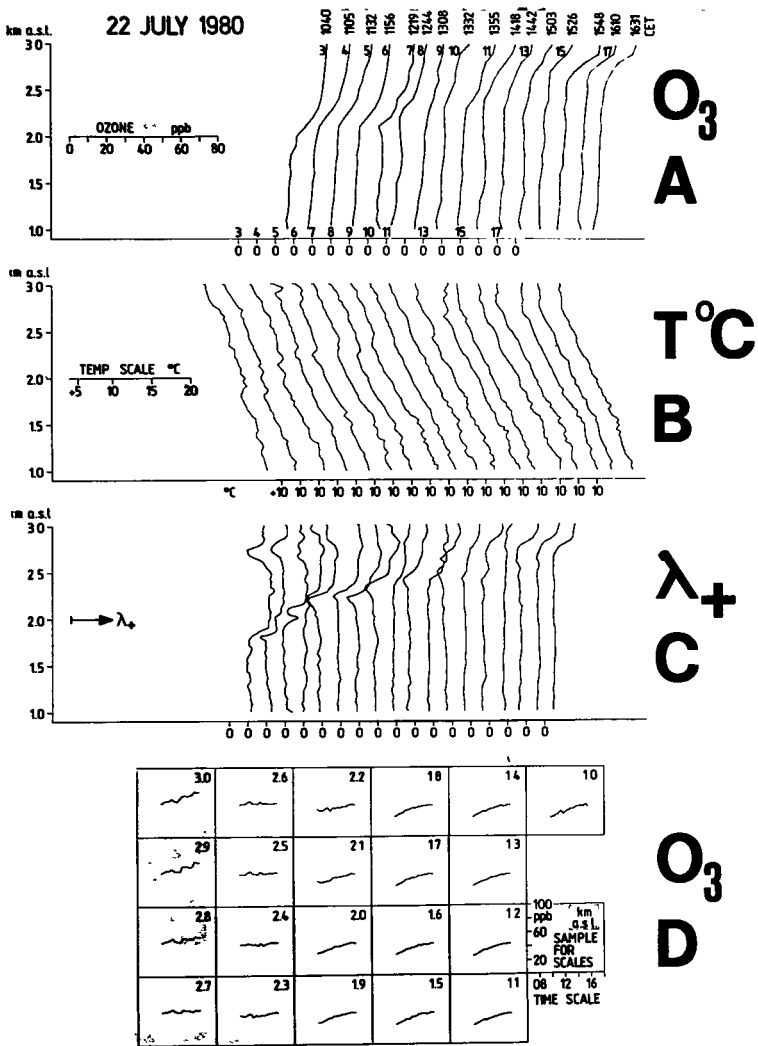


FIG.2



**FIG.3**

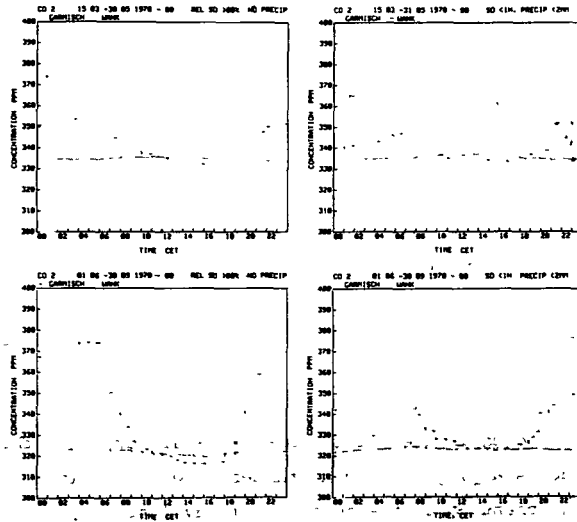
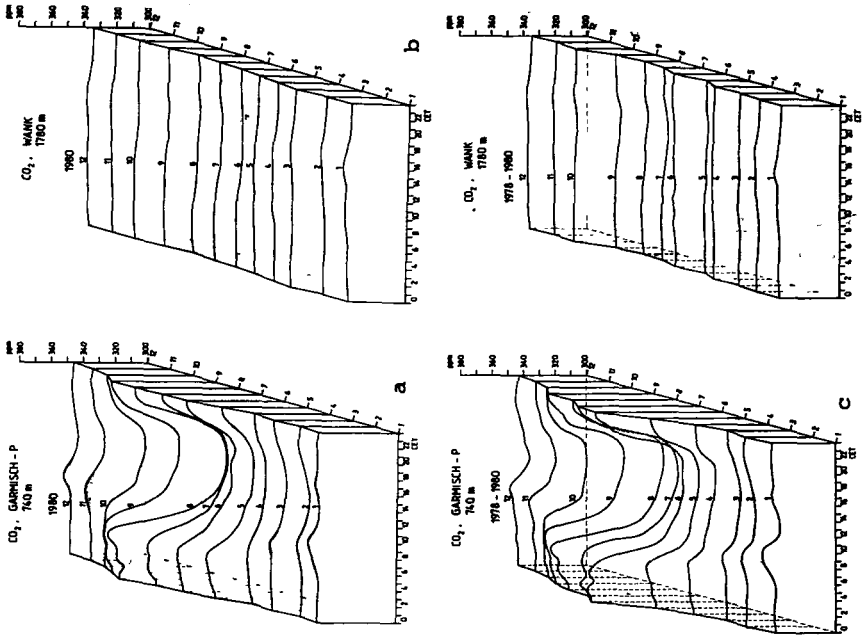


Fig. 4

Contractor : A.R.M.I.N.E.S, 60, Bd St-Michel, 75272 PARIS CEDEX 06  
Contract n° : 337 Env F  
Project Leader : D. DI BENEDETTO  
Title of project : A Study of Air Pollution on the Mediterranean Coast

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#### OBJECTIVES OF THE RESEARCH

After different measurements had indicated high ozone concentrations (particularly at Marseille) and taking account of the presence of other factors which favour smog formation, the Ministry of the "Quality of Life in France" decided, several years ago, to undertake a study of the oxidant pollution on the Mediterranean coast. The objectives of this study were :

- a continuous monitoring of different parameters of the problem
- from this overall measurements, to obtain a sufficiently detailed knowledge of the phenomenon to permit modelisation and to use this model to raise the alarm wherever necessary.
- to monitor the change in the phenomenon with time, in order to control it and this avoid passing a threshold limit which would be practically irreversible.

#### MATERIALS AND METHODS

##### 1) The measurement sites

Set up and controlled by the services of the InterDepartemental Direction of Industry and the Ministry of Health, a series of six stations has been implanted along the Mediterranean coastline. The measurement stations were initially set up at Nice Marseille (urban stations) Port de Bouc and Sète (Industrial stations) Ile de Porquerolles and the summit of the Luberon (reference stations with "zero pollution").

##### 2) On-site analysers

Each station is equipped for the following automatic analyses.

- Measurements of different standard meteorological parameters (P,T, humidity, wind speed and direction....)
- Measurements of the following chemical parameters : hydrocarbides, nitrogen oxides, sulfur dioxide and ozone.

##### 3) The calibration kit

In order to be able to calibrate all the on-site analysers with the same standard, it was necessary, given the distances between stations, to use easily portable equipment. Since the commercially available systems were im-

practicable, the Ecole des Mines de St-Etienne, has developed, in the course of this study a portable system for generating standard gases based on the principle of gas phase permeation. This system, whose second prototype has been constructed is very reliable, easily transported and can be used on site.

#### 4) Data treatment

All the data collected on the measuring sites was transmitted to the ARLAB Company for a statistical treatment to bring out the correlations between the different parameters. These correlations were used to guide the modelisation of the development of the oxidant pollution.

### RESULTS

#### 1) Quantitative Problem

Far too much data has been lost on the sites, this problem can have several causes :

- isolated and therefore vulnerable stations, or a badly designed station, these problems have been progressively corrected,
- technical difficulty in data collecting, too many recording equipment breakdowns due either to a poor choice of the equipment or a maintenance problem.
- problems with certain types of measuring instruments, in general the analysers are very vulnerable on site.

All this lost data has strongly pertubated the validity of the statistical treatment which is applied. Furthermore some data has been quite simply omitted from the treatment since it was of very poor quality. On the other hand some data was treated although it was known to be a doubtful values.

#### 2) Qualitative problem

It should be mentioned that, in general, the only parameters to which a high degree of reliability could be accorded were :

- The meteorological parameters except for the irradiation levels.
  - The NO/NO<sub>x</sub> parameters and to a certain proportion the SO<sub>2</sub> parameters.
- One should note that a regular calibration of the measuring stations allows, assuming a relatively high frequency, the detection of hidden faults in the equipment (and there by an increase in the reliability of the measurements).

#### 3) The case of a pollution "episode"

During a case of pollution in the FOS-BERRE region which lasted about ten days we were able to treat, by hand, the available data (all the problems which beset this study were found yet again - equipment breakdowns, unreliable data, no hydrocarbide measurements and only one ozone analyser.)

Innespective of these problems, there is no doubt that a large amount of pollution occurred, but it is improbable that this involved a case of oxidant pollution (a low ozone concentration was detected.)

#### 4) Attempts at statistical prediction of pollution peaks

We have tried to establish a predictive model for cases of pollution. We were again hampered by the lack of example of pollution peaks. A model has however been established and relates relatively low ozone concentrations ( $<100 \text{ g/m}^3$ ) to  $\text{NO}/\text{NO}_x$  and the atmospheric pressure.

#### 5) General Results

The general photolytic behaviour of ozone formation was confirmed and it was shown that the peak shape was much shapper than those encountered in the USA during pollution peaks. One should note in particular, the absence of correlation between ozone and hydrocarbides.

Theseresults would tend to prove that hydrocarbons do not interfere in the smog formation cycle. One should however keep in mind the poor reliability generally accorded to hydrocarbide measurements.

#### CONCLUSION

The different problems encountered in this study have revealed the necessity for reinforcing the reliability of the equipment used for these tests, and in particular the equipment used in high risk zones. Experience shows that Port de Bouc, characterised by a large scale industrialisation surrounding, a very wide stretch of water (which probably develops a micro-climate) would be this type of high risk zone.

As yet, if the risk of pollution is not negligible, especially in the region of Nice and Port de Bouc, it is not proven that this pollution is an oxidant one.

More detailed studies should clarify the problem. Such studies should also rely on specialists advice since we believe that the scale of such study, carried out for the first time in France, was perhaps initially underestimated.

Contractor : FIAT ENGINEERING S.p.A., Torino  
Contract no. : 155-77-1 ENV I  
Project leader : Dott. P. Gaddo  
Title of the project : Urban air pollution from photochemical smog  
in Turin

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#### Objective of the research

The project research intends to value the real entity of the photochemical smog phenomena occurring in the urban area of Turin and in the surrounding zones, related to the promoter agents and transformation products behaviour.

#### Methods

The research has been carried on during 1979-80 by means of :

- continuous precursor measurements, in the urban (Lagrange) and extraurban area (Eremo)\*; at ground and elevated levels;
- continuous measurements of solar radiation;
- random measurements both in the urban and extraurban zones of hydrocarbon species and peroxyacetyl nitrate.
- correlation between the observed pollutant patterns.

#### Results

The investigation carried out has allowed to draw some significant consideration on the characterizing trends of the photochemical smog phenomena in the urban and extraurban area of Turin.

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\* Eremo: extraurban hilly area at about 650 m above sea level.

The results emerging both from sampling at ground and elevated levels indicate that :

- ozone is at the same time tracer and activator of the photochemical smog. In fact it is present in the high layers of the atmosphere and in the extraurban zones at higher concentrations than those recorded in the urban centre (tab. 1 and fig. 1).

Therefore it looks reasonable to argue a tropospheric ozone intrusion in the low layer of the atmosphere.

The average ozone levels, reported in tab. 1, result meanwhile influenced by the solar irradiation degree;

- as far as nitrogen oxides and dioxide are concerned, the concentration levels strongly decrease in the summer time with respect to winter time and passing from urban areas to extraurban ones.

In fact the ratio  $\text{NO}_x/\text{NO}_2$  is minimum in the extraurban zones and during summer, while it is maximum downtown and in winter time, thus resulting influenced by the solar irradiation degree. In any case the  $\text{NO}_2$  values recorded in the urban and extraurban areas are very low and ranging from 0.026 to 0.04 ppm.

- the composition of reactive hydrocarbons found in urban areas shows a higher quantity of olefinic and aromatic hydrocarbons with respect to the hilly extraurban area, both in winter and in summer (tab. 3). This could suggest that in the hill area emissions sources different from the city ones may be present too (Tab. 3-4);
- the photochemical reactions involving reactive hydrocarbons seem to occur in the urban area during days with a strong solar irradiation. Only in these conditions it is possible to observe variations of atmospheric hydrocarbon composition, resulting in a decrease of olefins and aromatics during the day (Tab. 5-8);
- in any case the oxidated compounds ( $\text{O}_3, \text{NO}_2, \text{PAN}$ ) are present in the atmosphere at low concentration (tab. I, 2 e 9).

Only in the 20% of the examined samples the presence of PAN has been observed at concentration of 0.2-3.5 ppm, as final product of photochemical reactions.



Future activity

The knowledge acquired by means of the study on photochemical smog has allowed to interpretate a few general aspects of the phenomenon.

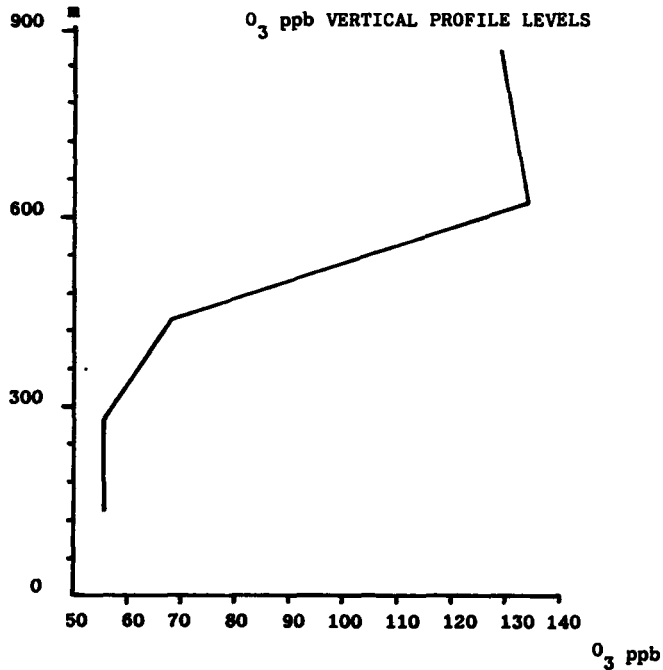
The investigation carried out have shown the complexity of atmospheric reactions and the substantial difficulty of correlating the promoting agents trends and the real entity of the phenomenon.

Therefore the research should be integrating the acquired experiences with :

- a systematic interpretation of the data acquired by means of statistical elaboration on a computer;
- the widening of the collected data by means of atmospheric transference and aerosols (acids, nitrate, sulphates, etc) measurements to be correlated with the patterns of the other pollutants.

Tab. 1 - Ozone mean values in the urban and extraurban areas

Period	Solar radiation cal/cm <sup>2</sup> g	V.Lagrange O <sub>3</sub> ppb urban Total average value	Eremo O <sub>3</sub> ppb extraurban Total average value
Summer	400	29.9	57.7
	400	23.2	50.5
Winter	200	11.5	32.9
	200	8.8	22.5



**Tab. 2 - Nitrogen Oxides mean values in the urban and extraurban areas**

Period	Urban Area			Extraurban Area		
	NO <sub>x</sub> ppm	NO <sub>2</sub> ppm	ratio NO <sub>x</sub> / NO <sub>2</sub>	NO <sub>x</sub> ppm	NO <sub>2</sub> ppm	ratio NO <sub>x</sub> / NO <sub>2</sub>
Summer	0.090	0.030	3	0.026	0.015	1.73
Winter	0.23	0.040	5.75	0.040	0.020	2

**Tab. 3 - Atmospheric Hydrocarbons average composition in the summer period**

	Hydrocarbon species %						Reactivity index	
	Paraffins		Olefins		Aromatics			
	z.urb.	z.extr.	z.urb.	z.extr.	z.urb.	z.extr.	z.urb.	z.extr.
Morning	88.75	93.45	3.8	1.86	7.45	4.69	0.5434	.4251
Afternoon	91.05	92.79	3.6	0.88	5.35	6.33	0.5060	.4194

**Tab. 4 - Atmospheric hydrocarbons average composition in the winter period**

	Hydrocarbon species %						Reactivity index	
	Paraffins		Olefins		Aromatics			
	z. urb.	z.extr.	z. urb.	z.extr.	z. urb.	z.extr.	z.urb.	z.extr.
Morning	90.84	94.24	4.90	4.11	4.43	1.62	0.3764	.2965
Afternoon	92.62	95.58	3.44	2.38	4.18	2.02	0.3154	.2369

**Tab. 5 - Atmospheric Hydrocarbons composition in strongly irradiated days**  
 ( > 400 cal/cm<sup>2</sup> day) Summer period

	Hydrocarbon species %						Reactivity index	
	Paraffins		Olefins		Aromatics		z.urb.	z.extr.
	z.urb.	z.extr.	z.urb.	z.extr.	z.urb.	z.extr.		
Morning	86.0	93.2	5.5	2.2	8.5	4.7	0.6	0.445
Afternoon	88.5	92.5	4.8	0.75	7.6	6.75	0.48	0.432

**Tab. 6 - Atmospheric hydrocarbons composition in strongly irradiated days**  
 ( > 200 cal/cm<sup>2</sup> day) Winter period

	Hydrocarbon species %						Reactivity index	
	Paraffins		Olefins		Aromatics		z.urb.	z.extr.
	z.urb.	z.extr.	z.urb.	z.extr.	z.urb.	z.extr.		
Morning	89.64	96	5.28	2.44	5.21	1.54	0.4220	.1205
Afternoon	91.85	96.6	3.53	1.70	5.06	1.70	0.3458	.1661

**Tab. 7 - Atmospheric hydrocarbons composition in weakly irradiated days**  
( < 400 cal/cm<sup>2</sup> day) Summer period

	Hydrocarbon species %						Reactivity index	
	Paraffins		Olefins		Aromatics		z.urb.	z.extr.
	z.urb.	z.extr.	z.urb.	z.extr.	z.urb.	z.extr.		
Morning	89.7	94.1	4.2	0.9	6.1	4.95	0.46	0.364
Afternoon	89	93.2	4.6	1.1	6.4	5.7	0.48	0.396

**Tab. 8 - Atmospheric hydrocarbons composition in weakly irradiated days**  
( < 200 cal/cm<sup>2</sup> day) Autumn-Winter Period

	Hydrocarbon species %						Reactivity index	
	Paraffins		Olefins		Aromatics		z.urb.	z.extr.
	z.urb.	z.extr.	z.urb.	z.extr.	z.urb.	z.extr.		
Morning	92.22	91.78	4.23	6.46	3.55	1.75	.3248	.5431
Afternoon	93.50	94.15	3.34	3.35	3.18	2.49	.2811	.3362

**Tab. 9 Peroxyacetyl nitrate samplings**

Zones	Sampling numbers	PAN presence n° (%)	Concentration (ppb)
Urban	208	45 (21.6%)	0.2 + 3.5
Extraurban	16	0	0

Contractor : UNIVERSITA' DEGLI STUDI DELL'AQUILA ( Facolta'  
d' Ingegneria Istituto di Elettrotecnica )

Contract n. : ENV/440 I

Project leaders: M. CARLEVARO, D. PAOLETTI

Title of project : Correlation between the presence of  
pollutants and air ionization

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#### OBJECTIVE OF THE RESEARCH

This study aims :

- 1) To find a proof of a possible correlation between the density of industrial pollutants in the atmosphere and the state of air ionization, defined by the volumetric density of ions of different sizes and mobility.
- 2) To develop a method of measurement based on such correlation
- 3) To test the capability of the method under all climatic and meteorological conditions.

Several techniques, which measure traces of gas, within a range of very small densities (units or fractions of p.p.m) have been developed. These are based traditionally on chemical, electrochemical and photochemical methods which imply complicated processes and the use of expensive, and sometimes complex, instruments. All the systems use the principle of analyzing in delayed time, sample taken from the environment by means of pneumatic instruments and filters. It is thus advantageous to dispose of a high sensitivity instrument which permits the analysis of the pollutants in real time in order to obtain a diagnosis and to control systematically the efficiency of pollutants filtering systems.

#### PRINCIPLE OF THE METHOD

Almost all gaseous pollutants deriving from domestic and industrial activities are released into the atmosphere at the ionized state.

The low frequency electrical conductivity of the atmosphere depends entirely on its ion content. Atmospheric ionization is the result of conflicting processes, namely recombination of positive and negative ions, coagulation of small ions with airborne particles or droplets of water to form large ions, diffusion and capture of ions by solid and liquid conductive particles.

The physics and the kinetics of small and large ion formation and recombination have been studied by NOLAN and POLLACK and their colleagues during the past 40 years and have been extensively reported in the literature [1] [2] [3].

plume diffusion.

As it was shown by many studies, there are many properties of plume behavior, which depend on the actual values of plume pollutant densities, rather than on the time averaged densities normally measured. The technique proposed in this study will allow the development of a detection instrument having a fast response to rapid density changes, high sensitivity and an unlimited life of the sensor.

#### METHOD OF MEASUREMENT

The current response to a linearly varying voltage of an ideal condenser in series with a resistor is a square pulse. If the dielectric of the condenser is a weakly ionized gas, the top of the pulse will be a slowly increasing current, the slope being proportional to the conductivity of the gas. The proportionality constant is the same one tying the amplitude of the initial step to the dielectric constant of gas. Non linearity of the conductivity will result in variations in slope of the current response. The analysis of this response by means of simple algorithms allows to get information about the parameters  $N_j$ ,  $u_j$  of the ions contained in the gas. Wider exposure of the method may be found in [5].

#### EXPERIMENTAL APPARATUS

The laboratory prototype consists of a probe, in which the armatures of the cylindrical aspirant condenser are constituted by thin iron tubes of the following sizes:

internal diameter of outer armature	75 mm
external diameter of inner armature	65 mm
length of the armature	400 mm

The inner armature supports are in teflon, shaped to minimize the dispersion conductance between the armatures. This condenser is fed by a ramp voltage generator, incorporated in an electronic electrometer. The voltage drop caused by the condenser current across a precision resistor is recorded. The velocity of aspirated air is 3,5 m/s.

A 6 mc mixing chamber was built, in wooden fabric in order to minimize ion absorption. This volume is sufficient to obtain a quasi-omogeneous air pollutant mixture with the possibility of introducing water vapour also.

The chamber is connected through a pipe to the central room of a dry-box, utilized in open circuit. The probe, and the apparatus for temperature and relative humidity recording, during the experiment, are placed in this box. An aspirator ensures air circulation and its expulsion outside. In the mixing chamber, the transit time of the environment air and the polluting gas, suitably dosed, is about 10 sec. This is sufficient for the expected modifications of ionic composition of the air, due to the presence of a pollutant, to be completed. The output unit is being modified by the addition of a sampling unit and an

The average drift velocity at which an ion moves through a gas submitted to an electric field, is proportional to the intensity of the field itself ( $V_i = uE$ ). It is therefore possible to define, for each type of ion in given conditions of the gas chemical purity, of the pressure and of the temperature, a mobility  $u$  [(m/s)/(V/m)] essentially dependant on the relation between the charge and the mass of the considered ion.

Air conductivity in presence of a multiplicity of ions of different types, characterized by mobilities  $u_j$ , and volumetric densities  $N_j$  results from the sum of the individual contributes to the charge transport.

Therefore :

$$\sigma = |q_e| \sum_j N_j u_j \quad \Omega^{-1} \text{ m}^{-1}$$

where  $q_e$  is the elementary electric charge. This expression clearly shows the dependence of conductivity on the distribution of ionic densities  $N_j$ . In polluted atmosphere with many solid and liquid particles or gas, small ions are rapidly transformed into large ions having minor mobility and the air conductivity is thus reduced. The mean life of a small ion varies considerably depending on the rate of ion formation and on the density of gaseous or otherwise pollutants in the air as well as on meteorological parameters such as humidity and air motion. It results, and has also been proved experimentally, that the ionization state of the atmosphere shows a marked instability; this makes the situation more complicated: a small ion may survive as long as 5 or 6 minutes in clean air, but a few seconds only where high pollutant densities are present.

The association of ion concentrations with the occurrence of specific atmospheric pollutants, gas and particulates, was first studied by Steigerwald [4], who emphasized apparent correlation between heterogeneous polluting factors, and no apparent correlations between large ion density and climatic-meteorological parameters.

Hence, the use of measurements at ground level of atmospheric electric parameters which are more strictly dependent on the ionization state of the air at the testing site or in the layers immediately above, is justified.

Air conductivity measurements may in fact show, through altered values of the potential gradient, the presence of ions of given mobility, which may be correlated to particular pollutants at the testing site, while measurements of atmospheric potential gradient may show with their possible anomalies the presence of abnormal ionic densities in the layers above the observation point.

In conclusion, the measurement of some electric parameters may furnish an alternative to, or, at least, an integration of conventional techniques of density measurement, proposed by various authors, in order to study the fundamental phenomena of



analog/digital converter of the output signal. The development of the processing software, to be implemented into a dedicated in line microprocessor device, is being carried out. This will allow the direct reading and the printing of processed values of air conductivity, volumetric density and of the mobility of every type of ion present.

#### EXPERIMENTAL RESULTS AND DISCUSSION

Analogical recording on relatively long periods of time at laboratory, were carried out in order to define slope and duration of the voltage-wave, most convenient for the purpose of the measurement, and to prove the capability of the method in different meteorological situations.

These recording had to be limited to typical winter climatic situation; the result cannot therefore be considered conclusive. The apparatus for precise quantitative pollutants dosage is not yet perfected, but qualitative proofs carried out by introducing small quantities of SO<sub>2</sub> for about 1 sec. into the mixing chamber, have shown noticeable deviation in the patterns recorded, when compared to the average behavior

The modalities of these qualitative tests do not allow a rigorous interpretation of the corresponding measurement. However these results encourage the continuation of the experiments.

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Quaderno di istituto No 1 -L'AQUILA- 1980

Contractor : Institute of Astrophysics  
University of Liège, Belgium

Contract n°: 302-77-11 ENV B

Project leader : R.J. ZANDER

Title of project : Contribution to the study of the pollution  
of the atmosphere by solar spectrometry

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#### OBJECTIVE of the RESEARCH

The main objective of the research was to make infrared solar observations of very high quality from the ground and by balloon, and to analyze these in order to :

- 1.- establish the presence, the concentration and the temporal behaviour of atmospheric gases which are either primary pollutants or species issued from the decomposition of these last ones.
- 2.- to measure the concentration in the stratosphere of "natural species" whose role is important for the aeronomy of the upper atmosphere.
3. to show that a number of molecules whose measurements are likely to help to assess the preoccupations related to the ozone shield depletion problem can be detected and monitored from the ground, using the remote technique of solar absorption spectrometry.

#### METHODS and MATERIALS

The observations have been carried out :

- 1.- at the International Scientific Station of the Jungfrau-joch (Switzerland, 3580 m altitude), using a 7.5 meters focal length grating spectrometer operating in a double pass configuration and providing a spectral resolution of  $0.02 \text{ cm}^{-1}$ .

- 2.- at the Kitt Peak High Altitude Observatory, Tucson, Arizona (USA, 2064 m altitude), with a Fourier transform interferometer, which achieves a spectral resolution of  $0.008 \text{ cm}^{-1}$ .
- 3.- on board a balloon gondola carrying a 2.5 meters focal length grating spectrometer operating in double pass and having a spectral resolution of  $(0.04 \pm 0.01) \text{ cm}^{-1}$

Prof. L. Delbouille and Dr. G. Roland were responsible for all ground observations during 9 stayings at the Jungfraujoch and 3 at the Kitt Peak-HAO.

Balloon observations were carried out from the NCAR-Balloon Facility, Palestine, Texas, USA ; float altitudes ranged from 30 to 37 km.

All material reported here was deduced from recordings in the near- and middle infrared, between 2 and 5.2 microns, using modern cryogenically cooled detectors (InSb and Ga-Ge bolometers). The molecules of interest here which have characteristic absorption bands in the spectral region mentioned above and could be detected from the ground are : HF, HCl,  $\text{CH}_3\text{Cl}$ ,  $\text{N}_2\text{O}$ , NO, CO,  $\text{CO}_2$ ,  $\text{CH}_4$ ,  $\text{H}_2\text{O}$ ,  $\text{O}_3$ ,  $\text{O}_4$ , OCS, HBr ; from balloon altitudes, the following species were looked at : HF, HCl,  $\text{CH}_3\text{Cl}$ , HOCl,  $\text{N}_2\text{O}$ ,  $\text{CO}_2$ , CO,  $\text{H}_2\text{O}$ ,  $\text{CH}_4$ ,  $\text{O}_3$ ,  $\text{N}_2$ .

## RESULTS

It is impossible to report here about all the results deduced during the period covered by this project; the "Final Report" to this contract, dated August 1980, may be consulted for more details and conclusions. However, we shall give, here below, a typical example and related comments for each of the 3 points noted in the paragraph "Objective of the Research".

- 1.- The hydrofluoric acid, HF, is a stable "end product" arising principally from the photodissociation of  $\text{CFCl}_3$  and  $\text{CF}_2\text{Cl}_2$ . It is generally admitted that HF measurements are appropriate for monitoring the amounts of chlorofluoromethanes which have been destroyed in the stratosphere.

The HF ground results deduced since 1976 are displayed on Figure 1, assuming that all HF is located above 20 km altitude (this is a simplified but not unrealistic presentation). Our HF measurements by balloon have led to average mixing ratios equal to  $(3.6 \pm .7) \times 10^{-10}$  ppv above 27.9 km in 1976;  $(4.8 \pm .3) \times 10^{-10}$  ppv above 30.3 km in 1978 and  $(6.2 \pm .5) \times 10^{-10}$  ppv above 36.8 km in 1979. All together, the balloon results indicate an increase of the HF mixing ratio out to at least 37 km altitude. The question about whether or not all our results reflect a temporal trend of the HF concentration in the stratosphere involves some speculation on the real HF distribution; more observational data are needed to answer definitely that crucial question.

- 2.- Aeronomical reasons calling for CO measurements in the stratosphere are that CO has removal capabilities of the important OH radical in the lower stratosphere, followed by a series of reactions which may ultimately lead to an increase of  $O_3$ ; furthermore, the significance of a production of CO via the  $CH_4$  oxidation chain in the stratosphere demands reliable measurements of CO above 20 km altitude.

Balloon observations made in 1978 and 1979 have allowed us to obtain the first reliable CO concentration profile between 30 and 48 km altitude :  $(1.0 \pm .25) \times 10^{-8}$  ppv between 30.6 and 36.2 km,  $(2.0 \pm .4) \times 10^{-8}$  ppv from 36.2 to 41 km and  $(4.7 \pm .9) \times 10^{-8}$  ppv above 41 km. Aside of being predominantly of natural origin, CO is, after  $CO_2$ , the most abundant pollutant released at the ground, mainly by combustion of fossil materials; it has to be taken into consideration in the context of long term global climatology investigations.

- 3.- It is believed (since Molina and Rowland's suggestions in 1974) that chlorine free radicals released by the photolysis of a large number of chlorinated compounds of both natural and anthropogenic origin may amplify the rate of ozone destruction in the upper stratosphere through homogeneous gas phase catalysis. Once formed, HCl is subject to reactions with OH,  $HO_2$ , ..., which reform the reactive chlorine radicals Cl and  $ClO$ , establishing a rapid chemical exchange between HCl and those radicals. Measurements of the mean concentration of HCl versus altitude

will provide insight into both the total chlorine budget of the stratosphere and the existence of competing reservoirs such as HOCl and ClONO<sub>2</sub>.

Our ground measurements indicate no measurable temporal trend of the HCl column density above the sites of observation but significant variability which we believe is due to changes of the tropospheric HCl content associated with horizontal transport of air, of either oceanic or continental origin. Balloon observations have provided information on the HCl concentration between 22 and 37 km altitude; the 1978 mixing ratios yields  $(4.5 \pm .6) \times 10^{-10}$  ppv at 21.7 km,  $(7.5 \pm .7) \times 10^{-10}$  ppv at 27.5 km,  $(2.1 \pm .4) \times 10^{-9}$  ppv above 30.5 km; observations in 1979 imply an average HCl mixing ratio above 36.8 km of  $(2.4 \pm .4) \times 10^{-9}$  ppv. These balloon data indicate an increase of the HCl mixing ratio out to at least 37 km altitude but no measurable temporal trend of its concentration above 30 km.

#### CONCLUSIONS

The results raised or presented here are typical of what we feel should be done regularly and over a substantial period of time (at least one decade). From the ground, the techniques used are definitely applicable for monitoring programs of species such as CO<sub>2</sub>, CO, CH<sub>4</sub>, N<sub>2</sub>O, NO, OCS, HF, HCl. If various absorption lines with different temperature and pressure dependences were investigated, significant distributions versus altitude could be obtained.

Our proposed research program included observations in the 5 to 13 microns region, where important species such as ClO, ClONO<sub>2</sub>, CCl<sub>4</sub>, HNO<sub>3</sub>, ... present favourable absorption bands. These investigations could not be carried out during the time covered by this contract; they will be undertaken in a near future.

During the next decades, despite important contributions which can be expected from space programs, it is certain that ground and balloon observations will remain vital, not only

for data validations but also for searching new species in our environment, using the most powerful instruments existing or under development; these cannot yet be operated on board of space vehicles.

This work was supported by the Commission of the European Communities, by the Belgian Government, by NASA, by the Chemical Manufacturers Association and by the Air Force Geophysical Lab., Bedford, Mass. USA.

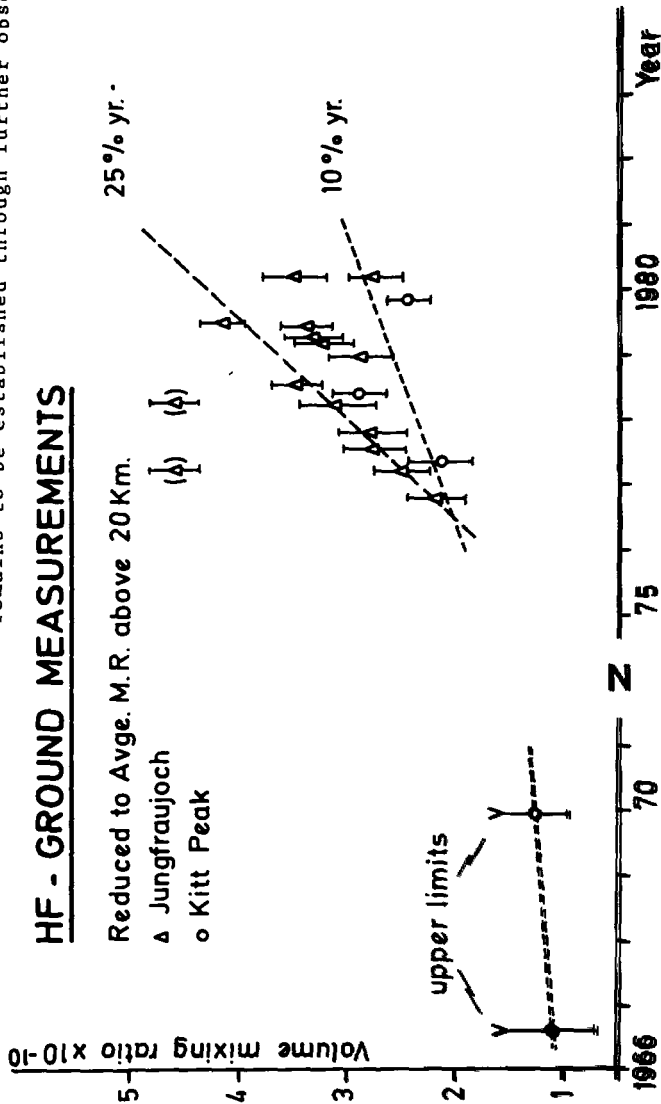
#### LIST of PUBLICATIONS

- Observational Aspect Related to the Chemical Evolution of our Atmosphere, R. Zander, to appear in the 1980 Proceedings of the Solvay Conferences.
- Concentration of Carbon Monoxide in the Upper Stratosphere, R. Zander, H. Leclercq and L.D. Kaplan, to appear in Geophysical Research Letters, 1981.
- Recent Observations of HF and HCl in the Upper Stratosphere, R. Zander, to appear in Geophysical Research Letters, 1981.

#### ORAL PRESENTATIONS

- Monitoring of minor telluric species by high resolution infrared solar observations from the ground and by balloon, R. Zander, G. Roland and L. Delbouille, Second CIRP Conference, Zurich, Switzerland, 1979.
- Complementary aspects of ground-based and balloon-borne spectroscopic measurements of minor telluric species : applications to HF, HCl, CH<sub>3</sub>Cl, etc..., R. Zander, Max Planck for Aeronomy - Workshop on Stratospheric Trace Gas Measurements, Bad Lauterberg, Germany, 1979.
- Balloon-borne spectroscopy of stratospheric constituents, R. Zander, L.D. Kaplan, Quadrennial International Ozone Symposium, Boulder, Col., USA, 1980.
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Figure 1. Graphical representation of ground observations of HF over the last years. The mixing ratios plotted on the graph are the values obtained when assuming that the total quantity of hydrofluoric acid deduced from each measurement is uniformly distributed above 20 km altitude (57 mb). The two values between parentheses were obtained when peculiar meteorological conditions were responsible for the transport of HF-enriched stratospheric polar air towards mid-latitude lower altitudes. The dotted lines are indicative of two hypothetical average increases per year. A definite conclusion about any temporal variation of HF remains to be established through further observations.







**TOPIC 15 : AIR QUALITY**

.... **Dispersion models**

Contractor: Ente Nazionale per l'Energia Elettrica (ENEL)  
Via G.B. Martini, 3 - 00198 ROMA

Contract n° ENV/324 I

Project Leader: Ing. D. Borgese

Title of project: 'Atmospheric pollutant diffusion models in sites with large electric power plants

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#### Objective of the research

In the frame of its Environmental Research Programmes, the Commission of the European Communities in Brussels has been promoting, since 1975, field exercises where teams from CEC countries have had the opportunity of working cooperatively with the common aims of:

- a) evaluating their advanced techniques for remote sensing of gases and aerosols;
- b) assessing their suitability for atmospheric pollution studies;
- c) comparing their results.

The fourth campaign at Turbigo (I) benefited greatly, both scientifically and organisationally, from experience gained at the three previous campaigns, held at Lacq (F), Drax (UK) and Cordemais (F). This report summarizes the results obtained during the Turbigo exercise, in which some 100 people, coming from 7 european countries, participated with the most advanced instrumentation for remote sensing of the structure of the atmospheric boundary layer and of the concentration field of gaseous and particulate pollutants.

Most of the apparatuses were installed on mobile or fixed laboratories, each of them fitted up with facilities for automatic recording and treatment of data.

This document is not a detailed account of all measurements taken and all situations dealt with. Instead, it is limited to four days which between them represent the main characteristics of the conditions encountered during the campaign and illustrate the most important results obtained.

The particular climatic and meteorological conditions at Turbigo and the topographical features of the area present two distinct local breeze regimes, both of which are decoupled from the general synoptic circulation.

In the first, for wind speeds less than about  $1 \text{ m s}^{-1}$ , there is large wind shear both in direction and speed.

In the second, for wind speeds in the range  $3 - 4 \text{ m s}^{-1}$ , the direction is better defined and more steady, although considerable shear

can still occur, especially at the top of the mixing layer. The campaign was directed to a study of both situations. The objectives were:

- 1) to measure meteorological conditions (wind profiles, stability, ecc.) under low wind unstable conditions and apply the results to plume trajectory models, comparing the calculated trajectories with those actually measured in the field. This to be done under conditions of large wind shear, where plumes from sources of different height have distinctly different trajectories and produce different patterns of ground level concentration;
- 2) to measure trajectories and dispersion when plumes from stacks of different height and emission characteristics merge to form a single plume;
- 3) to derive and compare atmospheric stability categories from different types of measurement (temperature profiles, standard deviations in wind direction, etc.) and to investigate their applicability to models of dispersion;
- 4) to compare dispersion parameters obtained from measurements with different instruments (ground level sensor, lidar, etc.)
- 5) to measure the effectiveness of changing the sulphur content of the power station fuel on reducing ground level concentrations of  $SO_2$ .

These objectives, together with quality and quantity of the data collected, determined which days have been chosen for consideration in this report.

The 11th and the night from 17th to 18th of September were considered the most representative of undetermined situations, with low winds, sheared flows, development of convective mixing layer (11th September) and radiative inversions (17th-18th September).

On the other hand, on the 13th, a typical breeze flow developed; well determined up-valley wind flew in the afternoon, with regular counter-clockwise rotation of the wind direction, during the transition from down-to up-valley breezes around noon.

On the 15th of September, at last, the power plant had three sections operating and could perform on each of them a cycle of fuel switchings from high to low and again to high sulfur content.

$SO_2$  ground level concentrations were further reduce by canceling the emission from "ponente" sections.

#### Results and conclusions

Analysis of lots of data gathered during the campaign so far is restricted to only a small percentage of total.

Other more detailed analysis of particular measurements have been already presented elsewhere by single teams, while more analysis will follow (produced by the participating teams).

With respect to the five specific objectives of Section 4, the conclusions so far are:

- 1) Given measured wind and stability profiles, it is possible to calculate plume trajectories with reasonable accuracy, even under conditions of light variable winds with strong shear.
- 2) Merging plumes were measured in a variety of situations. The conditions under which merging occurs are being investigated and the effect on ground level concentrations are being analysed.
- 3) Several methods of assessing stability categories were examined. In general the best correlation with measured dispersion is that derived using stability criteria given by Smith and Pasquill. Those obtained from instantaneous temperature profiles gave the least satisfactory agreement.
- 4) In general different methods of measuring cross-wind plume dispersion agree reasonably well although electric field measurements show a systematic trend of indicating larger dispersion than correlation spectrometers and ground level samplers.
- 5) Measurements of ground level and total overhead burden of  $\text{SO}_2$  agree well with the reduction of sulphur content of the power station fuel.

Other results, not contained in the previously mentioned five objectives are:

- a) The evolution of stable or mixing layers has been found well correlated with theoretical models, temperature profile measurements, wind shear profiles and sodar indications.
- b) The  $\text{SF}_6$  tracer experiment made possible to assess the contribution to  $\text{SO}_2$  fall-out from some specified chimneys.

The campaign was succesful because of the good organization (particularly radio communications) and many simultaneous measurements, which produced a kind of synergistic effect on results and comprehension of phenomena.

Several new technique underwent extensive comparison with more conventional techniques. They were:

- PBL package - UCL (Belgium)
- Radio Acoustic Sounding System (RASS) CNR/ICG (Italy)
- Fluxmeter JRC - Ispra
- 3 D Doppler sodar - JRC Ispra
- 3  $\lambda$  Lidar IAU - (W. Germany)
- 2  $\lambda$  Lidar JRC - Ispra

- Aircraft lidar - DFVLR (W. Germany)
- Mobile DIAL - CERL - (U.K.)
- Mobile electric fieldmill - ELF (France)

All system, as we already said, operated very efficiently, giving consistent results.

The mobile electric fieldmill showed particularly interesting for its ability to evaluate lateral dispersions parameters and plume heights in a very simple way; its larger  $\sigma_y$  could both be due to different instrument thresholds and, possibly, also to the  $(\frac{1}{r})^2$  effect influencing space charge measurements.

An interesting feature of electric field observation is represented by the shift between the peak concentration of space charge and the corresponding one of  $SO_2$  burden lateral profiles.

Another very promising mobile unit, able to detect and localize plumes as far as 20 km and to give information on  $SO_2$  concentration distributions in them, is represented by DIAL. The complete development of this technique could make obsolete the conventional Lidars for aerosol, which showed in the past very useful in determining rise and dispersion of plumes and depths of stable layers, but always failed in plumes measurements outside the range 1000-1500 m (at last, in the Po Valley and in other Italian regions). The only interesting development of aerosol Lidars seems to be the multiwavelength Lidars, allowing evaluation of size distributions of plume aerosol.

Finally, an original coupling of acoustic and electromagnetic radars gave rise to a new PBL sounder, the RASS, for remote measurements of vertical profiles of air temperature as far as 1000 m in height. Together with 3 D doppler sodars, able to measure from ground fixed positions the vertical field of wind as far as 1000 m, they could represent a very powerful means to forecast air pollution episodes and for the correct management of industry emissions during unfavourable weather conditions.

The turbulence data obtained by the turbulence probe of the UCL, compared with the 3 D Doppler Sodar and with the JRC fluxmeter, is expected to provide very useful information on stability conditions of boundary layer during the campaign.

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Contractor: Danish Meteorological Institute 81 °n , (olon  
Contract No. 306-77 ENV DK  
Project Leder: Dr.scient. Lars P. Prahm  
Title of project: Two-dimensional Global Dispersion Modelling

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Two-dimensional global models with both vertical and meridional resolution and the improved representation of transport, compared with one-dimensional models, are the ideal instrument for diagnostic and prognostic application for atmospheric experimental programs. Comparison between model simulations and measurements of different atmospheric traces, however, has shown that dispersion parameters which are adequate for simulation of some tracers are inadequate for others.

The present research focuses on the physical theory and numerical method for the dispersion computations. Improved procedures are tested and compared with conventional methods to give an estimate of the range of some errors originating from assumptions in the dispersion computations, and to assess the advantages of future application of the spectral diffusivity theory and pseudospectral numerical method for 2-D global dispersion simulations.

A two-dimensional meridional global dispersion model is developed. The model is based on the new spectral diffusivity theory. The main feature of this theory is the introduction of diffusivities as a function of the wave number of the spectral components of the concentration distribution. This makes it possible to describe the turbulent diffusion as a scale-dependent process, but in an Eulerian framework.

In the case of dispersion on a global scale, eddies of a size of the order of 10.000 km play an important roll. A dispersion model based on the gradient-transfer formulation of eddy fluxes can not, in this case, be expected to yield satisfactory results because the characteristic extension of the considered tracers might be smaller than the size of the eddies. A properly designed scale-dependent dispersion model is thus of great importance.

Studies are presented of simulation of atmospheric dispersion of radioactive tracers resulting from nuclear explosions in the stratosphere. Preliminary results show a reasonable agreement between the model simulations and the existing measurements. Comparison is also made with modelling based on the gradient-transfer theory formulation. The model is being tested on ozone dispersion with a simplified linearized chemistry.



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Berkowicz, Ruwim, and Lars P. Prahm, 1980. On the spectral turbulent diffusivity theory. Journal of Fluid Mechanics, 100, part 2, 433-448.



**TOPIC 15 : AIR QUALITY**

**Remote sensing**

Contractor: Fraunhofer Institute for Atmospheric Environmental Research,  
D-8100 Garmisch-Partenkirchen

Contract n° 142-77-ENV D

Project Leaders: Reinhold Reiter and Walter Carnuth

Title of Project: Absolutely Calibrated Double and Three-Frequency Lidar  
for Remote Aerosol Sensing

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## 1. OBJECTIVE OF THE RESEARCH

Continuing the work during the 1st phase of the 2nd Environmental Research Programme of the EC, the objective of the research was the further development and application of the multiwavelength lidar method for absolute remote sensing of atmospheric aerosols in the troposphere and stratosphere. A stationary, vertical pointing lidar system with 347 and 694 nm wavelengths, developed under a preceding contract n° 013-74-1 ENV D, is being in operation since 1974, delivering vertical aerosol profiles under various atmospheric conditions up to 30 km altitude. A second, mobile lidar system with 347, 530 and 694 (recently also 1060) nm wavelength has been completed under the current contract late in 1978. Both systems were calibrated using aerosol and aerological data obtained at the institute and at surrounding mountain stations at 1800 and 3000 m altitude above MSL. In 1979 the mobile system has been prepared and proved for the participation in two field measuring campaigns, the 4th CEC campaign at Turbigo/Italy and the MESOCLIP experiment in the Rhine valley near Speyer, Germany, both in September 1979. A new unmanned mountain station at 1650 m altitude and only 2 km distance from the institute, providing meteorological and aerosol data (the latter by a Knollenberg optical particle spectrometer), offered good preconditions for studying the ability of the three-frequency lidar for quantitative remote sensing of aerosol concentration and size distribution by firing the mobile lidar along a slant path toward that mountain station which was the principal aim of the work during 1980. Simultaneously, the tropospheric and stratospheric aerosol monitoring by the stationary lidar should be continued.

## 2. MATERIALS AND METHODS

As the technical status of the lidar systems, especially the stationary system, has undergone only minor changes and improvements during the second phase of the programme, the systems may be specified only briefly. A more detailed description is given in the references [1].

### 2.1. The stationary lidar system

**Transmitter:** Ruby laser with frequency doubling, wavelengths 347 and 694 nm, output energies .12 and 2 Joules maximum, respectively, pulse repetition frequency 1 per sec, electromechanical chopper for rejection of ruby fluorescence during stratospheric measurements, energy monitor for both wavelengths.

**Receiver:** Vertical pointing 52 cm Cassegrain telescope, interference, neutral density and polarization filters, S-20 photomultiplier tube (EMI 9816 for tropospheric measurements, RCA 8852 for stratospheric measurements by photon counting) with range gating.

**Data acquisition, processing and storage:** Biomation 8100 transient recorder for tropospheric data or 64-channel photon counter for stratospheric data, on-line computer for real-time display and hardcopy of normalized backscatter profiles and fully-automatic operation of the system, data output for final evaluation on floppy disk and punch tape.

## 2.2. The mobile lidar system

**Transmitter:** Ruby laser with 347 and 694 wavelength like in the stationary system, additional neodymium-glass laser with 530 nm wavelength generated by frequency doubling, prepared for 1060 nm also. Output energy up to 2 Joules in 694 and 1060 nm, .1 Joule in 347 and 530 nm. Repetition frequency 4 per min. for both lasers. Energy monitors for 347, 530 and 694 nm, for 1060 nm in preparation.

**Receiver:** 30 cm telescope, S-20 photomultiplier (EMI 9816) for the first three wavelengths, S-1 tube (EMI 9684) for 1060 nm, interference filter wheel, no range gating.

**Data processing:** Biomation 8100 transient recorder, on-line computer, real-time plot of normalized backscatter profiles, data storage on tape cassettes or, during stationary operation, recently also on floppy disk.

Transmitter and receiver are mounted on a two-wheel trailer, the other equipment in a van. Power generator operation is possible.

## 2.3. Instrumentation for auxiliary data

As already mentioned, the network of measuring stations at different altitudes has been extended by an unmanned station at 1650 m, at 2 km distance from the institute, which is equipped with instruments measuring temperature humidity, wind, aerosol (Knollenberg particle spectrometer), and, in the near future, visibility by an integrating nephelometer. A small radiosonde may be fixed to a near-by cable car for assessment of profiles of aerological data. Another cable car sonde is operating between 1000 and 3000 m altitude. Further data are provided by mountain stations at 1800 and 3000 m and by radiosondes.

## 2.4. Data evaluation

During the first phase of the research work a mathematical scheme has been developed for the evaluation of the two-wavelength data from the stationary lidar, which has been already described previously: Approximation of the particle size distribution by a two-parameter model consisting of two log-normal distributions centered at .4 and 2 microns diameter, with fixed standard deviations but variable number concentrations  $N_1$  and  $N_2$  in both size ranges, iterative procedure for solving the integral-type lidar equation for derivation of absolute backscatter and extinction profiles and for deduction of profiles of particle concentrations  $N_1$  and  $N_2$ . Experiences and problems arising from the application of this scheme are discussed in the last chapter of this report.

## 3. RESULTS

### 3.1. Stationary system

The series of routine measurements of the vertical distribution of the tropospheric and stratospheric aerosol has been continued in the last two years. More than 1400 tropospheric two-wavelength profiles have been acquired and stored. Apart from the evaluation problems discussed below, two examples demonstrating the capability of the two-wavelength lidar method for distinguishing aerosols of different size distributions shall be presented here. On December 7, 1979, we had a conspicuous influx of Sahara dust in a layer between 1500 and 2000 m altitude, identified by an unusual whitish color of the scattered sunlight, increasing turbidity with altitude etc. and deduced from the general weather situation. This aerosol from the Sahara desert is known to have an enhanced concentration around 2 microns diameter. In this case the fine and coarse particle concentrations in our model  $N_1$  and  $N_2$ ,

can be derived approximately without application of the complete computer routine, if a lidar measurement a few days before during extremely high visibility and predominant Rayleigh scattering is used for calibration of the system. Our inversion scheme, based upon Mie calculations and applying a refractive index  $1.62 - .02i$ , delivers a model distribution shown in Fig. 1a (A), together with an actually measured size distribution measured by a five stage impactor at 1800 m altitude (B). Fig. 1b, on the other hand, presents a similarly deduced model distribution of an aerosol of local origin (A), together with a simultaneously assessed Knollenberg particle spectrum from ground level (B). The diagrams show satisfactory agreement between the model distributions and the measured size spectra, both with respect to the shape of the distributions and the absolute concentrations.

The series of stratospheric lidar soundings has also been continued. After the violent eruption of the St. Helens volcano in USA in May 1980 it was possible to observe the formation of remarkable aerosol layers in the stratosphere over the institute site, as shown in the diagram Fig.2. For more details see [2].

### 3.2. Mobile system

During the 4th CEC campaign at Turbigio a total of 83 backscatter profiles of the power plant plume were obtained. Most of them were recorded for obtaining plume geometry data together with the other lidar teams and measured in 964 nm only, but in one afternoon a series of useful multi-wavelength data of the plume could be obtained. Unfortunately the neodymium laser (530 nm) was out of operation due to a damage, but in 347 and 694 nm we got some interesting results. It is known that the extinction of an isolated aerosol could like a smoke plume be deduced from the reduction of the backscattering level of the surrounding air behind the plume, without requiring an absolute calibration of the lidar. We found extinction coefficients decreasing rapidly with distance from the stack due to dilution. The ratio of the extinction coefficients in 347 and 694 nm was 5.6 just above the stack and decreased to 3.4 at 1 km range. An attempt was made to derive information on the particle size from these data which cannot be presented in detail here, rendering a most probable particle diameter between .25 and .4 microns. According to the Mie-theory, the above mentioned extinction ratio does not vary very much with the refractive index in this size range.

During the MESOCLIP-experiment about 100 three-wavelength lidar profiles were obtained showing the vertical aerosol distribution as governed by the aerological structure. Results are presented in [3]. About 250 profiles were obtained at the institute, in each wavelength, mostly with the lidar pointing towards the 1650 m station.

### 4. CONCLUSIONS

There is no doubt that a calibrated lidar is able to obtain not only a qualitative picture of the spatial aerosol distribution but also at least approximately quantitative aerosol data. The derivation of these data from the lidar signals, however, is a very complicated procedure and requires not only the calibration of the system, but two additional steps, namely solving the integral-type lidar equation to get extinction-free backscatter profiles, and inversion of the true backscatter (or extinction) data into aerosol data. Since we were able to derive sufficiently accurate and stable calibration factors, step one can be regarded as solved. Also our method to solve step three by the bimodal model distribution seems to work under certain premises, as shown above. We must admit, however, that our iterative

procedure for step two, the integration of the lidar equation, which was designed to do with as few a priori assumptions as possible, is not stable enough for a routine evaluation of the lidar data. This instability with respect to signal errors and noise and especially to errors in the backscatter to extinction ratio, which is very common to all lidar experts, often leads to negative or absurdly large aerosol scattering coefficients and concentrations and requires quite arbitrary looking modifications of the data for correction. Nevertheless, we are regarding our lidar data, especially those obtained by the mobile system along the slant path with Knollenberg spectra available at both ends, worthwhile to look for a better method for the inversion of the lidar equation. Such a method, for example, is presented in a recent paper by Klett [4]. Whereas in our procedure the profiles in the two wavelengths are treated simultaneously with no preconditions about the relationship between backscatter and extinction, it is probably better to solve the problem for each wavelength separately, maybe using Klett's method. The further discussion of these problems must be restricted to a more detailed report.

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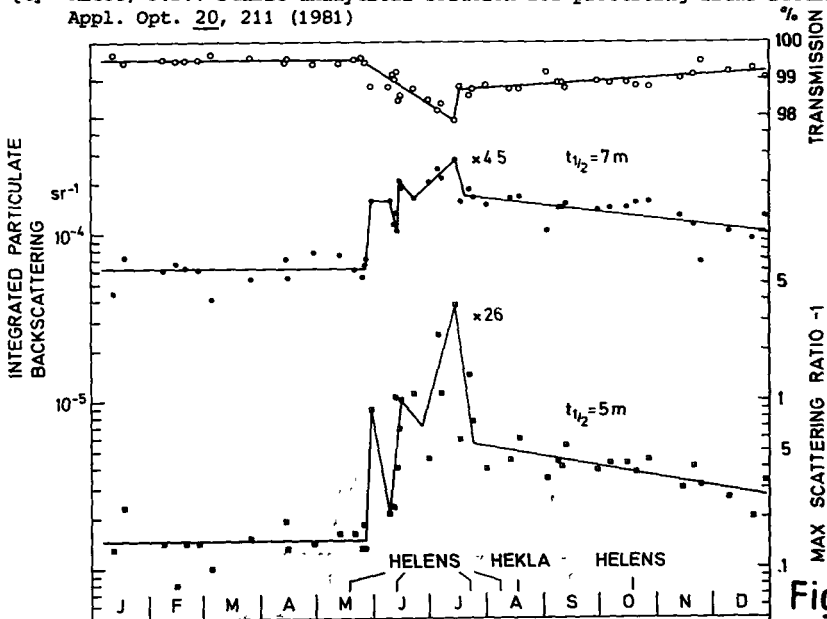


Fig. 2

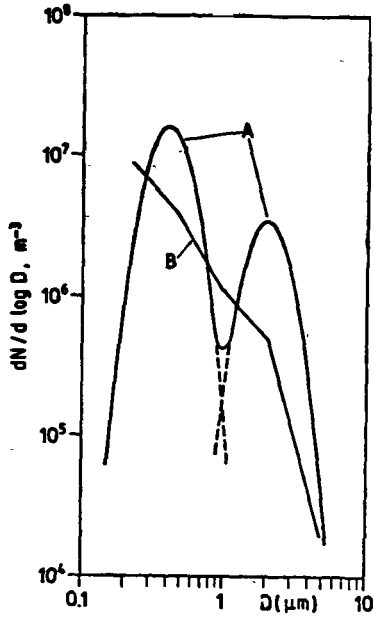


Fig. 1a

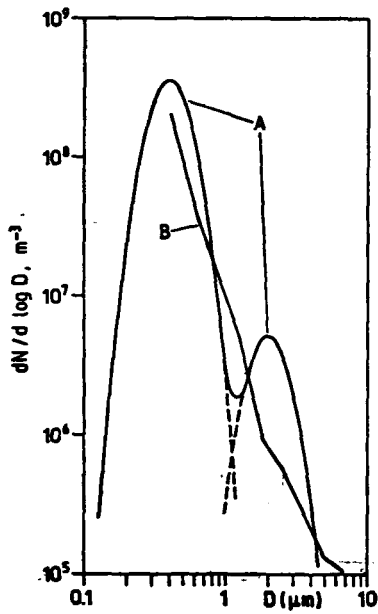


Fig. 1b



Contractor : Nuclear Research Center - GRENOBLE

Contract n° 189-77 - ENV - F

Project leader : J. COMERA

Title of project : Remote sensing of atmospheric pollution using a CO<sub>2</sub> double frequency laser : prototype adaptation to continuous measurements in a network ; results.

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## 1 - OBJECTIVE OF THE RESEARCH

The objective of this study is to compare pollution measurements obtained from differential absorption of two carbon dioxide laser beams with those from a point sensor in a pollution monitoring network. The differential absorption method gives the average pollutant concentration over a path. Its principle is the following [1], [2] : the absorptions of two laser beams with nearby wavelengths are measured. One wavelength is strongly absorbed by the pollutant studied, the other one is little absorbed. Both wavelengths are equally absorbed by the natural components of the atmosphere (water vapor, carbon dioxide).

The comparison has been done on ethylene monitoring, because the two systems can measure it and with comparable sensitivities (the network is equipped with an hydrocarbon sensor).

## 2 - MATERIALS AND METHOD

### 2.1 - Network

The ASCOPARG (Association pour le Contrôle de la Pollution Atmosphérique de la Région Grenobloise) network is composed of 5 monitoring stations connected to a computer. The monitoring stations measure the strong acidity, chlorine, hydrocarbons, temperature and wind speed and direction.

The measurements of the sensors are read every minute, and average values are sent every quarter of an hour to the computer. The average values over one hour are summarized in a daily record (including the laser measurements).

The hydrocarbon sensor has an analogic output and the comparison of the two measurements can be done on a chart recorder.

## 2.2 - Laser system

The two wavelengths laser and the beam propagation optics used in this study have been designed previously for pollutant monitoring in workshops [3]. They have been slightly modified in order to improve the concentricity of the two beams and to reduce the beam distortions [4]. The main features of the laser system are : size : 20 x 20 x 60 cm<sup>3</sup> (20 kg) for the laser and the beam propagation optics ; output power : 300 MW ; beam diameter : 50 mm ; sequential emission of the two wavelengths at 50 Hz.

After passage through the atmosphere, the beam is sent back to the laser by a cat's eye retroreflector.

The laser signals are received by pyroelectric detectors, and an analogic electronics gives the value of the pollutant concentration with a time constant that can be set between 1 and 30 seconds.

## 2.3 - Setting up of the laser

The ASCOPARG station is a small (2,5 x 2,5 x 2,5 m<sup>3</sup>) concrete shelter. The laser rests on bearings fixed against the wall, and the beam is transmitted to the retroreflector through a thin (5 microns) mylar foil. The retroreflector is situated on a metallic tower, 5 meters above ground, and 100 meters from the laser. The temperature of the shelter is kept constant ( $\pm 1^{\circ}\text{C}$ ) in winter but can vary strongly in summer.

## 3 - RESULTS

### 3.1 - Laser behaviour

#### - Reliability

The laser has been operating almost continuously (24 hours a day) for about one year without important troubles. The beam alignment is stable, and the laser system operated most often for weeks without maintenance. The few failures were always caused by secondary components, and the laser was rarely stopped for more than a few hours.

#### - Influence of atmospheric conditions

Rain has usually no effect. Strong rain only increases the system noise. Fog can bring a perturbation of the system if the visibility is less than a few ten meters. Weak snow falls have no effect on the system, but very

intense snow falls can result in a strong noise increase, and even stop the system. An example of neutral absorption is given in figure 1.

- Long term drift

In order to evaluate the long term drift of the laser system we have done tests for about ten days, with the laser operating on lines little sensitive to ethylene (ten times less sensitive than in normal operation). During all that period the settings of the laser system were not changed. For those tests the results were corrected for the electronics drifts, and for the water vapor differential absorption ( $< 10$  ppb  $C_2H_4$ ). For all that period the drifts correspond to less than  $\pm 7$  ppb when transposed to the lines used to monitor ethylene.

Those tests were done during a cold weather period, with little sun. During warm and sunny periods, distortions of the wall bearing the laser cause fluctuations as high as  $\pm 3 \cdot 10^{-4}$  radians in the beam direction, and can result in drifts of  $\pm 20$  ppb. Effects of that kind should be considered when setting up the laser.

- Selectivity

Though more than a few tens pollutants have been identified on the site where the laser was settled, selectivity never seemed to be a problem. A better check of the selectivity should require a comparison with an other sensor capable of selectivity monitoring  $C_2H_4$ .

3.2 - Comparison with the point sensor of the network [4]

Some elements complicated this study and prevented us from making a systematic comparison between the results of the two apparatus :

- there is a main road between the ASCOPARG station and the pollution sources. Thus, when there is a lot of traffic on the road, the signal from the hydrocarbon sensor gives the pollution along the road, which can be different from that given by the laser (the beam path is perpendicular to the road).
- the hydrocarbon sensor monitors the total hydrocarbon pollution, while the laser systems selectively monitors ethylene.

This is why we did the comparison in two types of situations only : small traffic on the road and peaks of pollution given by the two systems and weather conditions favorable to a strong accumulation of pollutants.

In the first case we found two main types of situations, one with a ratio of 1 between the concentrations read by the two systems, corresponding to ethylene alone, and the other one with a ratio of about 2.5 corresponding to a mixture of hydrocarbons containing a large amount of ethylene. Those ratios of 1 and 2.5 are obtained from the average values of pollution peaks lasting between a few minutes and a few hours, and with usually very large differences between the instantaneous values given by the two systems. An example of this type of situation is given (figure 2).

An example of the second type of situation is given on figure 3. It shows, during an episode of temperature inversion, the evolution of the pollution after a change in the wind direction bringing the polluted air from the town to the monitoring site.

#### 4 - CONCLUSION

The nearly continuous operation of the laser for about one year on the ASCOPARG network has shown that routine operation of this apparatus is possible.

The reliability of the system is good.

The sensitivity is that expected from laboratory measurements (< 20 ppb for C<sub>2</sub>H<sub>4</sub> and a 100 meter path between laser and retroreflector).

Setting up the apparatus on a monitoring station is not a problem, and the optical alignements are stable over long periods (months).

During this study a systematic comparison between the two apparatus was not possible because the hydrocarbon sensor could not selectively monitor ethylene.

#### PUBLICATION

J. JAUSSAUD. Differential infrared absorption for SO<sub>2</sub> monitoring. Contact Group Meeting Remote Sensing of Atmospheric Pollution. BRUSSELS, 29-30 April 1980.

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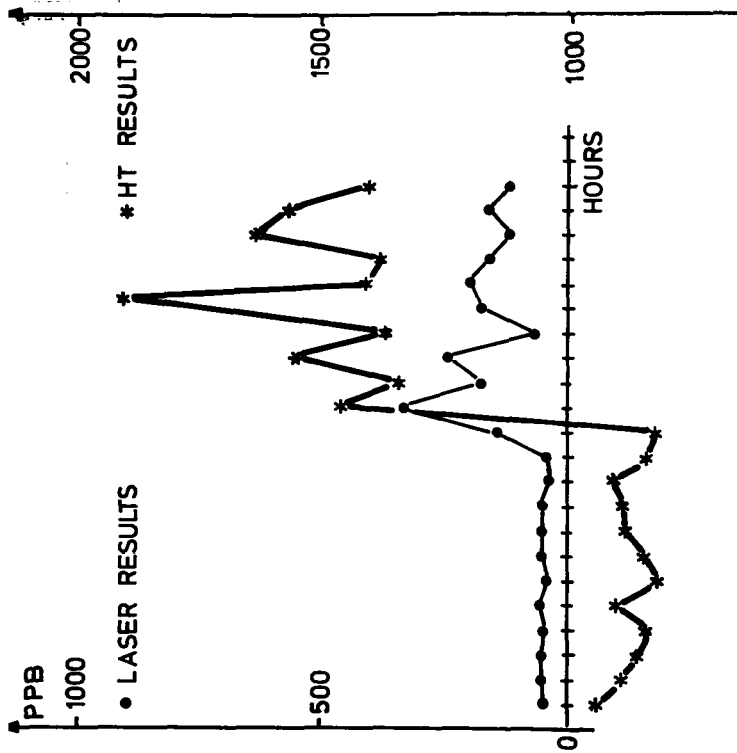


Fig. 3 - Comparison between laser and total hydrocarbon sensor data during a pollution episode, as recorded by the network.

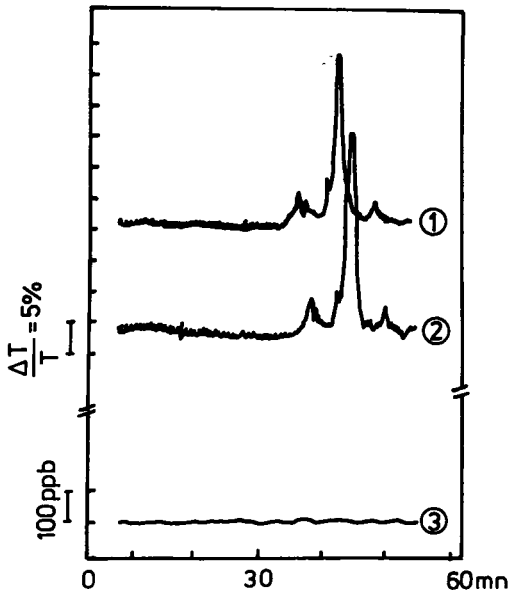


Fig. 1 - Neutral absorption effect (fog)

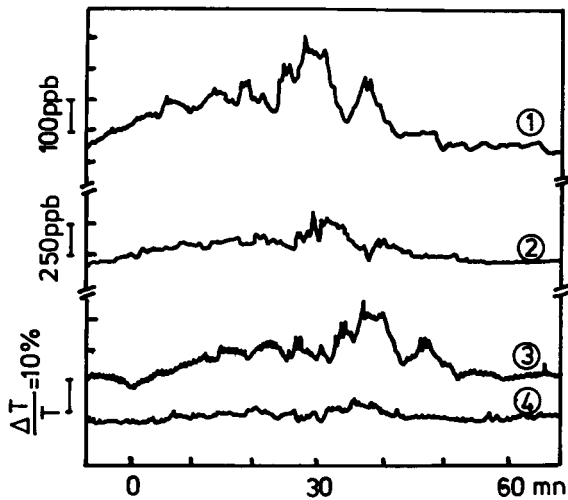


Fig. 2 - Ethylenum puffs recorded with the total hydrocarbon sensor (curve 2) and the laser (curve 1). Curves 3 and 4 indicate the atmospheric transmission of the absorbed and non absorbed lines.

Contractor            SERVICE D'AERONOMIE DU CNRS  
                         BP 3 91370 Verrières-le-Buisson, France  
Contract n°            ENV/339 F  
Project leaders        M.L. CHANIN, G. MEGIE  
Title of project       LIDAR MEASUREMENTS OF THE OZONE CONCENTRATION PROFILE  
                         IN THE TROPOSPHERE

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LIDAR MEASUREMENTS OF THE OZONE CONCENTRATION PROFILE IN THE TROPOSPHERE

J. Pelon, G. Mégie, M.L. Chanin

I. Research objectives

The recent development of powerful tunable laser systems has opened a new experimental field in spectroscopic studies of atmospheric trace constituents. The prime objective of the research described here was thus to define a lidar system for the measurement of the ozone distribution in the troposphere which will be of particular interest for such studies as :

- the continuous monitoring of the ozone vertical profile in the troposphere and lower stratosphere which is a basic requirement for the understanding of the mechanisms of the troposphere-stratosphere exchanges and of the ozone global budget ;
- the measurement of the ozone mixing ratio distribution over polluted areas taking advantage of the high spatial and temporal resolution of the lidar technique.

Upper atmospheric Lidar measurements are performed at the Haute-Provence Observatory since 1970 using high energy flashlamp pumped tunable dye lasers. A new facility has been set-up in January 1980 at the same location, more oriented towards tropospheric and stratospheric measurements using the Differential Absorption Lidar technique (DIAL):

Ozone measurements have been performed during several field experiments in 1980, which already demonstrate the capacity of the lidar system to perform accurate vertical soundings on a routine basis. The development of a ground based station to be used for the ECC field experiment in FOS sur MER (France) during the year 1983 can thus be undertaken using the theoretical and experimental expertise of our group.



## II. Methodology : The Differential Absorption Lidar technique

The basic principle of the DIAL technique involves the sequential emission of two laser pulses, the wavelengths of which are respectively tuned to  $\lambda_1$  and  $\lambda_2$  resulting in a difference in the ozone absorption cross sections :  $\sigma_1 - \sigma_2$ . Measurements are performed using the ozone UV absorption band from 285 nm to 305 nm. The signal backscattered by the atmospheric gas (Rayleigh scattering) is collected by a telescope and detected using a photomultiplier. The altitude resolution is directly related to the pulse laser duration and does not require any further mathematical treatment. By processing four different signals on the backscattered laser echoes, which include two wavelengths ( $\lambda_1, \lambda_2$ ) and two altitude steps ( $z, z + dz$ ), one derives the mean (local) value of the ozone concentration :  $[O_3]$ , between  $z$  and  $z + dz$  as :

$$(1) \quad [O_3] = \frac{1}{2(\sigma_1 - \sigma_2) \Delta z} \text{Ln} \frac{N(z + dz, \lambda_2) N(z, \lambda_1)}{N(z, \lambda_2) N(z + dz, \lambda_1)}$$

where  $N$  is the backscattered signal (in photons). The relative accuracy of the measurements is given by

$$(2) \quad \frac{\delta [O_3]}{[O_3]} = \frac{1}{2(\sigma_1 - \sigma_2) [O_3] \Delta z} \left\{ \sum_{i,j} N^{-1}(z_i, \lambda_j) \right\}^{1/2}$$

where  $z_i$  stands for  $z$  or  $z + dz$  and  $\lambda_j$  for  $\lambda_1$  or  $\lambda_2$ , in the case of a shot-noise-limited signal to noise ratio as expected for nighttime measurements.

A detailed analysis of the DIAL technique shows that a measurement is optimized when the differential optical thickness between the ground and the altitude  $z + \Delta z$

$$(3) \quad \tau(\lambda_1, z + \Delta z) - \tau(\lambda_2, z + \Delta z) = \int_0^{z+\Delta z} [O_3] (\sigma_1 - \sigma_2) dz$$

is equal to  $1.1^5$ , provided that the same number of laser pulses is emitted at both wavelengths. This important result leads to the conclusion that a given pair of wavelengths is fully optimized only for a unique level  $z$ . In fact, a compromise has to be found between the expected measurement accuracy and the overall experimental requirements. Nevertheless, in any case a single pair of wavelengths will never allow an accurate measurement of the ozone concentration from the ground up to stratospheric levels. The results which are presented here have been obtained by using a pair of wavelengths adapted to tropospheric measurements :  $\lambda_1 = 290$  nm,  $\lambda_2 = 295$  nm. In addition to the value of the differential optical thickness, other parameters have to be optimized with respect to the DIAL measurements. One has to take into account the temporal variations of the scattering medium and the wavelength dependence of the scattering processes as far as they are considered as identical for two successive laser pulses. To avoid any interference due to fluctuations in the aerosol content or to cirrus clouds at the tropopause level, the switching between the two wavelengths  $\lambda_1$  and  $\lambda_2$  has to be rapid enough. On the other hand, the dynamical range of the backscattered laser echoes between the ground and 15 km is large (3 to 4 orders of magnitude due to the altitude decrease of signal and atmospheric density) and requires various modes of photon detection. All these experimental requi-

rements and an expected accuracy on ozone concentrations better than 5% led us to specific achievements in the various elements of an operational lidar system.

### III. Experimental set-up

The experimental set-up, as schematically represented on figure 1, includes a laser pumped dye laser : the pump laser output ( $\text{Nd}^{3+}$ : YAG, Quantel 480 C, 300 mJ, 15 ns, 10 Hz, 532 nm) is converted to longer wavelengths by a Rhodamine 6G dye oscillator-amplifier system (Jobin-Yvon) with an overall energy conversion efficiency of 40 %. Spectral narrowing and tuning of the dye laser oscillator emission is performed by a grating near grazing incidence and results in a 5 pm linewidth in the 600 nm range. The output wavelengths of the dye laser are continuously monitored by a Fizeau interferometer and servo controlled by a mini computer (PDP 11-34) with the aid of a feedback electronic loop. This system allows a stability better than  $10^{-6}$  on a shot to shot basis and an automatic switching between the "on" and "off" emission wavelengths\* (corresponding respectively to  $\lambda_1$  and  $\lambda_2$  previously defined) with a preselected time interval. In order to obtain a laser emission in the 300 nm range, the dye laser output is frequency doubled using two KDP crystals, one for each wavelength. This frequency doubler configuration is designed to allow a rapid mechanical switch of the crystals when the dye laser emission is tuned from the "on" to the "off" wavelengths. The overall time of the wavelength change, preprogrammed and computer controlled, requires less than 0.5 s. The backscattered photons

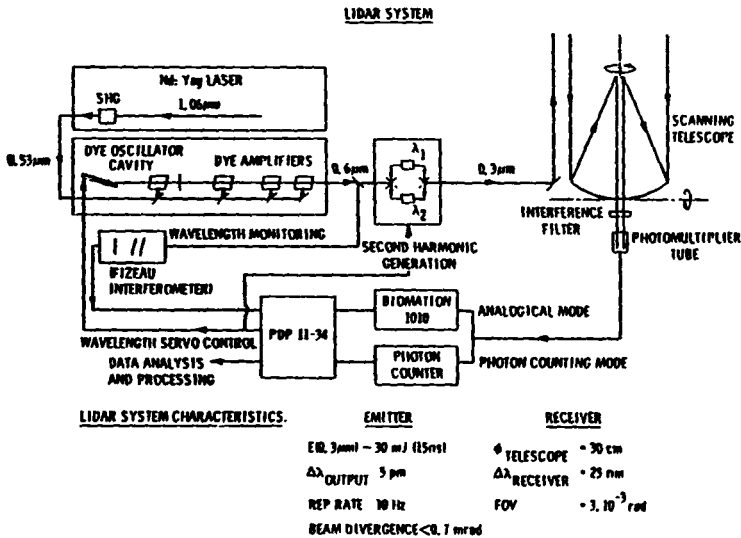


Figure 1 - The lidar experimental set up.

\* The "on" and "off" denomination refers to discrete absorption line spectrum and was extended to continuum spectrum where the "on" line corresponds to the strongest absorption.

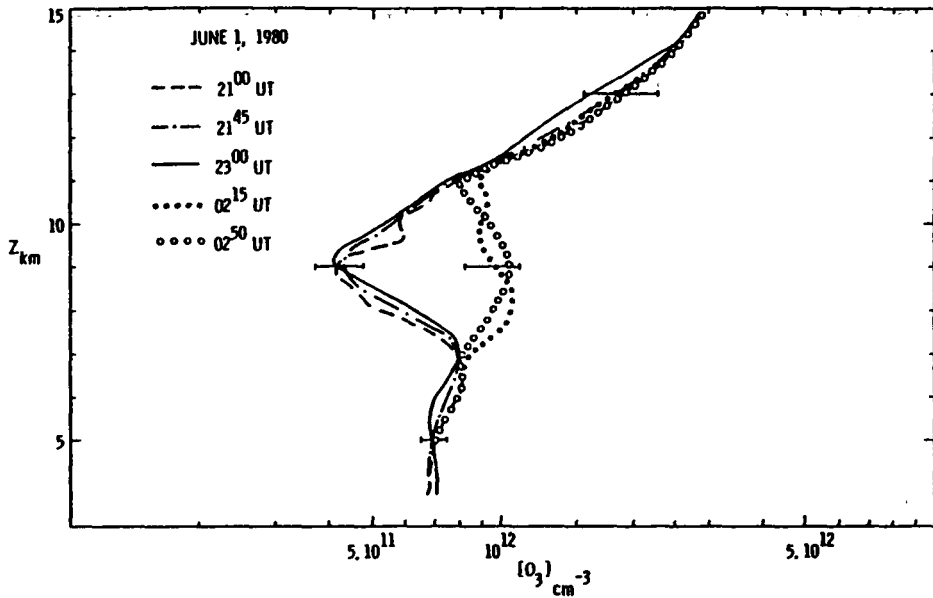


Figure 2 - Ozone concentration profiles during the night of June 1st, 1980.

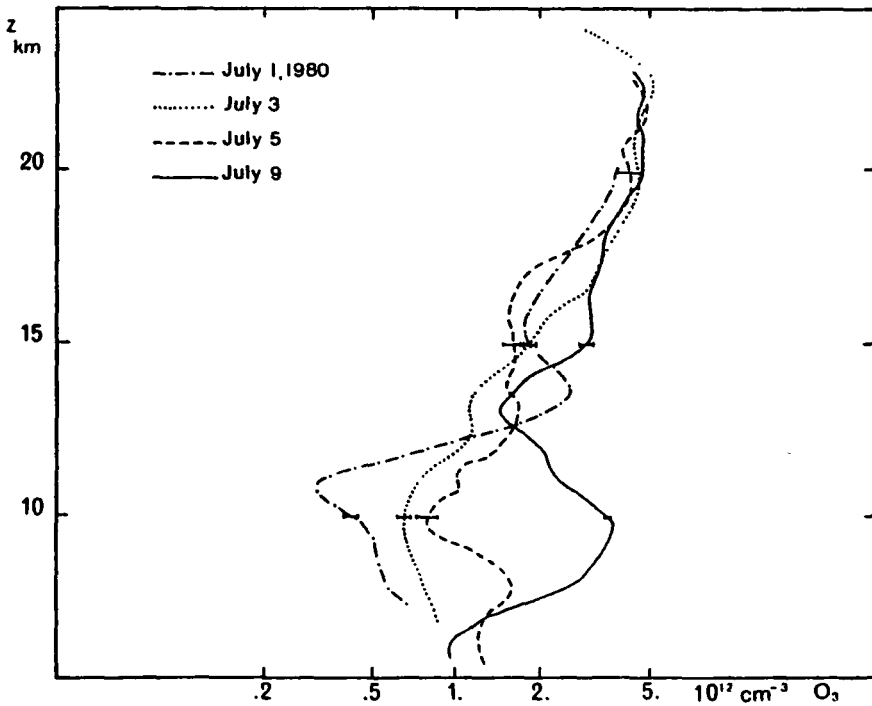


Figure 3 - Ozone concentration profiles, July 1980.

are collected by a 30 cm diameter telescope and detected through an interference filter by a photomultiplier tube. To take into account the large dynamical range of the signal, as previously mentioned, two detection modes are worked out : an analogical mode using a waveform recorder at 10 MHz (10 bits) and a photon counting integrator at 0.25 MHz. The altitude resolution of the lidar system is respectively 600 m in the photon counting mode and 150 m to 300 m in the analogical mode where a least mean square fitting of the signal is made using 10 to 20 experimental points to improve the relative accuracy. The real time data averaging and processing is made by the PDP 11-34 minicomputer.

This lidar system is in operation at the Haute-Provence Observatory since January 1980.

#### IV. Experimental results

Several field campaigns have been conducted during the year 1980 corresponding to a total of 35 nights of observation. The vertical ozone profiles between 2 and 20 km are obtained within a 30 minutes integration time. The vertical resolution which is a basic property of the lidar system, is always less than 1.2 km. The relative accuracy on the ozone concentration is better than 5 % in the troposphere and decreases down to 15 % at the 20 km level for the above temporal and spatial resolutions. The statistical analysis of the measured ozone profiles shows the existence of very large variations in the ozone concentration below 16 km, even within short observation periods.

As an example of this short term variability, successive profiles recorded during the night of June 1, 1980 are represented on figure 2, showing an increase of the ozone concentration by a factor of two at the 10 km level. It is important to note that the tropopause height, as determined by radio soundings data from the nearby meteorological station of Nîmes 120 km east of the O.H.P., is about 12 km. Another example is shown on figure 2 where different profiles recorded in the upper troposphere are plotted. These variations which are related to the horizontal transport are also observed on a day to day basis (figure 3). Events such as the ozone bulge present at an altitude of 9 km on July 9, 1980 have also been observed twice in March and June. An analysis of the meteorological charts at the 300 mb level during the days preceding and following these events, show that intrusions of stratospheric air masses of polar origin are responsible for the ozone concentration increases observed at tropospheric levels at the latitude of the Haute-Provence Observatory (44°N). A quantitative evaluation of the total ozone mass introduced in the troposphere show that these events might be responsible for between 1 to 2 % of the total annual ozone mass exchange between the troposphere and the stratosphere.

#### V. Conclusion

An operational lidar system has been evaluated and its potentiality for continuous monitoring of the ozone vertical profile has been demonstrated. Further improvements to be conducted in 1981 will include :

- comparative measurements with balloon borne ozonosondes and other ground laser IR spectroscopic techniques ;
- daytime measurements ;
- ground truth and complementary experiments for satellite borne ozone measurements.

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Communication presented at the 10th International Laser Radar Conference, Silver Springs, USA (October 1980).
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- (4) J. PELON, G. MEGIE  
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Submitted for publication in Applied Optics (1981).
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Contract n° ENV/340 F

Project leader : Jacques CARBONNELLE - CEA - CEN/SACLAY - DPr/SPT - 91191  
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Title of project : Ultra-violet videometry

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## 1. OBJECTIVE OF THE RESEARCH

The use of modern remote-detection systems on industrial pollutant emissions generally requires a knowledge of the rates of transfer of these pollutants.

By the use of suitable treatment, television image (video signal techniques serve to measure this parameter. Initially, the process uses light absorbed or diffused by the mechanism to be investigated, in the wavelength band corresponding to the visible range.

Interesting developments emerged in practice, in the area of the analysis of the spread of pollutants, provided that they can be displayed.

This work was based on the display of SO<sub>2</sub>, requiring the development of a technology for the formation of images in the wavelengths corresponding to the near ultraviolet.

## 2. MATERIALS AND METHODS

As video ultraviolet equipment does not exist on the market, it had to be invented.

To obtain images in this spectral range implies the resolution of several problems corresponding to the three main functions of the unit :

- optical function,
- filter function,
- receiver function.

A spectroscopic system derived from the Ebert-Fastie system was selected for the two first functions.

The image formed by a spherical mirror, and the spectral range is selected by slits and a grating.

The existing prototype system at the CNRS Space Astronomy Laboratory was modified to increase its aperture, and was fitted with a video tube sensitive in the absorption wavelength of  $\text{SO}_2$ , of about 310 nm.

Since the sensitivity of this tube is insufficient for the aperture of the optical system, the signal obtained is processed by integrating a variable number of video signal frames.

These frames are accumulated on a magnetic disc memory. All the recordings are stored on a standard videorecorder.

### 3. RESULTS

It has not yet been possible to proceed with systematic tests. However, an initial attempt succeeded in operating the optical and electronic systems.

Some images were obtained on the plume of the EDF power plant at Martigues, near Marseille.

The first results are encouraging, and show a considerable increase in contrast of the plume in relation to the sky background.

This is shown by photos 1 and 2 of recordings concerning the visible range in the first and at 300 nm in the second.

### 4. CONCLUSIONS

The initial results are encouraging, although they were obtained in difficult conditions (low height on the horizon giving rise to a low level of ultraviolet radiation, and mediocre insolation).

It remains to measure the sensitivity of the system (visibility limit of the plume). This will be carried out in the near future on a known source, by comparison with the measurements of a correlation radiometer operating in the same spectral range.

**Contractor:** Consiglio Nazionale delle Ricerche (CNR) - Istituto  
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Italy

**Contract n°** ENV/348 I

**Project Leader** Giuseppe Bonino

**Title of project:** Radioacoustic sounding system for the measurement of  
temperature vertical profiles in the planetary boundary  
layer during fog conditions

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### 1. Objective of the research

During day time, when convective turbulence is well developed and strong vertical mixing is assured, or in windy conditions with low or moderate insolation (neutral conditions), the dynamic and thermodynamic structure of the PBL can be satisfactorily assessed. This situation generally occurs when synoptic weather conditions are such as to allow sensible heat and momentum to be transferred at a constant rate along the vertical coordinate.

However, other situations are frequently encountered, which may lead, for example, to nocturnal stable inversion layers, subsidence inversions, fog layers due to water vapour condensation processes. For these cases no prognostic model exists. Consequently, the need for more reliable data from PBL observation is felt, for which knowledge of the vertical thermal profile become imperative.

In particular, one of the most difficult structure of the PBL to be measured is represented by a radiative fog layer capped by an elevated thermal inversion of unknown depth and intensity. This situation draws its origin in synoptic anticyclonic weak circulations, with clear sky, supporting the development and growth of nocturnal radiative inversion. When such a situation persists for several days, the relative humidity increases and may eventually reach the saturation value, causing the formation of fog below the inversion layer.

The relevant data needed in this case are: a - thickness of fog layer; b - intensity of radiative thermal inversion at the top of fog; c - atmospheric stability parameter above the fog-capping inversion, in the height range 500 - 1000 m. Use of SODAR may reveal a), but not data b) or c).

These last data may be assessed with disposable radiosondes. However, economy and air traffic regulations impose severe limitations on the use of this method.



The above considerations show the interest in the development of advanced experimental devices, able to perform remote sensing of the PBL structure from ground based stations; avoiding the previously quoted limitations. The Radio Acoustic Sounding System (RASS), described in this report, represents a powerful methodology of this kind, our group has developed and tested in the frame of the CEC environment programme.

## 2. Materials and methods

The radio-acoustic sounder consists in its essence of a powerful acoustic source beaming a short burst of sinusoidal waves toward the zenith. The upward speed of this pulse is proportional, at every height, to the square root of the local temperature. The pulse speed is continuously measured from the ground by means of a Doppler radar.

The radar echo is due to the change in the refractive index of air intensely compressed by the acoustic wave. The faint echo is maximized by choosing an acoustic wave in Bragg resonance with the radio-wave. The record of the measured sound speed as a function of the delay leads to the acquisition of the temperature vertical profile.

The main parameters of the Turbigio RASS are listed in table I.

Table I.

Parameter	Acoustic	Radio
frequency	360 Hz	159.000 MHz
wave-length	0.94 m	1.88 m
power	200 W	25 W
array	9 folded horns	8 dipoles with reflector (each)
beam	25° x 25°	30° x 30°
location of the antenna	half-way between radar antennas	~ 60 m between antennas

The acoustic antenna has floor and walls built in solid concrete, which present a plane, firm surface to the acoustic reflection.

The radar transmitting array is fed by a c.w. quartz-controlled transmitter. The radar receiving array feeds an autodyne-type receiver, followed by an audio amplifier which includes a steep-edged filter having a band width of 20 Hz.

The distance between the two radio arrays is such that, while the

two zenith-directed beams are sharply decoupled on the ground, and they start to overlap at a height just above 100 m.

The frequency of the Doppler echo is measured by means of a precision counter and memorized on the photosensitive paper of a high-speed-galvanometric recorder.

The Doppler frequency exhibited by the counter is the result of the averaging of 16 consecutive soundings. The temperature, in °C, correlative to every height considered, is derived from these averages by using the equation:

$$t = \left[ \frac{4.7 \cdot 10^2 f_D}{(1 + 4.714 \cdot 10^{-5} (H - 30)) \cos(\arctg(d/z))} \right]^2 - 273.15$$

where  $f_D$  is the Doppler frequency, H the relative humidity in %, d the half-distance between the two radar antennas and z the height of observation. In the above expression, the term  $\cos(\arctg(d/z))$  is used to convert radial velocities into vertical velocities. The numerical constants are peculiar to the Turbigo site geometry and assume a speed of sound linearly related to H within the relative-humidity range 30 to 100%.

The time required for the measurement of one vertical profile varies from few seconds with a disposable radiosonde to few minutes with a tethered balloon. RASS soundings could be repeated at a rate of one every 2 minutes. At this rate a detailed study of the thermodynamic evolution of the PBL may be envisaged.

### 3. Results and conclusions

As an example a comparison of thermal profiles measured with the RASS and with other methods (performed during the 4th CEC Campaign of Turbigo) is shown in fig. 1. It is evident from the inspection of these diagrams that all RASS data compare favourably with all other readings. Any local disagreement in the plots is contained within 1 °C and may be justified in all cases by differences in the footprint of the sounding, a few km, and/or in the instant of measurement. During the campaign approximately 100 soundings have been compared.

As far as fog layers are concerned, temperature profiles measured by RASS during fog conditions are reported, as an example, in fig. 2.

Other examples referring to synoptic weather conditions marked by fog in the Po Valley in connection with a high levelled pressure over Mediterranean area, between 8 and 14 February 1980 are shown in fig. 2 of the article of G. Bonino, P.P. Lombardini, A. Longhetto and P. Trivero entitled "Radio acoustic measurement of fog-capping thermal inversions", Nature, vol. 290, p.121 (1981) (Ref. n. 2).

Comparison of RASS measurements with other tropospheric soundings

has demonstrated 1) RASS ability to produce vertical thermal profiles in the range of altitudes 150 to 1000 m with a temperature accuracy and a height discrimination comparable with conventional soundings, 2) RASS qualities typical of a remote sensing device, i.e. short time of measurement, minimum cost of operation, possibility of performing soundings in rapid succession under the control of a single attendant.

During the Turbigio campaign the limitation of conventional sounding methods was also put forth. Due to the proximity of the Malpensa International Airport, tethered balloons and model aircraft had to be grounded every time an airplane left or reached a runway. Disposable radiosondes were limited in their use by the high cost of the individual sample.

The interest of air pollution control focuses on the use of the RASS as an alarm device against sulfur dioxide air pollution.

When an atmospheric thermal inversion is located above the effluence height of smokestack plumes, these are liable to diffuse below, thus enriching the air layers near ground with the pollutants they have in suspension. When, however, the plumes rise beyond the inversion boundary, they are prevented from falling by the stability situation existing there.

The examination of vertical temperature profiles reveals the presence of an elevated thermal inversion, the height of its base, its altitude span and its gradient. By introducing these parameters in a theoretical model, it is possible to forecast the behaviour of a plume flowing out of a smokestack of a given height.

Elevated thermal inversions are often accompanied by fog near the ground. In the Po Valley, the incidence of fog is very great: about 10% of the year. In foggy weather, low visibility bars the use of tethered-balloon sounders.

Hence the unique advantage of using the RASS as a tropospheric vertical sounder in a location of great fog incidence, as throughout the Po Valley.

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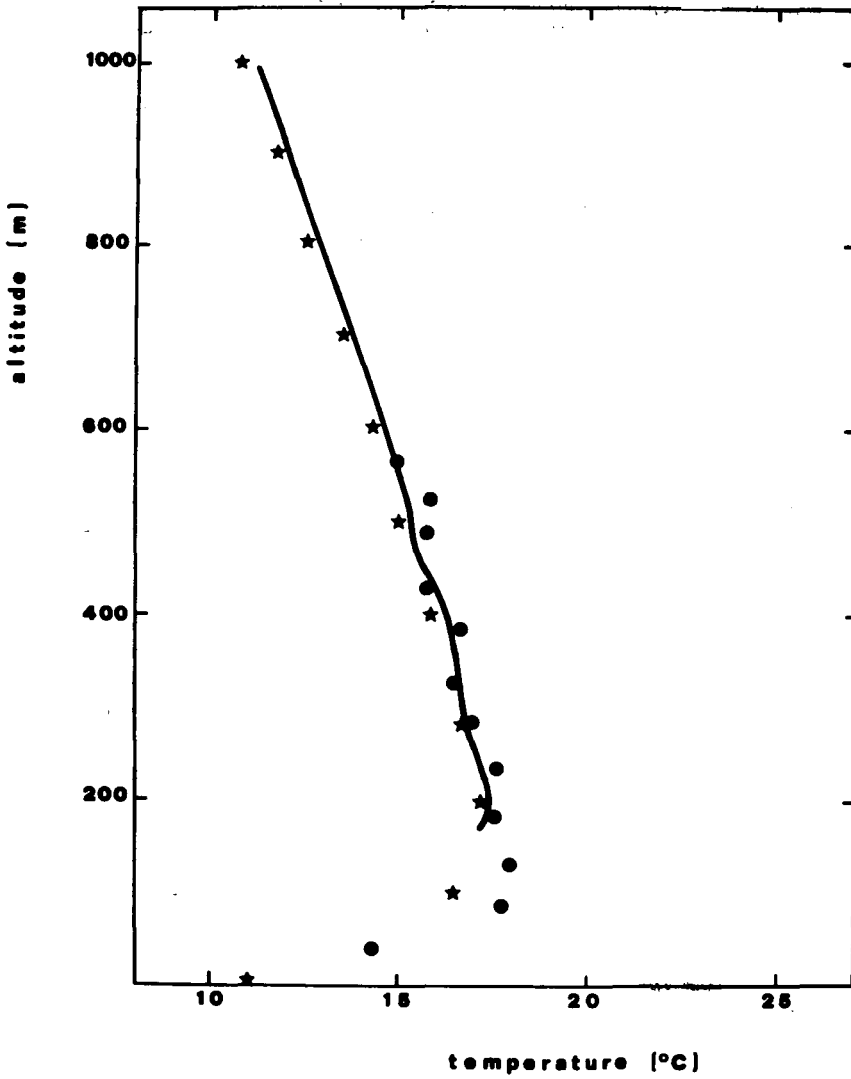


Fig. 1 Comparison of thermal profiles, September 17, 1979, 22 h 05 min: — RASS, ● radiosonde on tethered balloon (Inst. Cosmo-geofisica), ★ radiosonde on disposable balloon (Joint Research Center). (Figure taken from Ref. 1.).

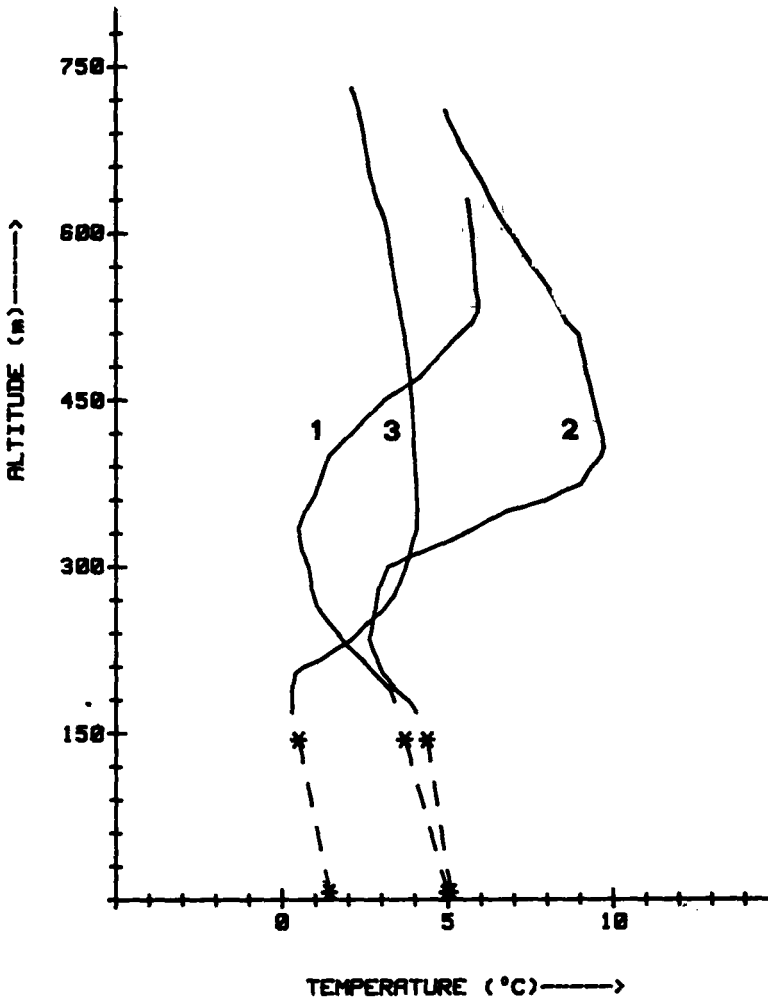


Fig. 2 - Atmospheric temperature vertical profile: — RASS measurements; \* measurements at 2 and 145 m height along a smoke stack of the power plant; --- interpolation line connecting measured points.

RASS profile	Date (Nov.1980)	Time	Weather conditions	RH at g.l. (%)
1	21	2.00 p.m.	foggy	98.5
2	22	4.00 p.m.	foggy	98
3	23	8.00 a.m.	foggy	98

Contractor: CNR - LABORATORIO DI RICERCA E TECNOLOGIA PER LO STUDIO DEL  
PLASMA NELLO SPAZIO, FRASCATI, ROME (ITALY)

Contract n° ENV/369 I

Project Leader: G. FIOCCO

Title of project: APPLICATIONS AND DEVELOPMENTS OF LIDAR AND SODAR DOP-  
PLER SYSTEMS FOR THE STUDY OF THE DYNAMICS OF THE  
ATMOSPHERIC BOUNDARY LAYER

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#### Objective of the research

Objective of the research is the development of and the experimentation with either acoustic (Sodar) or optical (Lidar) remote sensing techniques, for the study of the dynamics of the planetary boundary layer. The research has been carried out in two phases: the first, under contract CEE 241/77/1 ENV-I, in the period 1977-78, the second under contract n. ENV/369/I, in the period 1979-80.

#### Results

During the initial phase of the research a monostatic Sodar not yet capable of obtaining wind velocity information, was implemented and operated with a Doppler Lidar at the laboratory site in Frascati. The Lidar was able of obtaining vertical profiles of the vertical wind component. These early experiments aimed at associating phenomena of increased turbulence and acoustic cross section with updrafts as measured with the Lidar (F. Congeduti et al., 1977).

In the month of July 1977 in collaboration with ENEL the acoustic sounder was utilised in a measurement campaign to characterize the site of La Spezia. During this period analog recording was added to the system to allow Doppler analyses to be carried out on the echoes, off-line.

During 1978 the effort was mainly directed to the development of software for the off-line analysis of the recorded Sodar data. The interface with a minicomputer type Laben 70 was built and programs for the harmonic analysis with the use of the Fast Fourier Transform and for the presentation of the data were solved (G. Mastrantonio et al., 1978, G. Fiocco et al., 1978).

At the end of 1978 the Sodar was mounted on the ship *Salernum* for participation to the First Global Garp Experiment in the Indian Ocean. For this special purpose a screen had to be built and mounted on board the ship and problems related to the ambient noise on board had to be faced. The campaign lasted from 31 December 1978 when the ship departed from Naples to 5 April 1979, when the ship returned. A portion of the data obtained during this campaign has been reduced: in particular those pertaining to the transit of the ship through the Suez Canal where boundary layer phenomenology was expected to be particularly intense (G. Fiocco et al. 1979a, G. Fiocco et al. 1979b, G. Fiocco et al., 1980).

During the early part of 1979, in view of a campaign sponsored by the EEC at Turbigo, a system (Fig. 1) consisting of three monostatic Sodar was developed, (A. Ricotta et al., 1980) and successfully and uninterruptedly operated during the campaign. Much of the subsequent activity has been dedicated to the analysis of the data obtained at Turbigo. The analysis of the triaxial system has demonstrated that it is possible in the presence of convection to obtain information on the horizontal wind component, when the vertical components of the wind velocity measured at the three positions are correlated (M. Berico, 1980).

Intensity measurements are less suitable for this purpose because they are related to the presence of thermal turbulence of scale  $\lambda/2$  where  $\lambda$  is the sodar wavelength; they are subject to a rapid loss of coherence while being horizontally advected. A fair amount of analytical work remains before the proposed method can be utilised to characterize the complete wind velocity field.

Recently in order to carry out the analyses in real time a HP 1000 system has been acquired and the transfer of software from the old to the new system is presently being carried out. Improvements in the time required to carry out the analyses have also been obtained by utilising a slower rate of digitalization thus reducing the bandwidth over which the FFT is performed. The operation is presently performed at a sampling rate of 1.25 KHZ, for a signal extending in frequency from approx 1600 HZ to approx 2050 HZ. This procedure has permitted to save computing time by about a factor of 20.

An analysis has been carried out of the precision and accuracy of the wind velocity measurement obtainable from a Doppler Sodar; a procedure has been tested which overcomes the difficulties of obtaining the first moment of a signal in the presence of noise when the bandwidths are large (G. Mastrantonio and G. Fiocco, 1981).

Also, during the last year, results of a new series of combined Sodar Lidar experiments were analysed and presented. Both systems have Doppler capacity and a comparison of the precision obtained with the two techniques has been obtained (F. Congeduti et al. 1981).

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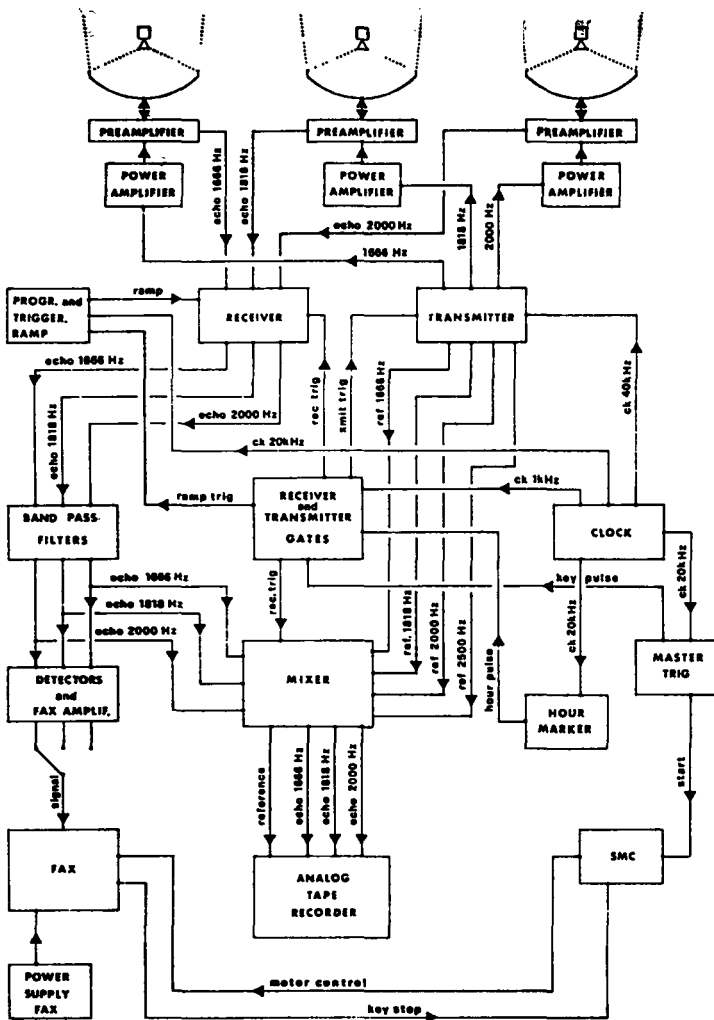


Fig. 4 - Sodar System

Contractor : National Physical Laboratory, Teddington  
Contract no. : 193-77-1 ENV UK  
Project Leaders : P T Woods, I H Curtis  
Title of project : Differential absorption system to monitor air pollution  
using tunable diode lasers

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### Objectives

To establish a long-path monitor for measurement of ambient concentrations of carbon monoxide and other pollutants in the open atmosphere using a tunable diode laser.

To investigate in detail those aspects of the method which bear upon the accuracy and validity of measurement, emphasizing potential sources of systematic error.

To evaluate the technique in field operation and to determine the extent to which this type of environmental measurement is valid and reliable.

### Method

A schematic representation is shown in figure 1. A commercial diode laser is used, operating in a closed-cycle cooler at temperatures between 10 and 50 K. The method can be applied to a variety of pollutants having infrared spectra; the initial measurements have been carried out on carbon monoxide using a Pb-S-Se laser emitting at approximately 4.7  $\mu\text{m}$ . A collecting lens focusses the laser output power onto a paraboloidal reflector which transmits a nominally-parallel beam to a retroreflector at a distance of between 100 m and one km. The returned beam is focussed onto a cooled detector via a germanium beam splitter which also provides a reference path for monitoring and controlling the wavelength. The angle of incidence at the beam splitter is minimized in order to reduce the effect on the output power of different polarizations of the laser modes.

In operation, tuning is achieved by adjusting temperature and driving current: the tuning range for a particular diode can be up to 250  $\text{cm}^{-1}$  with continuous tuning over approximately one  $\text{cm}^{-1}$ .

The laser emission is frequency-modulated and chopped and the conditions are adjusted to bring the frequency of a mode into coincidence with that of an absorption line of the pollutant of interest. The ratio of the derivative signal and the total power is proportional to the pollutant concentration.

The laser cold head, monochromator and reflector are on X-Y tables for ease of alignment. The cold head can carry two lasers; either can be brought into use by means of the transverse adjustment.

A small van is being fitted with a modified caravan body to serve as a mobile laboratory for field work.

### Field System

#### a) Mobile laboratory

An earlier report describes the arrangement shown in figure 2. The complete supporting structure has been tested and is satisfactory. Motors fitted to the periscope control the top mirror about vertical and horizontal axes. Weather protection for the mirror and mechanism is provided by the cowl which is isolated from the optical system but is driven round by the associated motor. Before operation of the system, airbags, pressurized by an electric pump, adjust the height of the complete structure while the legs are lowered; these also support it while in transit.

#### b) Experimental arrangements

Field trials were carried out from a laboratory window, using an atmospheric path of 110 m. This phase of the work was used to assess the problems which arise during field operation.

The equipment was set up on a honeycomb table as shown schematically in figure 1. The germanium lens focusses the laser output radiation for collection by the paraboloidal mirror. To transmit the nominally-parallel beam a single, plane mirror was substituted for the periscope system used in the mobile laboratory. The retroreflector was stationed on a convenient building beyond a road.

#### c) Measurements

Initial alignment of the "periscope", plane mirror was done using a visible beam. Subsequently, a hinging mirror and telescope enabled a signal to be

obtained very quickly. Optimum alignment was achieved by adjusting the plane mirror for maximum signal.

Modulation at 1 kHz over approximately two line widths was used, the resulting signal being ratioed with the total power chopped at 130 Hz. Records using both first- and second-derivatives were taken. Reference gas cells of atmospherically-broadened CO were inserted in the measuring path for calibration.

When first harmonic content was detected as a measure of pollutant concentration, the second harmonic was used to detect the flank of the absorption feature and lock the laser frequency. In the alternative arrangement the pollutant concentration was derived from the second-harmonic signal measured at the line peak, the first harmonic being used to stabilize the frequency.

#### d) Results

(i) General. The system described was used to monitor carbon monoxide over a period of time. Different laser operating conditions and detection methods were used. The frequency locking circuit worked satisfactorily, the stability was good and the system responded to passing vehicles.

(ii) Derivative spectroscopy and ratioing. Using the first-derivative signal, the system noise was 0.02 ppm at a time constant of one second. Use of the second-harmonic gave a three-times-worse signal-noise ratio.

The ratioing system showed a 2% change in the ratio when the beam obscuration was 50%.

(iii) Frequency stabilization. Using either method for deriving a feedback signal, the stabilization was satisfactory. The open-loop gain using second harmonic was about 50: that using the first harmonic was not measured at this time.

#### Laboratory measurements

##### a) Summary

(i) Mode structure. The mode structure of the diode laser output has been investigated. At threshold, the laser emits a single mode. Typically,

several modes exist simultaneously, the number increasing with power. Each mode tunes over  $\sim$  one  $\text{cm}^{-1}$  and has a half-power width of  $\sim 10^{-3} \text{ cm}^{-1}$ . All the modes are polarized randomly.

(ii) Angular distribution of power. Maximum power is not concentrated on the laser axis, in general. Power varies with angle in a random fashion and is emitted into a cone of greater than  $90^\circ$ . Different modes terminate at different angles, thus providing a possible filtering method.

(iii) Polarization. The major axes of the modes are orientated randomly and ellipticities up to 5 were found in the laser investigated.

(iv) Interfering species. Line widths are typically  $0.1 \text{ cm}^{-1}$ , so unwanted coincidences can be detected and avoided by an oscilloscope-display technique.

(v) Frequency modulation of laser output. This is caused by mechanical shock to the laser by the cooler mechanism operating at three cycles/s. It can be eliminated by the use of a modified cooler but the problem can be avoided by the use of a modulation frequency well away from the spontaneous frequency modulation and by a suitable time constant.

(vi) Aging. Pb-S-Se lasers can have lives of several years depending partly on the number of cooling cycles. The threshold current rises while power and operating range decrease. An otherwise-useless laser may produce power if pulse-operated.

(vii) CO spectroscopy. Laboratory reference spectra have been obtained using both a broadband source and a diode laser. An evaluation of these, in conjunction with the spectra of potential interfering species and the laser mode spectra, showed that it is possible to operate at any convenient frequency in the range P(1) to P(7) of the CO vibration-rotation spectrum.

Additionally, absorption coefficients for the CO lines at atmospheric pressure have been measured. These are necessary for systems not having built-in calibration methods.

(viii) Selecting suitable operating conditions. The complex behaviour of the laser modes, together with random changes between one laser cooldown and the next, necessitates the use of some fast method of displaying the power spectrum in order to find some suitable operating point. A continuous display method using an oscilloscope was developed.

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### Comments

(1) Ratiocing. The immunity of the signal-power ratio to power changes may depend on close matching of time constants in the synchronous detectors and on the optical alignment. These are being investigated further.

(ii) Choice of detection method. Although we have found so far that first-harmonic detection has a better signal-noise ratio, the second-harmonic method has advantages of zero output for zero concentration of pollutant, whereas first harmonic has finite value because, in general, the laser power is a function of frequency. Second-harmonic detection at the peak has the added advantage that reduced pressure in the gas cell sharpens the line thus giving sufficiently good stabilization using the first-harmonic feedback with less electronic gain.

(iii) Single-mode operation is preferred but in practice it is not always easily achieved. Multi-mode operation presents some calibration problems and a mode selector of some form may be incorporated but the present system has the advantage that maximum-available power is transmitted.

### Conclusions

The instrumentation for the field system has been shown to operate satisfactorily leaving only detailed electronic and optical effects to be explained and eliminated.

Successful field experiments have to be made on carbon monoxide. The projected sensitivity of the completed system is a few parts in  $10^9$  and the range is 0.1 to 1 km.

The system has potential for measuring a range of pollutants. Eg.,  $N_2O$  at  $4.5 \mu m$ ,  $NO$  at  $5.3 \mu m$ ,  $NH_3$  at  $10.5 \mu m$ ,  $C_3H_4$  at  $10.5 \mu m$ .

The mobile laboratory consists of a light commercial vehicle fitted with a modified caravan body.



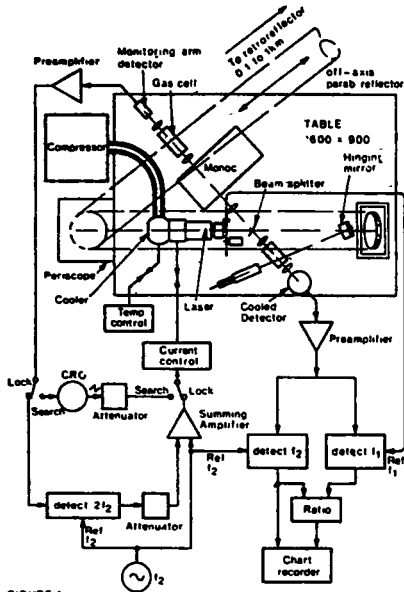


FIGURE 1

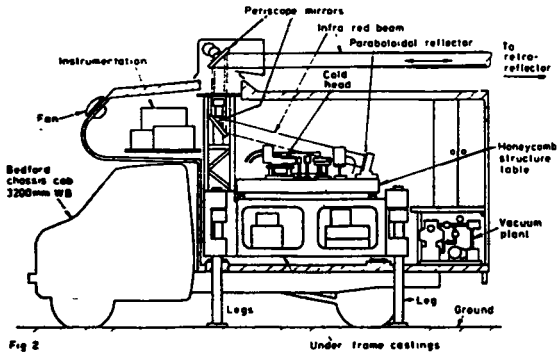


Fig 2

Under frame castings

Contractor: Central Electricity Generating Board, Leatherhead  
Contract No: 290-77-1 ENV UK  
Project Leader: Dr R.H. Varey  
Title of Project: Development and application of remote sensing for measurement of airborne materials

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#### OBJECTIVES

The aim of this program is to construct a mobile differential lidar for the measurement of SO<sub>2</sub>, NO<sub>2</sub> and other gases in the atmosphere. Subsequently the instrument will be compared with other similar apparatus such as the Hull University infra-red system (contract 141-77-1 ENV UK) and used in an operational mode to make pollutant measurements.

#### MATERIALS AND METHODS

The project has been carried out in two stages, first the production of a non-mobile prototype and second the installation and development of the system in a vehicle to provide a full mobile facility which can be used in the field to traverse industrial plumes. The prototype development was described in the previous report on the first phase of the Second Environmental Research Programme 1976-80. The main components of the system mounted in the vehicle are a tuneable dye laser, a system to receive back-scattered radiation, a data processing system and the vehicle itself.

The laser is a Chromatix CMX-4 producing 0.2 mJ at 300 nm with a pulse length of 1.2 μs at a repetition rate of 20 Hz. Between each pulse the output is switched by electromagnetic transducers between two wavelengths about 0.6 nm apart corresponding to strong and weak absorption by SO<sub>2</sub> in the atmosphere. The laser and frequency switching system have been reliable and trouble free. Care has to be taken with pulses as long as those produced by the CMX-4 that the shape at both wavelengths is the same or at least reproducibly different. If it is not then it results in the measurement of spurious SO<sub>2</sub> concentrations. Also, problems can arise in data analysis when the decay time constant of the tail of the laser pulse is of the same order of magnitude as the characteristic decay of the return signal from the atmosphere. Then the low energy tail back-scattered close to the receiving telescope interferes with the return from the high energy centre of the pulse scattered from a greater distance. A pockels cell has

been used with partial success to solve these problems by chopping the tail of the pulse.

The receiving system is a 0.5 m diameter cassegrain telescope and a standard EMI photomultiplier. A 3.5 nm, 20% transmission, interference filter is used to cut out background radiation and for day-time operation this is backed up with a 10 nm band pass filter.

The data processing system comprises a 20 MHz Data Labs 8 bit transient recorder interfaced to a PDP 11-34 computer. Also interfaced to the computer are energy and sensitivity monitors. The backscattered signals from, typically, 400 shots at each wavelength are averaged and normalised with respect to the total energy transmitted at each wavelength as measured by the energy monitors. Background radiation and systematic electrical pick-up are removed by blocking every other laser pulse and subtracting the measured signal. The logarithm of the ratio of the averaged data at each wavelength is used to calculate the SO<sub>2</sub> burden. Route position data is obtained from a distance logger and a software map of the route travelled.

The vehicle is a 9.3 m long, British Leyland Boxer. It is fitted with a 25 kW generator, air conditioning and radio communication equipment. A photograph of the vehicle is shown in Figure 1.

## RESULTS

The system was used for the first time in the field during the CEC exercise at Turbigio in September 1979. It worked successfully in a moving vehicle, measuring SO<sub>2</sub> with a performance close to theoretical predictions. For example, with atmospheric visibility of 5 km a sensitivity of 30 ppb can be obtained at a range of 1 km with a range resolution of 150 m. The laser and data processing system have performed to specification in the moving vehicle. Subsequently the system has been used extensively at various sites in England. Several methods have been developed to display the results. The first is a conventional display along the actual route followed by the vehicle. This is illustrated in Figure 2. It is often more useful, for visualisation and for flux calculations, to project the results on to one or more straight line approximations to the route, usually perpendicular to the local wind direction, Figure 3. A major reason for using a differential lidar system is to obtain the vertical distribution as well as the horizontal spread of the species being measured. Figure 4 shows a typical set of results for SO<sub>2</sub> in power station plumes in which there was considerable wind shear at different heights.

So far the laser pulse has been directed only vertically upwards. Facilities exist for computer controlled scans in elevation and azimuth but these require further mechanical development. No attempts have yet been made to measure NO<sub>2</sub> but the indications are that this will also be achieved at close to theoretical limits.

Comparisons have not yet been made with the Hull infra-red system since that is not yet operational.

#### CONCLUSIONS

Differential lidar has been shown to have a performance close to that predicted theoretically for the measurement of SO<sub>2</sub> in the ultra violet. The system works well in a moving vehicle, a mode of operation which is very effective for surveys of industrial sources.

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Figure 1 CERL Differential Lidar Vehicle

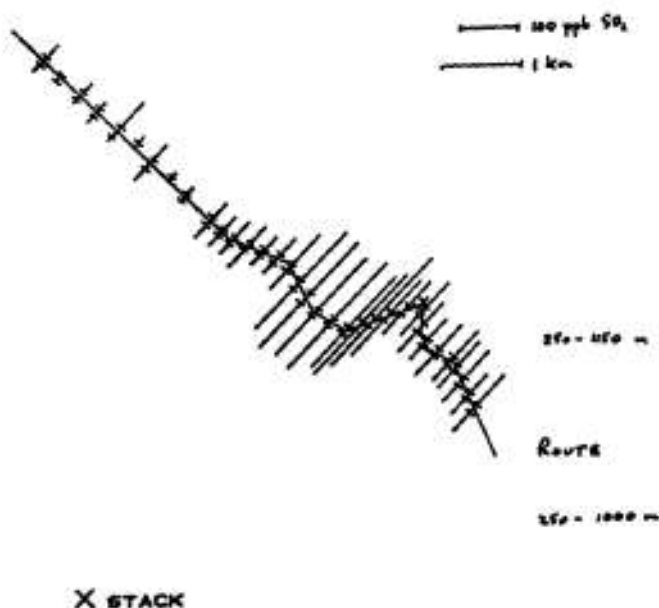


Figure 2 Route map. Mean SO<sub>2</sub> concentration between the heights indicated. Estimated errors shown by cross hatch.

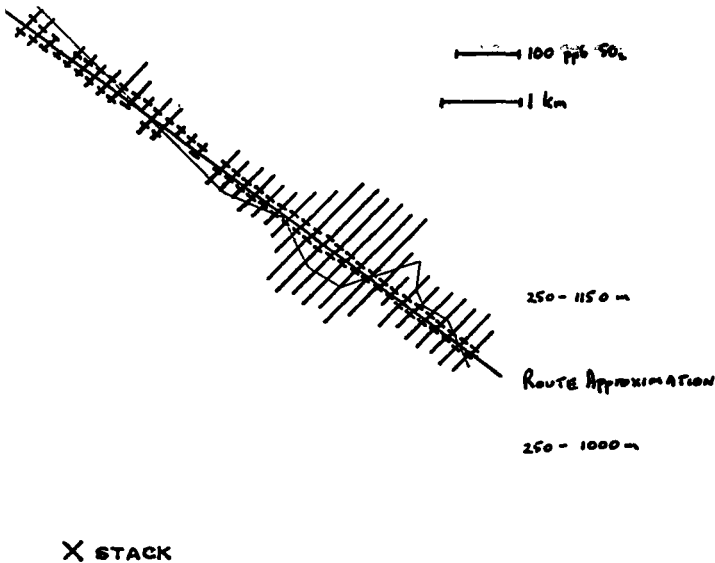


Figure 3 Projection of results on to straight line approximation to route

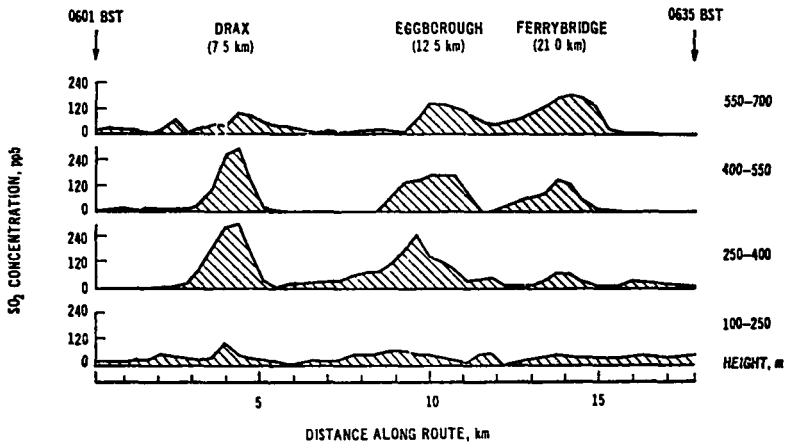


Figure 4 Traverse of plumes from Drax, Eggborough and Ferrybridge power stations. The plumes are at the distances indicated from their respective stacks.

Contractor : Department of Applied Physics, University of Hull  
Contract no. : ENV/321 UK  
Project leaders : E. L. Thomas, B. J. Rye  
Title of project : An infrared DAS system for the remote sensing of air pollution

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Following the work of Schotland on the measurement of vertical water vapour profiles using differential absorption lidar (DIAL) many groups have developed visible and ultraviolet DIAL systems for trace gas analysis. Since relatively few gases absorb in these parts of the spectrum it soon became apparent that more versatile DIAL systems could be built that operated at infrared frequencies. Provided that coherent detection could be used, several other advantages also accrue. These include very much enhanced range capability, output powers within eye safety limits, better all-weather performance and a detection technique that is not background (or therefore night-time) limited. Apart from trace gas analysis, a coherent lidar is also suitable for making, at long range, meteorological measurements such as wind velocity, relative humidity, cloud base height, aerosol, plume and cloud particle characterisation etc. The objective of the work described here was to demonstrate feasibility of an infrared DIAL system using lasers which were readily available. With the benefit of the ensuing experience and analysis, we are now in a position to design an operational system.

The report contains amounts of the work directly funded by the CEC and some results of related work stimulated by the CEC contract funded by other agencies. In the first section the principles of coherent lidar are discussed with reference to DIAL systems. In this application the system is required to estimate the mean irradiance of the backscattered return (or more strictly, the ratio of irradiances returned at different wavelengths from a single range element, or from different ranges at a single wavelength) so the capability of the technique depends on the form of the lidar equation that describes this mean return and on the statistics of the return. For coherent lidar each of these differs in important respects from its counterpart in direct detection lidar. Computer programs have been developed for calculating the normalised return taking into account the effects of speckle and the aberrations of the transmitter and receiver optical systems. The latter can include spherical or on-axis or off-axis paraboloidal mirrors

with or without central observation in a coaxial or bistatic configuration, the contribution of refractive turbulence to the mean return is readily included for the bistatic geometry, and a simplified model developed for the coaxial case led to the conclusion, since confirmed by computer calculations done elsewhere, that in this case the near field return in moderate turbulence can be greater than for free-space (or zero turbulence) transmission.

Further modelling work has been directed at calculating the atmospheric attenuation and backscatter coefficients using data from the McClatchy tapes in the former case and a model developed here for the aerosol structure - in particular the real and the imaginary parts of the refractive index - for the latter. It is shown that aerosol absorption makes the backscattering coefficient a sensitive function of frequency and relative humidity. The sensitivity to humidity changes is such that for example the backscattering coefficient can vary by as much as 50% at a given wavelength for a humidity change from 50 to 95%, the direction of the variation being a function of wavelength and aerosol composition assumed. This effect is naturally of importance for both coherent and direct detection lidar and is a further factor to be taken into account together with interference effects when designing differential absorption experiments in the infrared.

A further result of the analysis of the coherent DIAL technique indicated that the system should be capable of acquiring statistically independent bits of data as rapidly as possible. Even though this was known early in the project, budget constraints forced us to make certain choices of equipment which we would not have made otherwise. For example, the rate of data acquisition was expected to be severely curtailed by our detector bandwidth. In turn, the detector bandwidth made it necessary to restrict the chirp of the  $\text{CO}_2$  laser transmitter. In this way several system parameters were influenced by the choice of detector. For these and similar reasons, some system parameters turned out to be less than ideal. However, the system which was constructed (see Fig. 1) enabled us to



demonstrate feasibility and some encouraging results were obtained.

(i) The laser transmitter: A hybrid, TEA CO<sub>2</sub> laser was chosen as the transmitter. This laser consisted of a pulsed, TEA section in the same resonator as a CW section. The resonator is mounted on a steel girder framework and is mechanically isolated from the pulsed laser section. Since it would have been too expensive to passively stabilised a two metre resonator using invar rods, only active stabilisation has been used. If a simple TEA, CO<sub>2</sub> laser had been used as the transmitter, the laser output frequency would have chirped at a rate of about 7MHz.μsec<sup>-1</sup>. For a 1μsec. pulse, the chirp would represent a significant proportion of the detector bandwidth and would lead to a serious loss of signal. With hybridisation, it has been shown that the chirp has been reduced to about 100kHz. Hybridisation also leads to a longer, more symmetric pulse. The hybrid laser is capable of delivering at least 100mJ per pulse in a TEM<sub>00</sub> mode at a repetition rate of 100Hz. The frequency stability of the hybrid laser is discussed in detail.

(ii) The local oscillator: The local oscillator is a low pressure, CW, CO<sub>2</sub> laser which is capable of delivering over 1 watt on many of the CO<sub>2</sub> laser lines. The resonator is passively stabilised using invar bars and actively stabilised using a piezoelectric cylinder. Since we are only seeking a frequency stability equal to the reciprocal of the pulse duration over a ranging operation, simple peak stabilisation has proved adequate.

In addition to being the local oscillator, this laser is also the frequency standard for the system. The CW section in the hybrid laser is both locked to, and offset from, the local oscillator. Since this laser radar has also been used in range-resolved anemometry experiments, the offset was necessary in order to remove the sign ambiguity when making Doppler measurements.

(iii) The transmitting and receiving antennae: Whilst a transceiver is probably the preferred antenna configuration for a coherent laser radar (particularly CW systems), advantage can be taken of the obscuration in a coaxial telescope system

to reduce the dynamic range of the return signal. Also, it is easier to isolate the transmit and receive channels in a coaxial telescope system. Consequently, it was decided to construct a coaxial telescope system using a 30cm diameter, f/6 parabolic mirror and a 15cm diameter, f/8 parabolic mirror as the primary mirrors in the receiver and transmitter respectively. The mirrors are figured to  $\lambda/10$  in the visible and are front surface aluminised. The telescope, in its present form, points in a fixed direction. The expected efficiency of this coaxial mirror system is discussed in detail

(iv) The data acquisition and processing system: The I.F. signal from the heterodyne receiver is filtered, amplified and digitised using a Biometion 8100 transient digitiser. Since the digitiser is an 8-bit instrument, it has been possible to speed up the cycle time for transferring and pre-processing the data in the PDP 11/10. Arrays of all possible squares and fourth powers of the digitiser output have been stored in firmware. The value of the output is an address in one of the arrays and the contents of the address is either the square or fourth power of the output as required. Under these conditions, the laser and not the computer limits the repetition rate of the equipment.

Data processing is carried out in the PDP 11/10 (28K words) which is backed by an RL-01 hard disc and run under Version 3 of the RT-11 foreground/background operating system. The hard disc was found to be necessary because all the operating system software could not be included on one floppy disc. The system software includes Version 3 of RT-11, Plot 10 packages for the VDV and plotter, extended Fortran IV and some plot-package extensions for handling unformatted data files. The details of the DIAL data acquisition system are discussed

Results: Some typical results are shown in figures 2, 3 and 4. Figure 2 shows the direct detection signal from a topographic target about 2km away. Optimising the direct detection signal was found to be a convenient way of aligning the two

'telescopes.' Figure 3 is the heterodyne return from the same topographic target. As can be seen, this is a strong signal which has been clipped by the digitiser. Figure 4 is an example of the return signal from the aerosol scattering. The digitiser output has been biased away from zero so as to show the amplitude variation of the signal from the topographic target. The return from behind the topographic target is a measure of the noise signal.

When these preliminary results were obtained they were probably the first observations of aerosol returns using pulsed TEA lasers. Since then a similar system operate at NOAA, Boulder (USA) has routinely acquired returns at ranges in excess of 20km for wind velocity measurements; the S/N of their returns over the initial 2km of range are comparable to our own. Our system has since been augmented by provision of a double TEA laser enabling pulses to be transmitted at two wavelengths simultaneously (or with variable delay) and by improvement of the optical alignment methods. Commissioning the new system is currently in progress.

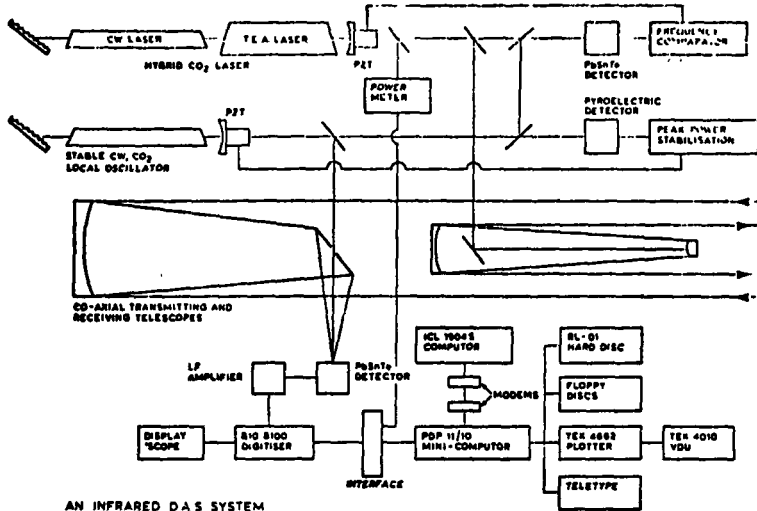


Fig 1 AN INFRARED D.A.S. SYSTEM USING HETERODYNE DETECTION

Fig 2 CAL TARGET - DIRECT DETN.

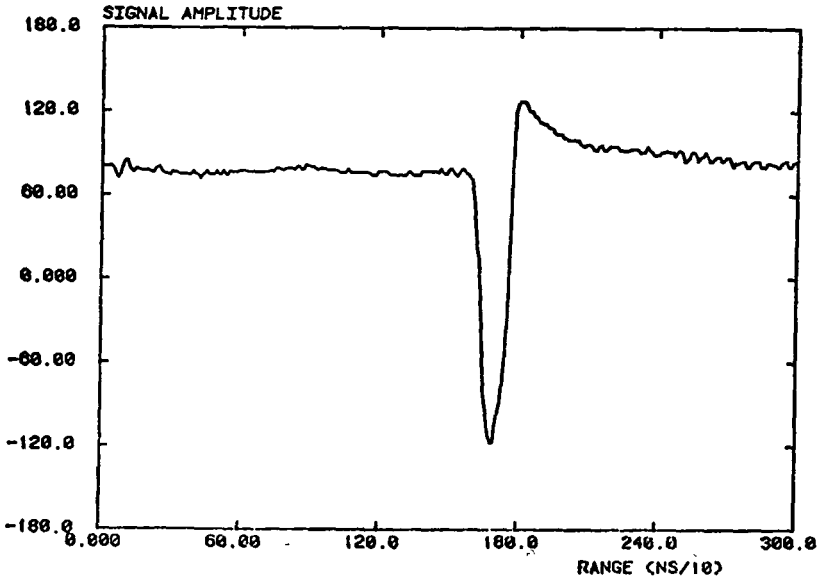


Fig. 3 TOPOGRAPHIC RETURN - SINGLE PULSE - 1.

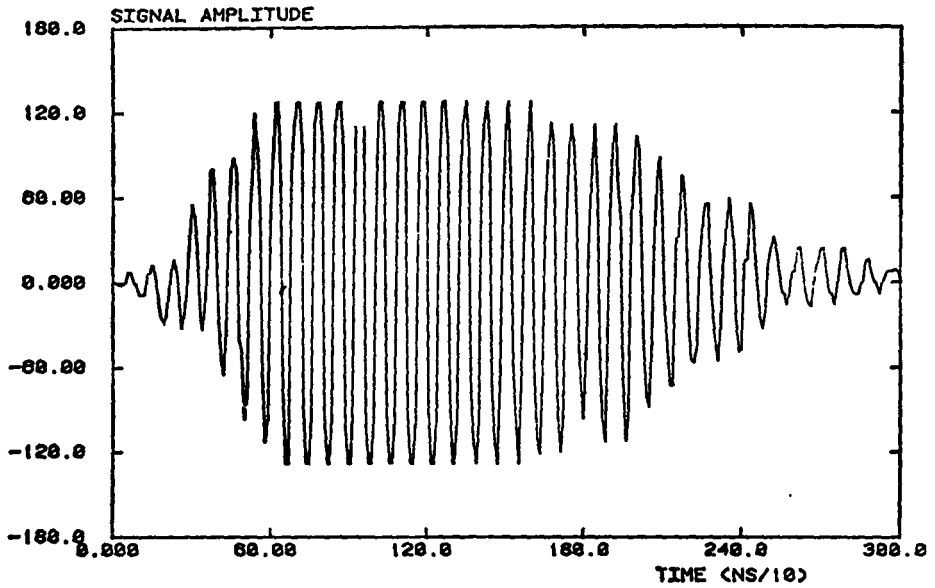
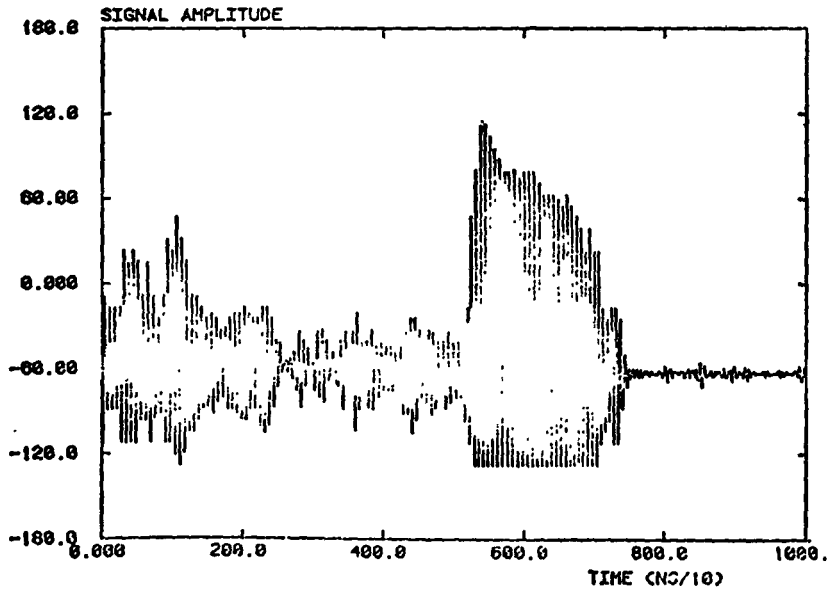


Fig. 4 HET DETN. - D31032.006



**TOPIC 16 : WATER QUALITY**  
**Ecology**

Contractor:                   Universität des Saarlandes  
                                  D-6600 Saarbrücken 11  
Contract n<sup>o</sup>:                   242-77-1 ENV D  
Project Leader:               Prof. Dr. H. Kaltwasser  
Title of Project:              Ecological effects of canalization and pollution on  
                                  microorganisms in river ecosystems

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#### Objectives of the research

Effect of thermal pollution on adaptation of heterotrophic, aerobic bacteria in the River Saar at a cooling water discharge of a power plant.

Mechanisms of adaptation at regionally increased concentrations of heavy metals in photoautotrophic microorganisms.

Distribution and destiny of fecal indicators in river systems studied in order to investigate the suitability of *Clostridium perfringens* as indicator bacterium. Significance of microorganisms within the sediment/water interaction with respect to possible effects of river canalization, studied by determination of concentration and turnover rates of low molecular metabolites, known to lead to methane.

Effect of anaerobic processes on the river water are determined, measuring the concentration and the activity of methane-, hydrogen-, and sulfur -oxidizing bacteria.

#### Materials and methods

At five sampling stations located up- and downstream from a cooling water discharge 750 bacterial strains were isolated. These strains were morphologically and physiologically analysed determining 105 to 134 characters and grouped by means of numerical taxonomy techniques.

Photoautotrophic microorganisms were cultivated under sterile conditions according to (3) and heavy metals determined by atomic absorption spectroscopy according to (4).

Concentrations of *E. coli* and coliform bacteria in water and sediment were determined using standard methods. The numbers of *Clostridium perfringens* were determined by membrane filtration according to (6) and by the Hungate-technique according to (10). Coliphages were concentrated from river water by adsorption to hydroxylapatite, membrane filtration and elution with beef extract according to (27). The concentration of coliphages was determined by the plaque test, using the double layer technique.

Methanogenesis, desulfurication and methane oxidation were determined according to (17, 19 and 25). The number of sulfide-oxidizing bacteria was determined by

the MPN-method and sulfur oxidation potency was measured according to (8). Concentrations of low molecular metabolites in sediments were determined gas chromatographically after extraction by acid, salt, and ether. Labelled methane and carbon dioxide were determined according to (25).

## Results

A numerical comparison, using the simple matching coefficient and single linkage clustering revealed several distinct groups of bacteria at the five locations near the cooling tower discharge. Some of them coincided with the thermal pollution of the river, while others disappeared and were restored after temperature had decreased again.

The influence of heavy metals on unicellular green algae was studied with a *Chlorella* strain isolated from the polluted River Rossel and *Chlorella fusca* 211-8b (Göttingen). In permanent light Cr caused a decrease in chlorophyll content with Cr (VI) more toxic than Cr (III). The wild strain was more sensitive. It exhibited, however, a limited uptake of the metal. Toxic effects on cell division were only observed with *C. fusca*. Under a light/dark regime, Cr was found to be more toxic for both strains than in permanent light. The trace metal vanadium was found to be inhibitory to cell division of *Chlorella*, the point of impact being an interference with nuclear division. Vanadium was also found to interfere with starch production, chloroplast development and photosynthesis. Vanadate revealed to be less toxic than chromate. The uptake of nitrate and urea was followed with time in presence of Cd, Cr, Ni, Pb, and Zn. In the dark, nitrate uptake turned out to be generally more sensitive to metal toxicity than in the light. Dark inhibition of urea uptake was much lower with the wild strain than with *C. fusca*. The difference was most pronounced in the case of Cr and Zn.

Studying fecal indicators, longitudinal profiles of the entire River Saar did not reveal identical distribution of *Clostridium perfringens* and coliform bacteria. Both organisms occurred at high concentrations indicating a severe fecal pollution. Near the source, no fecal indicators were detected. Downstream the numbers increased constantly, finally reaching a nearly constant level in the water. In the sediments two orders of magnitude more clostridia and coliform bacteria were found than in the water phase. A response to canalization was observed with *C. perfringens*, but only in the sediment.

The feasibility of coliphages as indicator for fecal pollution was studied also. It turned out that coliphages became inactivated very rapidly in water and sediment. For this reason they may be well suitable to indicate immediate pollution. In longitudinal profiles, the concentration of coliphages was clearly affected by canalization. Due to the adsorption of coliphages to particles their concentration was 30 times higher in the sediment than in the water of the canalized part, since



particulate matter settles more rapidly in evenly flowing waters. In the non-canalized part, where sedimentation did not occur to a comparable extent, more coliphages were detected in the water than in the sediment. These water/sediment ratios of coliphage concentrations were independent of the concomitant level of fecal pollution. Correlation studies on *C. perfringens* exhibited similar results which, however, were not as pronounced as those obtained with coliphages.

In addition, the dispersal of fecal indicators was analysed in the narrow surroundings of stations, where sewage water was discharged into the River Saar. In the canalized part most of the indicators introduced into the river were found in the sediment, 150 m downstream of the inflow. In the non-canalized part, however, the sediments were not contaminated to such an extent. Due to the high current velocities most of the fecal organisms rather remained in the water phase.

Experiments on the activity of *C. perfringens* in the sediment revealed that the bacterial hydrogenase was longer active in the anaerobic sediment of the polluted Saar than in those of little well aerated streams. Glucose added at low concentrations to sterile sediment was not fermented by *C. perfringens*, since an increase in typical fermentation products (butyrate, acetate, hydrogen and ethanol) was not observed.

The interrelation between methanogenic and desulfuricating bacteria which was observed in the anaerobic sediments of the impounded River Saar during a period of 18 months, was further investigated in the laboratory. Tracer experiments with labelled immediate precursors of methane (acetate, bicarbonate) revealed that acetate accounts only for about 4 to 13 % of the methane produced. The major part (87-96%) of the produced methane originates from  $\text{CO}_2$  and  $\text{H}_2$ . These tracer experiments supported results obtained during kinetic studies of methanogenesis, in which substrates were added. The turnover rate of acetate in the Saar sediment was about three times slower than in lake sediments where acetate accounted for 70 % of methane formed.

Due to sulfate, which is always found in the Saar sediment, desulfuricating bacteria interfere with the acetate metabolism of methane producers. Per gram of sediment  $5.5 \cdot 10^5$  acetate-utilizing desulfuricants were detected, while acetate-decarboxylating methanogenic bacteria accounted only for 1% of all methanogenic bacteria detected. This inhibition of methanogenic bacteria by sulfate was overcome by the addition of organic substrates. According to these experiments the organic substrates available in the river were limiting and thus responsible for the observed competition. This was particularly true for deeper layers of the sediment.

The close interrelation between methanogenic and heterotrophic bacteria was simulated by mixing pure cultures of *Methanobacterium spec.* and cellobiose-degrading *Bifidobacterium* and *Fusobacterium* strains. The main product of the hetero-

trophic strains was lactate, but in syntrophy with the  $H_2$ -utilizing methanogenic bacteria cellobiose was further fermented to acetate. Concomitantly methane was produced.

The numbers of thiobacilli were determined in longitudinal profiles in water and sediment of the Saar. At the sediment surface two to five orders of magnitude more thiobacilli were detected than in the free water. In the canalized part more thiobacilli were found at the sediment surface than in the non-canalized parts. The high concentration of thiobacilli observed at the sediment surface was due to the presence of sulfide. In water almost no sulfide was detected whereas sulfide production in the sediment was high, particularly in the canalized river part. The activity of thiobacilli, assessed by sulfur oxidation potency, coincided well with their numbers.

Methane oxidation turned out to depend on substrate concentrations. In the running water the methane concentration was low. At the sediment surface the number of methane- and hydrogen-oxidizing bacteria was about two orders of magnitude higher and the methane concentration was three to four orders of magnitude higher than in the free water.

In addition, methane and sulfide oxidation was studied in the oligosaprobic Brook Netzbach. At one sampling station, gas from a subterranean coal vein escapes from the bottom into the brook, consisting mainly of methane and hydrogen sulfide. Increased concentrations of sulfide in water and sediments were only detected at the point of ebullition and 30 m downstream thereof. Methane was present at high concentrations at all stations below the ebullition. In the sediment maximum numbers of thiobacilli and of methane-oxidizing bacteria were detected at the station of gas ebullition. In the water, the numbers of these bacteria increased due to a wash-out from the sediments. The oxidation potency of thiobacilli corresponded well with their numbers as determined in water and sediment. Methane oxidation was extremely low in the water of the unpolluted brook, but high rates of methane oxidation were determined at the surface of the sediment, indicating that the surface of sediments were a more suitable environment for methane-oxidizing bacteria.

### Conclusions

Numerical taxonomy was shown to be an efficient tool in characterizing bacterial populations; this techniques turned out to be well applicable in limnological studies. Comparative studies with two *Chlorella* strains indicated that the wild strain had adapted to the high concentration of heavy metals and urea present in its natural environment. *Clostridium perfringens* turned out to be well suitable to indicate fecal pollution, especially in sediments.

In canalized parts of the river, anaerobic microorganisms were shown to exhibit

high metabolic activities in the sediment. Due to the presence of high sulfate concentrations, desulfuricating bacteria oxidize fermentation products, thus increasing decomposition of organic matter. Sulfate-reducing bacteria, which simultaneously produce hydrogen sulfide, compete with methane producers for available acetate and hydrogen. The fermentation products methane, hydrogen, and hydrogen sulfide, thus formed in the sediments, are oxidized by aerobic microorganisms in the water. In the river water this oxidation proceeded at low rates and did not significantly affect the oxygen budget of the river. At the surface of the sediment, however, the numbers and the metabolic activities of these organisms were two to five orders of magnitude higher. In this region these bacteria contribute markedly to the disappearance of oxygen from the river.

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Contractor : U.E.R. d'ÉCOLOGIE, Université de METZ, FRANCE

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Project Leader : J.C. PIHAN

Title of project : Freshwater ecosystem studies and ecotoxicology

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I. DEFINITION OF THE LEVEL OF HEAVY METAL'S CONTAMINATION IN AN AQUATIC SYSTEM USING MOLLUSCS AS BIOINDICATORS
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OBJECTIVE OF THE RESEARCH

The main object of this study is :

- . to realize a map making of the level of contamination in metal especially Zinc, Lead and Cadmium in an aquatic system, taking account molluscs as bioindicators,
- . to develop an in situ intoxication experiment with first bilvalves like test-organisms : *Anadonta cygnea* L. and *Dreissena polymorpha* Pallas.

RESULTS

1. Map-making

For the first year, the river MOSELLE has been chosen as a river-test because of an important metallic pollution brought by a siderurgical industrialization which is especially developed. We have monthly sampled from march to october 1979 several points distributed both along the river MOSELLE and its affluents.

For heavy metal's contamination, the river MOSELLE is separated in two distinctive areas :

- . an upstream zone of the ORNE (left tributary) moderately or not polluted
- . a downstream zone strongly contaminated.

For the physico-chemical parameters, the impact of the ORNE is particularly clear with the suspended matters and still important at the frontier.

ORNE  
↓

	upstream	downstream
Zinc	582-1723	3983-7116
Plomb	150-350	1430-3100

Evolution of heavy metal's concentration in suspended matters (µg/kg dry weight)

The analysis of the dissolved salts doesn't show the same characteristic impact.

The repartition of the molluscs is disturbed by this metallic pollution. The snail *Bythinia tentaculata* persists in all the studied areas in spite of the strong contamination of the downstream zone. *Viviparus viviparus* is going out after the junction of the ORNE and reappears about fifteen kilometers downstream. The bivalve *Dreissena polymorpha* P. seems to be absent of the downstream zone.

The values of Lead and Zinc in the molluscs fluctuate like the concentrations in the suspended matters. The Zinc concentration is stronger in soft tissues than in the shells. In soft tissues, the metal concentration changes with the different species that we have classed like this :

Unionids > *Viviparus* > *D. polymorpha* and *B. tentaculata*

In the strongly contaminated area, shells have the highest Lead concentrations when it's in the opposite direction in an upstream zone.

For soft tissues, we have the following classification :

Unionids > *D. polymorpha* > Snails

These results show that we can find the most important heavy metals concentration in the molluscs living in the very industrialized area ; that's agree the choice of molluscs like bioindicators.

## 2. In situ intoxication experiments

A precise investigation of the polluted effluents and their importance, allowsto distinguish three areas along the river ORNE, main spring of metallic pollution in the MOSELLE :

• an upstream zone slightly contaminated which is located outside of the siderurgical area. The heavy metal concentrations are at the level of the limits of analytical detection : total Zinc (dissolved Zn + Particulate Zn) = 20 µg/l, total Lead 8 µg/l.

• a downstream zone separated in two sectors :

*a contaminated sector with respectively 100 µg/l of total Zinc and 20 µg/l of total Lead*

*a downstream sector strongly polluted with concentration of 2 mg/l for Zinc and 300 µg/l for Lead.*

In these three areas, specimen of *D. polymorpha* and *A. cygnea* are immerged. We make a survey of ten weeks with analysis of heavy metals (Zinc and Lead only) in soft tissues and shells of the two species and in organs of *A. cygnea*.

Concerning the biological parameters of the test-organisms, we have not observed a modification in the relation length-dry-weight of soft tissues for *A. cygnea*. On the other hand, we record loss of weight for all the lots of *D. polymorpha* except the control. However, for all the experimented organisms, we have good statistical correlations between the different biometric parameters.

The results that we have obtained present a great variability, however this observation is attenuated for *D. polymorpha* which experimented organisms are more abundant.

We have not found a significative correlation between size and metal concentrations, only an inverse relation.

For the molluscs immersed in the different zones of the river ORNE, the values of Zinc and Lead well indicate the level of heavy metal's contamination of the stream. In polluted area, the fixation of metal is generally a fast processus which goes to a maximum level at the end of the exposure time. This evolution is faster with *D. polymorpha*.

	WEST *	soft tissues	shell	soft tissues	shell	WEST	soft tissues	shell	soft tissues	shell	
control area	500	480	12	160	50	100	6.7	1.7	6.6	6.6	
polluted area	6500	1000	45	430	120	2050	10.5	10.3	30	25	
strongly polluted area	74000	2000	300	5500	700	27000	150	60	950	300	
		A. cygnea		D. polymorpha			A. cygnea		D. polymorpha		
		← ZINC →					← LEAD →				

\* The heavy metal's concentration suspended matter are more characteristic of the metallic contamination level than the dissolved salt values which are too slight especially for control point.

The metal concentrations are stronger in soft tissues, especially for *A. cygnea*. The distribution of Zinc in different organs of these species is not modified along the different stations. The stronger concentrations are in the gills which represent more than 50 % of total Zinc in soft tissues. So we obtain the following classification :

gills > mantle - viscera > muscle > shell

For Lead, we observe the same phenomen for what concern the organ distribution ; however, the shell represents the stronger percentage. The analysis of metal concentration gives the following classification :

gills > mantle - viscera > shell > muscle



## II. ECOTOXICOLOGY

### OBJECTIVE OF THE RESEARCH

The water's quality evaluation by study of bioindicators does not take into account the biological and physiological state of organisms. On the other hand, the toxicological tests are generally acute tests (DL 50 or DL 100 - 48 or 96 hours) in static or dynamic conditions. In all cases, results cannot be transposed directly to natural conditions.

For these reasons, after acute intoxications, we have carried on an experimental research about toxicity of heavy metals (Cd, Zn, Pb) in semi-chronical and chronical conditions upon two organisms of an experimental food chain of Crustacea : *Daphnia magna* and a fish *Leucaspis delineatus*, first for each organism alone, then on all the food chain including producers (*Chlorella vulgaris*).

The results allow us to propose a maximal concentration of micro-pollutant compatible with all the biological cycle of organisms, with regard on major parameters : concentration, lasting, t°, in well defined conditions (water quality, hardness, dissolved oxygen, photoperiod).

### MATERIALS AND METHODS

Each organism is studied by mean of different parameters

<u>Algae</u>	. growth rate
<i>Chlorella</i>	. concentration of micropollutant
<i>vulgaris</i>	. photosynthetic pigments
<u>Crustacea</u>	. growth, birth and mortality
<i>Daphnia magna</i>	. behaviour (swimming)
	. concentration of micropollutant
	. histological investigations
	. respiratory activity (VO <sub>2</sub> )
<u>Fish</u>	. behaviour
<i>Leucaspis</i>	. integument aspect
<i>delineatus</i> or	. hemorrhages
<i>Brachydanio rerio</i>	. histological investigations (optic, electronic, scanning, X microprob)
	. respiratory activity (VO <sub>2</sub> )

## RESULTS

### a) Isolated organisms

#### a1) Daphnia magna

We notice in all case a delay in the mecanism of birth. If cadmium is present only during few days (from day 0 to day 3, for exemple) at 10 ppb and 20°C, we observed a greater longevity and birthrate of Daphnia

If cadmium is present at 20 ppb, 20°C during more time or during all life long of Daphnia, we observe toxicological effects : increase death rate, decrease of birth rate and total sterility after 36 days. This is reversible if Daphnia is transfered in control water (without cadmium). Young Daphnias are released by mothers 6 ou 7 days after transfer.

Histological studies confirm the impact od Cadmium on ovary, ovogenesis and vitellogenesis. Moreover, Cadmium has an ovoid action. Some histological disturbances are observed in digestive-trac. An impact on Ecdysis is also discussed.

Il all cases, and with various heavy metals (Cd - Zn - Pb), an elevation of temperature increases the toxicological effects, Cadmium beeing more toxic than Zinc.

#### a2) Fishes (*Leucaspis delineatus* and *Brachydanio rerio*)

Acute intoxications of *Leucaspis delineatus* and *Brachydanio rerio* by Cd, Zn, Pb, have different impacts.

The first target-organ is the gill. We can observe an increase of mucus secretion and an invasion of macrophageous cells. The gill epithelium may, by side, come unstuck so we can see an exfoliation leading to a real abrasion of the secondary lamellae. The gill epithelium may also present an important hyperplasia so that the secondary lamellaes begin completely joined togoether. Some hemorrhagic areas and necrotic cells can also be seen.

The olfactory organ is also rapidly affected by metal intoxication, with comparative reaction to the gill process in invasion of macro-

phageous cells, increase of mucus secretion, necrotic cells in the olfactory epithelium leading to the dissociation of the lamellae.

The integument reacts by the same way : secretion of mucus and haemorrhagic process.

The kidney under massive intoxications shows a necrotic aspect of the distal part of the nephron.

Chronic intoxications with Cadmium have histological impacts :

- . on the female gonad : treated fishes showing abnormal oocytes, younger ones having a too vacuolised cytoplasm, older ones are getting a perturbed vitellogenesis so their cytoplasm is inequally loaded with vitellus,

- . the testis shows abnormal concretions between the male cells.

This amorphous concretions can also be seen in the spleen, in the kidney, pancreatic tissue and digestive tract.

So histology reveals us that acute and chronic intoxications act at different levels and allows to determinate the nature and the importance of the injury produced.

#### b) Organisms replaced in an experimental food chain

Experiments are carried out at five temperatures (10,15,19,24,28°C) and three concentrations 10,15 and 20 ppb of Cadmium. It appears very quickly that 10°C are not sufficient for algae-growth. In an other way, 28°C are much higher for Daphnia which are also killed by 20 ppb. So chronological experiments concern only 15°, 19° and 24°C in presence of 10 and 15 ppb of Cadmium.

##### b1) Algae

At 15° and 19°C, 10ppb of Cadmium decreases growth rate of the culture. At 24° and 28°C, temperature is predominant and unfavourable

in comparaisou to Cadmium.

b2) Daphnia

- . The longevity is maximal at 15°C, then decreases with the elevation of temperature.
- . Maturity is obtained more quickly when the temperature increases.
- . Birthrate is low at 15°C and maximal at 20°C.
- . The respiratory activity is also higher at 19°-20°C.
- . For these reasons, 20°C is considered as the optimal temperature for experiments on the food chain.
- . The presence of Cadmium in water has a greater direct impact that an indirect one by way food.
- . There is a synergist action of Cadmium and temperature. For example, at 24°C, a concentration of Cadmium higher than 10 ppb stops the reproductive activity.
- . Cadmium has an inhibitive effect on respiratory activity, effect which increases with the temperature.
- . Bioaccumulation of Cadmium in Daphnia has been observed and evaluated by atomic absorption.

b3) Fish

At the concentrations of Cadmium that we have choosen (10 and 15 ppb) the respiratory activity of fish is not disturbed. The temperature is predominant with a  $Q_{10}$  of 2 between 16°C and 26°C.

CONCLUSIONS AND COMMENTS

The study of Cadmium toxic effets upon all the biological cycle of the more sensitive organisms of an experimental food chain (in this case : *Daphnia magna*) permits us to propose the threshold concentration of a pollutant that respects the ecosystem's equilibrium.

For example, the threshold concentration of Cadmium in aquatic ecosystem may be very low : note more than 1 or 2 ppb in water of 20°C to 24°C, containing 50mg/l CaCO<sub>3</sub> ; for all the organisms which have the same sensibility than that of Daphnia choosed as the representative organism of zooplancton. This concentration is lower than that holded in the directive of C.E.E. for freshwater fisheries (10 to 50 ppb of Cadmium) only, experiments carried upon all the biological cycle may give a relative good information upon toxicological effects of micropollutants in field conditions. We propose to continue on this way of research with mixed micropollutants (Cd + Zn, Cd + Zn + Pb, for example) and to determine the locus and the way of toxicity in various tissues and more species of freshwater ecosystem (Mollusca).

In the future, the norms defining dissolved Cadmium concentrations in water would take into account the ability for the test organisms to perform their principal vital fonctions, but not only their survival. In the case of Daphnia, it would be nutrition, molt and reproduction.

### III. IN VITRO ECOTOXICOLOGY OF SILICON

The silicon ecotoxicology is an unexplored subject, nevertheless same authors work on toxicology of silica, silicon determination, and its physiological role in some organisms. Recently CARLISLE (1972) and SCHWARTZ (1972) found the essentially of silicon for warm-blooded animals.

The paper reports preliminary study and in particular a wide bibliographic review on the biological, physiological and biochemical aspects of silicon. Our results turn on the analytic determination of silicon and on the silicon ecotoxicity with freshwater species belonging to various trophic levels.

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Contractor : Freshwater Biological Association/ Natural Environment

Research Council, Cumbria

Contract n<sup>o</sup> 278-77-1 ENV UK

Project leader : Dr J. Gwynfryn Jones

Title of project : Denitrification in freshwaters

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#### Objective of the research

1. To determine the fate of nitrate in selected rivers.
2. To assess the effect of various effluents on the removal of nitrate in river water.
3. To determine the main path of nitrate removal.
4. To attempt to predict nitrate loss in a river from selected laboratory measurements.

#### Materials and methods

**Sampling:** Water samples were taken with Friedinger and Collins water bottles. Where appropriate, sediment samples were taken with a Jenkin surface mud corer. Dissolved O<sub>2</sub> and temperature were measured in situ with a combination oxygen electrode/thermistor. River flow was measured at the effluent discharge points and at measured intervals downstream.

**Analytical chemistry:** Nitrate, nitrite and ammonium nitrogen were determined according to the methods described by Jones, Downes & Talling (1980) and Jones & Simon (1981). Gas chromatographic analysis of N<sub>2</sub> and N<sub>2</sub>O was done according to Jones, Downes & Talling (1980).

**Enumeration of nitrate-reducing bacteria:** The numbers of bacteria capable of reducing nitrate to nitrite, ammonium and nitrogen gas were estimated by an MPN procedure on the medium described by Horsley (1979).

**Measurement of denitrification and nitrification:** Denitrification was measured by the acetylene inhibition technique with C<sub>2</sub>H<sub>2</sub> at a final concentration of 1% in the gas phase. The N<sub>2</sub>O which accumulated was determined gas chromatographically. The final quantity of N<sub>2</sub>O produced was calculated after corrections for its solubility under the conditions of the experiment. Nitrification was measured as change in ammonium, nitrate and oxygen concentration in the presence and absence of the specific inhibitor 2-chloro-6-(trichloromethyl) pyridine (N-Serve, Dow Chemical Co) at a final concentration of 5 mg l<sup>-1</sup>.

#### Results

These are presented in approximately the order in which the project developed.

The first phase of the project was an examination of the River Kent,

Cumbria, with a view to determining the effect of various effluents on the process of denitrification within the river. The effluents were varied and included those from a milk products factory, landfill leachates (containing heavy metals), a thermal output and a sewage treatment unit receiving mixed domestic and industrial waste. The rate of nitrate removed downstream of selected sites was determined as were basic chemical and microbiological features of the river. Unfortunately improvement work by the local water authority of various key sites, and plans to divert the major sewage effluent to another discharge point, hampered further work.

Several rivers in the north of England were then examined in an attempt to relate observed rates of nitrate removal in the field and laboratory measurements of denitrification by samples of plankton and benthos. No clear relationships emerged between loss of nitrate in the field and temperature, nitrate concentration,  $N_2O$  accumulation (acetylene inhibition) and observed  $NO_3^-$  uptake in isolated samples. Clearly more detailed studies of individual sites will be required if a reasonable understanding of in situ rates of nitrate reduction is to be obtained (see Conclusions and Comments).

During this phase of the programme concern was expressed about several river sites where high levels of nitrate and nitrite had been recorded. A microbiological investigation of this phenomenon was undertaken. Studies with mixed populations of naturally occurring denitrifying bacteria (eg pseudomonads) and fermentative bacteria (eg coliforms) revealed that the latter inhibited and/or diverted the nitrate metabolism of the former so that the major end product was nitrite rather than  $N_2$  gas. The population density of coliforms required to achieve this antagonism was relatively low. Examination of selected rivers in England which received effluents containing relatively high concentrations of nitrate and nitrite suggested that, at most sites, the ratio of pseudomonads to fermentative bacteria was sufficiently low to allow such an antagonism to occur. Although nitrite accumulated in the effluents there was no evidence of generation of nitrite within the river systems. Addition of cheap carbon sources, particularly methanol, to the effluents, resulted in a reduction in the nitrate and nitrite concentrations.

#### Conclusions and Comments

1. A study of the effect of various effluents on nitrogen transformations



in the R. Kent (Cumbria) was undertaken but had to be discontinued because of changes in effluent discharges and alterations to the river bed at several sites.

2. A wider study of several northern rivers investigated the possibility of predicting nitrate removal within river reaches from laboratory measurements of microbial nitrogen transformations. No significant relationship between the variables was obtained, and clearly field and/or laboratory methods require refinement before rates of river denitrification can be predicted on a national scale. Mathematical relationships derived at one site are unlikely to be applicable to other rivers. If detailed information on variability in nitrate concentration at a potential abstracting point is required then measurements of point source dilution and nitrate metabolism must be made upstream of each site. Dilution might be measured with suitable fluorochromes and in situ denitrification by the application of  $^{15}\text{N}$  or  $^{13}\text{N}$  isotopes as nitrate.
3. An experimental investigation of denitrification by mixed cultures of bacteria revealed that, in the presence of fermenters such as E. coli, the nitrate dissimilation of a pseudomonad may be diverted or inhibited so that nitrite rather than  $\text{N}_2$  gas is the major end product. This antagonism appeared to be fairly widespread and may be of significance in effluents or rivers receiving high concentrations of nitrate.
4. A number of river sites, known to contain relatively high concentrations of nitrite, were then examined to determine whether the relative numbers of interacting species were within the range likely to create a similar antagonistic response. Higher proportions of fermenters were found in treatment unit effluents which served large communities. Addition of cheap carbon sources, particularly methanol, usually caused a reduction in nitrate concentration. Such further treatment of effluents might be considered if the release of nitrate and nitrite into water courses is to be reduced. The accumulation of nitrite appeared to be associated with activity within the effluent treatment plant; there was no evidence for the generation of significant quantities of nitrite within the rivers themselves.
5. Other research at this laboratory has demonstrated that there is insufficient information to determine
  - a) The sources and rates of production of ammonia.

b) The importance of nitrification in regenerating nitrate in freshwater ecosystems.

Until these reactions are studied in detail our understanding of nitrogen cycling in rivers will be incomplete.

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#### Oral Communications:

The results of this research have been reported to Meetings of the EEC Freshwater Contact Group, DoE Inland Waters Biological Review Committee, the Society for General Microbiology and at several university seminars.

**Contractor:** The Water Research Centre, UK  
**Contract No:** ENV-399-UK  
**Project Leaders:** Dr S C Warren and Mr J A Cole  
**Title of Project:** Research into the effects of Effluent Recharge on  
Groundwater (Phase 1)

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**1. Objectives of the Research**

The Water Research Centre has been involved in a comprehensive investigation into the effects of sewage effluent recharge on the major aquifers in the UK<sup>(1)</sup>. The Whitchurch Sewage Treatment Works (Figure 1) have been studied by the Southern Water Authority, in collaboration with the Centre, since 1978.

The effluent recharge work at Whitchurch up to 1980 had been principally concerned with the processes and effects of inorganic pollution in groundwater. The present research concentrated on the organic aspects of groundwater pollution. The main objectives were:

- 1) To relate the organic pollution to the inorganic pollution plume.
- 2) To determine the chemical character of the organic pollution.
- 3) To determine the fate of individual organic species in the effluent.

**2. Materials and Methods**

Fourteen exploratory boreholes had been drilled within the study area by the Water Authority between July 1978 and May 1980 (1 to 14, Figure 1). The details of these boreholes vary and have been tabulated in the Interim Report of this work<sup>(2)</sup>. All were drilled by the percussion method and each, except Nos. 7 and 8, were cored at 1 m depth intervals to obtain pore-water samples for analysis. Boreholes 1 and 9 were back-filled on completion but holes 2, 4, 5, 6 and 11 to 14 were left open for sample points. These open holes were supported by 150 mm ID uPVC well-casing perforated below the water table. The completion of exploratory boreholes 3, 7, 8 and 10 was rather more complex; specially designed WRC groundwater samplers<sup>(3)</sup> were installed on these boreholes at selected depths and then the boreholes back-filled to the surface. The samplers had uPVC bodies and sampling tubes of nylon.

The arrangement up to 1980 thus gave a range of sampling points to define the Whitchurch pollution plume but these sampling points all

pre-supposed the target of the sampling was inorganic pollution. Each point contained material, uPVC, ABS or Nylon, capable of introducing organic leachates to a sample. Four extra boreholes were designed, therefore, to be organically-inert to provide suitable sampling points for the trace-organic investigation. Boreholes 19 and 20 followed the open exploration hole design but were lined with steel casing. Boreholes 17 and 18 were each cored at 2 m intervals then, on completion, equipped with three organically-inert WRC samplers with stainless steel bodies and PTFE sampling tubes.

Southern Water Authority drilled boreholes 15 and 16 to the open exploration borehole design to improve the definition of the inorganic pollution plume.

The organic analytical programme was divided into two parts: validation and groundwater analysis. The presence and overall levels of organics were measured as total organic carbon (TOC). The characterisation of the pollution was by gas liquid chromatography (GLC-FID) and the identification of the pollutants by mass spectrometry (GCMS).

Sampling techniques were investigated by laboratory experiments on potential organic leaching or adsorption in standard WRC samplers, Inert WRC samplers, Nylon and PTFE tubes and a uPVC well-casing. The laboratory work was supported by a comparison of samples from various types of sample points on site. The main analytical technique was TOC analysis backed-up by GLC-FID of selected samples.

The GLC-FID analyses involve an early extraction/concentration stage where the organics are adsorbed onto XAD-2 resin then extracted by diethyl ether. The ether eluate is then freeze-dried and concentrated to a 250 µl residue by evaporation. This process is clearly open to errors through losses at several stages and had to be validated. The validation procedure involved the GLC-FID analyses of 'spiked' water samples containing standard organic compounds at concentrations of 10 µgms/l. The range of spiking compounds was chosen by reference to a few existing GCMS analyses of Whitchurch effluent<sup>(4)</sup>.

The validation experiments pointed to weaknesses in the early techniques and led to improvements in procedure, particularly in the concentration by

vaporation of the ether eluate for GLC-FID analysis.

The field analytical programme comprised:

- 1) At least 1 TOC analysis from every sampling point.
- 2) A GLC-FID trace from: Each inert sampling point  
Groundwater from boreholes 14 and 15  
Sewage effluent  
Unpolluted groundwater (Whitchurch public supply well).
- 3) A GCMS analysis of: Highly polluted groundwater (No. 17 Sampler 3)  
Slightly polluted groundwater (No. 14).

Each sample used for a GLC-FID trace was spiked with dimethylnaphthalene (10 ug/l) as an internal standard.

### Results

The inorganic (chloride) pollution is quite limited in area (Figure 1). The new boreholes confirm that the pollution is also restricted in depth; ammoniacal pollution in borehole 17, for example, is restricted to the upper 40 m of the Chalk aquifer. The regional organic survey (TOC) shows that the organic pollution plume is coincident with the inorganic one but the diminution of pollution with distance from the source appears greater in the organic than inorganic plume.

The highly-polluted water immediately below the recharge site (Borehole 6 and Borehole 17 Sampler 3) contains numerous organic compounds at significant levels (see (1), Figure 6). These levels fall-off rapidly away from the recharge site. Slightly-polluted water from both borehole 20 and 14 shows similar GLC-FID characteristics; no organic compound has survived in significant concentrations and the 'spiky' profile of the highly-polluted samples is totally suppressed.

Using the internal standard, dimethylnaphthalene, as a reference, no discrete compound of higher boiling point can be distinguished. Two compounds (still to be identified) can be distinguished on the GLC-FID traces of 19 and 14 as well as 20. The traces of several samples also show a distinct low-boiling point fraction immediately following the solvent front. This

~~fraction has to be identified and its significance evaluated.~~

#### Conclusions and Additional Comments

The sampling and analytical procedures used in this investigation have been evaluated. Sampling from WRC standard samplers is unacceptable because of high levels of organics leached from Nylon tubes. All other procedures appear satisfactory.

The highly polluted groundwater beneath the effluent recharge site is very similar in chemical character to raw effluent although infiltration has reduced the TOC levels from above 100 to less than 10 mg/l. After 400 m of lateral travel in the Chalk aquifer the TOC level is reduced to below 1 mg/l and all compounds are almost entirely removed. A few persistent low boiling point compounds remain but these have still to be identified.

The greater part of the work through 1980 and 81 has been devoted to the validation experiments. These are now completed and it is recommended that the field experiments are continued to take advantage of this work.

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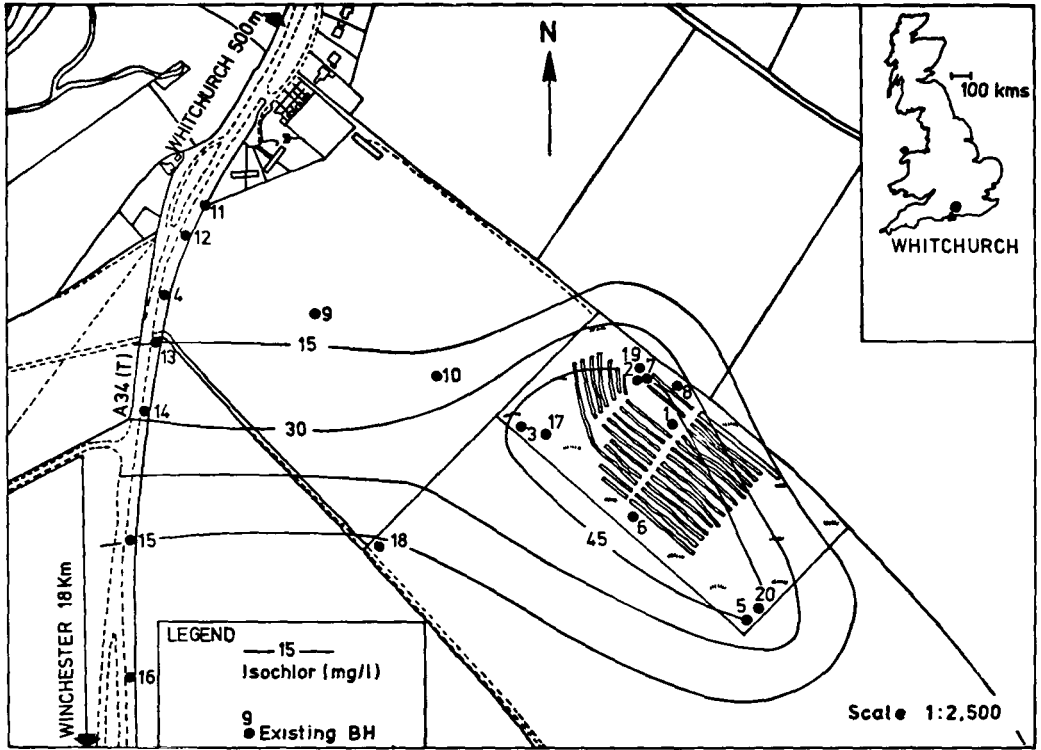


FIGURE 1. Whitchurch Sewage Treatment Works : Location Map with Groundwater Isochlors

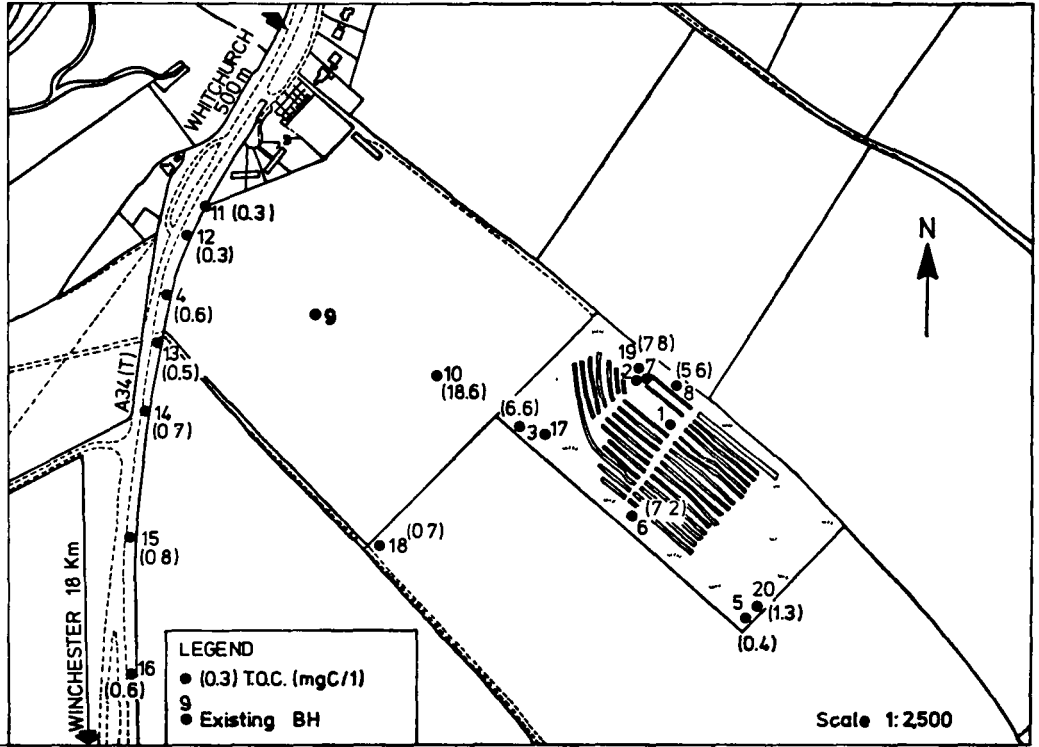


FIGURE 2. Whitchurch Sewage Treatment Works : Total Organic Carbon (TOC) in Groundwater



**Contractor:** Natural Environment Research Council , Wallingford  
**Contract No:** ENV 400 - 80 UK  
**Project leader:** Dr P G Whitehead  
**Title of project:** Operational Management of Water Quality in River Systems

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### OBJECTIVES OF RESEARCH

The research described in this report covers the first year of a three year research programme which is based on collaborative projects between the Institute of Hydrology and the Anglian and Thames Water Authorities. Partial financial support for the work is being provided by the Commission of the European Communities (CEC) through its Environment Research Programme.

The overall objective of the project is to develop integrated mathematical models of flow and water quality which can be applied to a spectrum of problems arising in the management of water quality. Financial support approval was obtained from the CEC on 1st January 1980.

The programme of research is centred on the application of mathematical models of flow and quality to water quality management problems which have arisen within the areas of the Thames and Anglian Water Authorities; these studies and their objectives are summarized here under the headings of 'the Anglian Study' and 'the Thames Study'. Progress achieved on both studies during the past year is summarized, followed by a description of proposed future work. A detailed description of the work carried out during the past year is given in the first annual progress report (Whitehead et al, 1981).

#### The Anglian Study

The collaborative study with the Anglian Water Authority is concerned with the short-term operational management of water quality in river systems. There is a major requirement by pollution inspectors and operational managers for information on the present condition of river systems and on likely future changes in water quality. Management must be able to respond rapidly to emergency situations in order to protect and conserve river water quality and maintain adequate water supplies for public use.

The application of particular interest in this study is the monitoring and

control of a critical section of the Bedford Ouse in South Eastern England. Effluent from the new city of Milton Keynes enters the river some 55 kilometres upstream of a major abstraction point at Bedford. An on-line water quality monitoring network has been established along the river and a telemetry scheme transmits data to a central control station. In order to improve data management and interpretation and to provide forecasts of water quality, a mini-computer is to be acquired and a suite of computer programmes developed which will enable the mini-computer to perform the following functions:

- (a) scan the automatic water quality monitoring stations and flow gauges at frequent intervals;
- (b) transform the basic data obtained from scanning to relevant physical and chemical parameters;
- (c) reduce and store the data and print summaries for management purposes;
- (d) calibrate water quality sensors and provide warning of instrument failure or drift;
- (e) provide alarm signalling in the event of poor water quality conditions;
- (f) forecast water quality at key locations and calculate travel times and measures of dispersion.

#### The Thames Study

In the Thames study the objective is to develop models for predicting water quality that take full account of how concentrations of pollutants vary with river flow, and to use these models to investigate alternative designs for controlling water quality in the Thames basin. The design problem arises primarily from the fact that nitrate levels in river waters are tending to exceed WHO and CEC limits for potable water which means that alternative strategies for keeping nitrate levels in potable water below these limits must be explored. Allied to this problem is the choice of a site for a new resource to meet the increasing demand for water in the London area.

Nitrate levels in the Thames have increased steadily over the past 50 years. The main causes of the upward trend are the extensive use of nitrogen based fertilisers in agriculture and the discharge of effluent from increased population and industrial activity. A number of options, however, are available to manage

the nitrate problem. These include the denitrification of abstracted water, the reduction of nitrates in effluents, the expansion of reservoir storage, the improvement of the mixing characteristics of reservoirs and the development of new resources such as groundwater schemes. The effectiveness and costs of all these options vary considerably and an integral part of the Thames study is the use of a water quality model of the TWA water resource system to select an optimal mix of alternatives for overcoming the nitrate problem.

#### RESEARCH PROGRESS

In a technical annex to the CEC contract, a programme of work for the first year was specified as follows:

- (i) Data Collection - the first task will be to assemble the relevant flow and water quality data already available. Further sampling programmes will be designed and initiated in collaboration with the Thames and Anglian Water Authorities. In the case of the Anglian Study data will be available from continuous water quality monitors located on the river system. Tracer experiments will be conducted to assess time of travel and dispersion characteristics.
- (ii) Hydrological Model Development - a multi-reach, multi-tributary hydrological model will be developed and applied to both the Thames and the Anglian River Systems. While the model structure will be essentially the same for both applications the time scales will be different. In the case of the Thames study a daily simulation model will be required whilst for the Anglian study short-term forecasts are required with the time scale of the order of hours.
- (iii) Water Quality Model Development - the hydrological models will be expanded to include important water quality determinands such as nitrate for the Thames and nitrate, ammonia, chloride and dissolved oxygen for the Anglian study. Again the time scales of these models will be different for the two river systems.
- (iv) Applications of the Models - during the first year it is intended to purchase the mini-computer for the Anglian study, interface the computer with the flow and water quality telemetry system and provide data reduction and modelling software. In the Thames study the emphasis in the first year will be on data collection and model development."

In each of these areas significant progress has been achieved in the last year as follows:

- (i) Data collection - in the case of the Thames Study, data on flow nitrate, chloride, silica, temperature and algae for all tributaries, effluent discharges and main river sampling and gauging sites for the years 1974, 75 and 76 have been transferred to the Institute of Hydrology (IH) and assembled in a data base on the IH computer. Additional information on flow-velocity relationships and reach

characteristics for the Thames have been obtained from hydrologists at Thames Water Authority. In the Anglian study, records of seventeen water quality variables collected at forty sites over a three year period (1973-75) have been transferred to IH and assembled as a data base on the computer. Additional data from the continuous automatic water quality have also been obtained. Tracer experiments have been conducted at a range of flow rates to establish velocity-flow and dispersion relationships.

(ii) Hydrological Model Development - A computer program has been written which uses numerical integration routines to solve the differential equations relating to the movement of flow down river systems. The program uses velocity-flow relationships derived from tracer experiments to describe the non-linear behaviour of river systems. Information on the river reach structure and reach characteristics is required in order to apply the model to a river system. In the case of the Thames the subdivision of the main river into reaches has been completed; this was particularly difficult because of the complexity of the system and the wide variety of factors affecting river flow and quality. In the case of the Bedford Ouse the reach structure has been chosen to meet the requirements of pollution inspectors who require information at key locations along the river.

The hydrological model for the Thames has been calibrated for the upper sections of the river using daily data for the years 1974, 75 and 76.

In the Bedford Ouse initial validation studies have utilised daily data for the year 1972, 73 and 74. However since the model is to be used for forecasting several hours ahead further validation at the shorter time scale is required.

(iii) Water Quality Models - The water quality modelling studies have taken from three forms. Firstly, some preliminary analysis of water quality data from the Thames system has been carried out to investigate the relationship between flow and mass loadings of nitrates. These studies have involved applying statistically based time series techniques to analyse the data and provide estimates of daily nitrate concentrations in the absence of measured data. Secondly, water quality data have been analysed to investigate and develop the structure of a nitrate water quality model. In the case of the Bedford Ouse this has taken the form of applying recursive estimation techniques such as the extended Kalman filter (EKF) to identify the structure of dissolved oxygen and ammonia models. In the Thames study instrumental variable estimation techniques together with the EKF have been used to investigate algal transport and growth processes. Thirdly the

hydrological model computer program has been extended to incorporate the mass balance equations relating to water quality. In the case of the Thames, preliminary chloride and nitrate modelling studies have been conducted for the upper sections of the river. At this stage only crude estimates of the model parameters (e.g. denitrification rates etc) can be made since information on the short-term pollutant loads must await the development of soil zone and groundwater models. In the Bedford Ouse the computer program has been expanded to include dissolved oxygen, conductivity, biochemical oxygen demand, nitrate and ammonia. The models for these have been based largely on previous estimation studies using daily data and further validation is required to ensure that they apply at the hourly time scale.

(iv) Applications - In the first year of the study the mini-computer has been purchased and interfaced with the water quality monitoring scheme on the Bedford Ouse. A suite of computer programs has been developed to scan the outstations and provide information for management such as daily, weekly and monthly water quality summaries, information on equipment calibration and failure, alarm signals in the event of a major pollution and forecasts of flow and water quality. These programs will require further refinements as additional equipment is installed at the outstations and improvements in modelling occur.

#### FUTURE RESEARCH

Further research will be concentrated under the following headings:

(i) Data Collection - In the Thames Study, additional flow data are required on the lower sections of the river for validation of the hydrological model. Water quality data are also required from soil zone and groundwater models to define more precisely the input loads of pollutants to the river system. In the case of the Bedford Ouse it will be necessary to collect further short-term flow and water quality data using the telemetry scheme for use in further model development.

(ii) Flow and Water Quality Modelling - Further research will concentrate on validating the flow and quality models for both the Thames and the Bedford Ouse. In addition to validating the flow model for the Thames along the lower sections of the river, further work is required to identify the nitrate and algal models for all reaches of the Thames and to estimate rate coefficients within these models. For the Bedford Ouse the flow and water quality models

will require validation at the hourly time scale using data provided by computer controlled telemetry schemes; again, model identification and parameter estimation studies will be required together with research into how forecasts can be updated efficiently as new data are provided in real-time by the telemetry system.

(iii) Applications - Having validated the Thames river system model it will be possible to investigate design options in relation to a new source for London and the likely options available for solving the nitrate problem.

In the case of the Bedford Ouse, it is intended to use the telemetry system to investigate operational management and control problems and in particular, the real-time blending and denitrification control problems at the water treatment centre.

#### References Relating to Research Project

- |   |      |   |
|---|------|---|
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#### Seminars and Lectures given at:-

International Institute of Applied Systems Analysis - Vienna, March 1980  
CEC Research Group Meeting - Dublin, November 1980.  
Water Quality Modelling Symposium, Wallingford - November 1980.  
University College, London University - September 1980  
Loughborough University - December 1980

Contractor: The Geological Survey of Denmark

Contract No.: ENV/412 DK

Project leader: Arne Villumsen

Title of project: Mapping the vulnerability of groundwater reservoirs due to surface pollution

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Objective of the research

The objective of the research is developing a method (tool) by which it is possible to predict the risk of groundwater pollution caused by substances induced to the surface. It is believed that there is a general need for this information in a.o. area planning and water planning. In Denmark a pronounced interest has turned up in this tool as it can be used for the decisions on

- placing of special polluting activities
- placing of waste-disposal sites
- licence to raw-material excavation
- the establishing of major cesspools.

The groundwater-vulnerability concept:

Groundwater vulnerability depends on series of parameters, dynamic as well as static.

Some of the important parameters are:

- thickness, permeability and water content of geological deposits above the groundwater table
- type of pollution on soil surface
- the ability of the deposits above the aquifer to neutralize, retain and delay the actual polluting compound
- water flow within the aquifer

- the pollution intensity in the aquifer
- the exploitation of the reservoir
- the volume of the reservoirs.

In many cases only a few of these parameters are known, and consequently the vulnerability can only be judged with limitations.

In the actual groundwater-vulnerability project only/mainly the more static parameters are included.

The basic idea of the research programme is as follows:

- a) The chemical composition of the ground water can be used for clarifying the hydraulic contact of the ground water with the ground surface.

Chemical elements and compounds indicative of a hydraulic contact with the surface are: nitrate ( $\text{NO}_3$ ), sulfate ( $\text{SO}_4$ ) and excessive carbon dioxide.

In areas with high contents of these elements in the groundwater zone the overlaying deposits are not able to prevent infiltration of pollutants soluble in oxidizing and slightly acidiferous environments.

The opposite situation is found when nitrate and excessive  $\text{CO}_2$  are absent, and the sulfate content is low.

- b) The geological conditions (e.g. porosity, permeability, lithology) of the layers above the groundwater reservoir are important for the vulnerability.

Areas with high permeability in the layers above the aquifer are more exposed to infiltration than areas with low-permeable deposits.

Chemical nitrate reduction is possible in the low-permeable clay deposits above the aquifer



- c) Hydraulic parameters, especially the groundwater potential, are essential for the vulnerability.

### Materials and methods

At The Geological Survey of Denmark a groundwater-quality file was established in 1930. The file contains at present 15,000 chemical analyses of ground water from all parts of Denmark.

At the same time a file containing geological, hydrogeological and technical information about more than 150,000 borings has been established. Following the Danish Water Supply Act (1973) these two files have been used for producing a.o. hydrogeological and hydrochemical maps. At present maps have been produced for most of Denmark.

The purpose of the maps is a localization of groundwater reservoirs and their chemical quality, thereby enabling the counties to plan the future water supply.

The maps and files have occasionally been used for planning groundwater protection in minor areas.

Widespread use of the maps and files to plan systematically the groundwater protection necessitates conclusive maps usable for control as well as for local planners.

In the groundwater vulnerability project a research on the possibilities of using the above-mentioned files and maps to predict the vulnerability of the groundwater reservoirs by surface pollutions is carried out. The aim is to produce so-called vulnerability maps.

The vulnerability maps benefit by the physical (e.g. geological) and chemical information from the files and maps to pre-

dict the probability of groundwater contamination in areas where sewage disposal, waste deposition and intensive agriculture are present or taken into consideration.

As test area Djursland in Jutland, Denmark, has been chosen.

The working plan is divided into the following 5 phases:

1. Preparations of the groundwater vulnerability project (literature study; preparation of detailed working plan; demarcation of study area; procurement of basic material).
2. Elaboration of maps showing basic information (reservoir maps, groundwater-potential maps and isopach map for geological formations above the aquifers).
3. Composite maps, including the aquifer/groundwater-catchment area, and recharge/decharge maps (R/D-map).
4. Combined chemical and hydraulic maps, percolation map and chemical reaction map.
5. Adjustment of the theoretical groundwater-vulnerability map (from phase 4) to actual chemical conditions. The result of this is the vulnerability map.

### Results

In the project the phases 1, 2, 3, and 5 (according to the working plan) have been finished.

The project will continue till 30.6.81, and at that time the preliminary vulnerability maps will have been finished.

### Additional comments

The preliminary vulnerability maps produced by the method described above seems to be a new and promising tool for future groundwater-protection management..

To secure a close collaboration with authorities responsible for water planning, area use etc.. The Survey has established an advisory group to ascertain that the vulnerability maps have a broad spectrum of applicability..

The Survey and the advisory group have agreed on the need for a continuation of the groundwater-vulnerability project, and proposals have recently been forwarded to the Commission for Financial Support in the third phase of the Environmental Research Programme..

The Scientific content of the vulnerability project - the present and the coming projects - has been co-ordinated nationally and internationally (for example with The Institute of Hydrology, Wallingford, England)..

### References::

Mapping of the vulnerability of groundwater reservoirs due to surface pollution. - Report for the period 1.2.80 to 1.9.80, p. 4..

Mapping of the vulnerability of groundwater reservoirs due to surface pollution. - Report for the period 1.9.80 to 1.3.81, p. 3..

### Oral lectures::

Report from The Geological Survey of Denmark to the Freshwater contact Group, 4. meeting, Dublin, 16.-17.10.80. A. Willumsen, DGU.

The vulnerability project. A new method for groundwater protection and management. Seminar 25.-28.11.80, Lyngby, Copenhagen. "Farming and Environment", The Dept. of Environmental Protection. O.S. Jacobsen, DGU.

**TOPIC 16 : WATER QUALITY**  
**Epidemiology**

Contractor: Federal Health Office (Bundesgesundheitsamt)  
Postfach, D-1000 Berlin 33  
Federal Republic of Germany

Contract No.: 196 - 77 - 1 ENV D

Project leader: Dr. M. Sonneborn

Title of project: Epidemiological Studies of the Influence of  
Drinking Water Ingredients upon Morbidity

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The influence of drinking water quality on mortality and morbidity of various diseases has been studied for more than 20 years. Those of the cardiovascular system have been playing a special role.

Some other investigations have shown correlations between e. g. the concentration of fluoride in drinking water and caries, iodide and goitre, nitrate and methamoglobinaemia. Cancer incidence caused by trihalomethanes will be discussed too.

Better and more specified information on the differences in the composition of drinking water may essentially contribute to a solution of such associations.

In more than 900 water supply areas in the Federal Republic of Germany the composition of the drinking water has been analyzed. From these data, areas of different water quality are to be selected for additional investigations of the problem of health relevance of drinking water quality. So far, the following constituents of drinking water have been measured: Na, Ca, Mg, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn, SO<sub>4</sub>, PO<sub>4</sub>, NO<sub>3</sub>, NO<sub>2</sub>, Cl, F, I, etc. The methods of analysis used were atomic absorption spectrometry, plasma emission spectrometry, and ion chromatography.

## I. Drinking Water Hardness and Cardiovascular Diseases

In epidemiological research, studies of the interrelationship between substances contained in drinking water and the mortality from cardiovascular diseases has been a favourite subject for more than 20 years. Parameters of morbidity have also found attention, although to a lesser degree. In view of the large number of epidemiological studies the results and the opinions on the pathogenicity of the "water factor" vary considerably. Frequently, the influence of water hardness, certain minerals or heavy metals contained in drinking water on morbidity and mortality is put in doubt.

Applying the same methodological criteria, all large-scale studies carried out in Japan, USA, England, Canada, Sweden, Finland have shown that there are statistical interrelationships between the degree of regional mortality due to cardiovascular diseases (mostly Nos. 390 - 458 of the ICD 1968) and certain ingredients of the drinking water in the same regions. However these interrelationships appear to be fortuitous. In some studies, they refer water hardness, in others, to calcium or magnesium and to a varying spectrum of other ions, heavy metals and trace elements. Moreover, it was frequently found that such relationships only existed in certain age groups or were restricted to the male or female sex. These interrelationships were found to apply, not only to the mortality due to cardiovascular diseases but sometimes also to the total mortality and further causes of death.

The thesis saying that "soft water" means high cardiovascular mortality and "hard water" low cardiovascular mortality can be regarded neither as confirmed nor as refuted. There are numerous studies which support this thesis. On the other hand, many studies have demonstrated that water hardness does not show a negative but rather a positive correlation to cardiovascular mortality. It must be ad-

mitted, however, that most of the studies known contain a number of errors. In contrast to this, a small number of convincing studies failed to find such relationship.

Also with respect to other substances contained in drinking water, valid, repeatedly confirmed interrelationships between them and certain diseases are not known. The concepts and methods of investigation used so far do not lend themselves to reveal the existence of one or several "water factors". Several reasons can be given for this:

1. Type and quantities of substances contained in water are measured by the water works. However, what matters is the water quality at the tap. The composition of the water at the user's tap may be quite different. Extension and material of the distribution network can be of great influence.
2. The consumption of drinking water varies greatly from person to person. The studies, however, do not allow for this.
3. Deaths are registered at the place of residence of the deceased persons. Whereas drinking water may have been consumed largely at the place of work. Many people satisfy their water requirement from other sources (mineral water, lemonades, beer).
4. The quantities of some minerals, trace elements and heavy metals ingested with food are much higher than those ingested with drinking water.
5. There are regional differences in the morbidity and mortality due to cardiovascular diseases. Many local conditions may be responsible, e.g. risk factors, the varying social situation of the population concerned, consumption habits. Many studies did not make allowance for other important pathological factors in addition to the substances contained in drinking water.
6. Whereas the regional quality of drinking water remains constant over long periods of time, cardiovascular mortality has shown pronounced trends during the last 20 years in the individual countries and this would rule

out a close relationship.

7. Together with the water factor, not only cardiovascular diseases were found to be related to mortality but also other diseases. Such an unspecific effect is not plausible.

For a number of local administrative units (Kreise) of the Federal Republic of Germany data on the distribution of mortality and substances contained in drinking water have been compared. The first evaluation has not shown the presence of interrelationships. Here again, some of the above reservation apply. A reliable answer to the question whether certain diseases and causes of death can be traced to the quality of the drinking water can only be expected from a prospective epidemiological study which allows for the quantity and type of the substances contained in drinking water consumed by persons over a extended period of time and for further disease-relevant factors. However, the limitation of the study to this single problem, does not appear to be justified.

## II. Diseases Caused by Inorganic Water Constituents

While in the case of cardiovascular diseases a significant influence of drinking water could neither be found nor confirmed, there is another disease associated with it which could be confirmed by our studies. It is a long established fact that everywhere in the world the occurrence of endemic goitre shows a preference for certain geographic areas. In the Federal Republic of Germany goitre is far more prevalent in the south than in the north: in Bavaria, the prevalence of goitre was 32 %, in Schleswig-Holstein only 4 %. Concentrations of iodide between less than 0.2 and 17.8 g iodide/l were observed in 108 drinking water samples in different localities of the Federal Republic of Germany. Although this study covered only a part of the entire country, a coincidence with the areas where goitre is known to be endemic is in-



icated. In some cases the prevalence of goitre found was compared with the corresponding iodide contents in drinking water. In the Vogelsberg area, well-known for its goitre endemicity, we found the lowest iodide concentration in drinking water.

Fluoride means another drinking water constituent that is of importance for a consideration of health aspects of the population. From the extremely large number of publications on this subject, it is shown how close together positive and negative effects of the fluoride content of drinking water may be. The fluoride concentration measured different drinking waters in Germany were found to be between less than 0.1 and 3.0 mg F/l. 4.5 % of all values were above 0.7, only 3 exceeded 1.5 mg F/l. Also the content of nitrate, varying over a wide range in the drinking water samples we examined, will effect human health. High concentrations presents a health risk to babies (methaemoglobin-aemia). Also nitrate in drinking water may contribute to the formation of carcinogenic compounds in man.

### III. Trihalomethanes in Drinking Water and Cancer

The present discussions of a possible association between trihalomethanes (THM's) in the drinking water and the occurrence of cancer in man are based on epidemiological studies conducted in the USA. For the present review, the results of 11 independent studies have been considered. Of these studies, 9 use indirect measures of the pollution of drinking water by THM's while 3 have used directly THM concentrations as measured in the water.

Where the populations studied had been classified by race and sex, correlations were not consistent or absent; rather, they appeared in the individual studies in a non-systematic manner and within varying subgroups. In the event of a causal relationship there should have been a better coincidence of results for specific localizations of cancer

in several studies and for race and sex if considered.

In the majority of studies, essential aspects were neglected:

1. Alterations of water quality within a given supply area;
2. Latent period between the exposure to a carcinogen and the occurrence of disease which can be estimated only in terms of decades;
3. Alteration of THM concentration in the water as a consequence of boiling;
4. Preceding exposure to chloroform from anesthetics, anti-tussitives, toothpaste etc.;
5. Known risk factors which are associated with regional differences in cancer mortality as occupational exposure (smoking, alcohol abuse), socioeconomic differences or food habits;

The determination of the THM content in a number of different drinking water within the Federal Republic of Germany shows in general very low concentration in contrast to the results found in USA and some other countries. These may be caused by different applications of drinking water chlorination. Therefore there are no signs for effects of the THM's to human health to be found in our investigations.

#### Publications

Hoffmeister, H., Schön, D., Junge, B. und Sonneborn, M.:  
Zusammenhänge zwischen Trinkwasserinhaltsstoffen und  
kardiovaskulären Krankheiten. Probleme der Analyse.  
SozEp-Bericht 3/1979, Dietrich Reimer Verlag, Berlin, 1979.

Hoffmeister, H. und Schön, D.:  
Trinkwasserhärte und Herz-/Kreislaufkrankheiten.  
Zbl. Bakt. Hyg., I. Abt. Orig. B 172, 67-81 (1980)

Sonneborn, M. and Mandelkow, J.:  
German Studies on Health Effects of Inorganic Drinking Water  
Constituents.  
Sci Total Env., 18 (1981) 47-60  
Elsevier Scientific Publ. Company, Amsterdam.

Contractors : CERCHAR, INSERM, IRCHA - LHVP  
Paris  
Contract nos. : 245, 249, 263, 270 ENV F  
Project leaders : P. LAZAR, R. CABRIDENC, R. FERRAND, B. FESTY  
Title of project : Health effects of drinking water organic micropollutants  
(coordinated study)

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### Objective of the research

The French cooperative study no. 2 was aimed to look for new tracks to determine health effects of drinking water micropollutants. The reasons for such a research lie on the main difficulties encountered in classical studies looking for long term effects (\*), among which modifications of the incidence of cancerous cardio-vascular diseases. Such studies are not often conclusive, mainly because of the long latency between exposure and suspected effect.

This latency increases the basic difficulties of current observational studies, mainly because of the large diffusion of exposure factors and of exposed populations along time and because of the multiplicity of possible inter-current confounders.

It was then decided to focus on human reproduction or early development, since it can be expected, for such phenomena :

- a) to deal with highly sensitive cells, tissues, individuals ;
- b) to observe shorter term effects.

Three main topics were explored, time-located at three different periods of developmental processes : (a) the risk of spontaneous abortion (through an indirect epidemiological way to measure it); (b) the risk of in utero chemical exposure (through the measurement of amniotic fluid organic contamination measured at birth); (c) the risk of height or weight growth modification during infancy according to the quality of water used for food cooking or for drinking.

As it could be expected, none of these investigations revealed to be highly conclusive at once. However all of them do open promising ways for further studies.

### Material and methods

#### 1. Study areas

Peri-conceptional studies (spontaneous abortions) and postnatal ones (statural development from 6 to 36 months) were conducted in Paris, taking

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(\*) including our no. 1 cooperative study

into account the information on the quality of water supply collected during the first phase of the EEC-sponsored studies (1977-1978). Let us recall that Paris is divided into 80 districts, 34 of them being fed with water coming almost exclusively from one of four sources: the rivers SEINE and MARNE (superficial waters) or rivers AVRE and VANNE (deeper waters). Main differences do exist between these rivers as far as organic micropollutants (OMP) are concerned, when they are highly comparable for hardness parameters.

For spontaneous abortions, all the areas were used, when, for developmental studies, 18 day care centres for babies (DCC) were selected according to the type of water supply (unique origin or mixtures of various sources).

Studies on the presence of OMP in amniotic fluids at birth were conducted from samples gathered in the maternity hospital of HAGUENEAU, a city in the eastern part of France (Alsace) where a previous survey of the quality of drinking water had also taken place during the first phase of the EEC studies. Information on the place where the samples women live were collected, in order to be able to look for local differences in relation with the quality of water which was measured in six areas within or around the city.

## 2. Sampling of water

For the DCC study, an average number of 10 samples of tap water were collected in each DCC during years 1979-1980. Each sample was treated by IRCHA for concentrating the OMP by "total" extraction and mixture of the extracts. The combined total extracts were then submitted to chemical analysis (IRCHA, CERCHAR) and to biological assays (LHVP). Water samples were also submitted to inorganic chemical analysis and haloform substances detection (LHVP).

A similar procedure was used for the treatment and analysis of the samples collected in Alsace.

At each sampling, a total volume of at least 250 liters was collected and treated.

## 3. Measurement of causative factors (main steps)

### 3.1. "Total extraction"

Let us recall it is a six steps procedure: three successive chloroform extractions at pH 7, then 9, then 2 and three adsorption-elution processes on IRA 68, XAD2 and Active Charcoal columns. Fractions 1+5+6 constitute the neutral fraction, fraction 2 the basic one and fractions 3+4 the acidic one. All together they constitute the combined total extract (CTE).

Reproducibility of this method can be experimentally assessed and is superior to .90. CTE purity, estimated from the measurement of the contaminants coming from the solvents, varies from about .85 to .99 or more, according to the degree of contamination of the water (the cleaner, the smaller). Sensitivity, defined as the ability to detect the presence and quantity of added substances, is also about .90.

### 3.2. Halofoms detection

Chloroform and bromodichloromethan were looked for from water samples by gas chromatography.

### 3.3. Chemical analysis of the CTEs

13 families of OMP were looked for, by thin layer chromatography aromatic amines, bio-amines, azocycles, chloro-phenols, chlorinated hydrocarbons, N-nitrosamines, organo-metallic substances, semi-polar phenols, phthalates, polycyclic hydrocarbons, organic phosphates, estrophenols, ureins and carbamates.

### 3.4. Inorganic micropollutants (IMP)

Classical IMP were looked for, among which hardness parameters, heavy metals, various salts.

### 3.5. Amniotic fluids analysis (\*)

After elimination of the protein-bound fraction, the same procedure as for CEE was applied. 8 families only were looked for, in order to avoid possible confusion with "natural" organic compounds. These were: aromatic amines, azocycles, chlorophenols, chlorinated hydrocarbons, N-nitrosamines, organo-metallic substances, phthalates and polycyclic hydrocarbons.

## 4. Selection of population

4.1. Spontaneous abortion study: all births (simple or twin) having occurred in Paris during years 1975-76 (52239 singletons, 379 like sex pairs, 152 unlike sex pairs).

4.2. Amniotic fluid study: a total sample of 131 consecutive (\*\*) births.

4.3. Statural growth study: 8572 children (4470 boys, 4102 girls) with up to 108 weighing and 33 height measurement per child, depending on his/her length of stay in a DCC. All the children staying for more than 3 months in the selected DCC were included in the data collection process.

## 5. Measurement of health status

5.1. Spontaneous abortion study: the indirect approach used consisted in measuring the rate of twins at births, which has been shown to be negatively correlated to the rate of spontaneous abortion in the population (\*\*\*).

5.2. Growth study: routine measurements of the DCC were used; values at ages (months) 3, 6, 9, 12, 18, 24, 30 and 36 were either collected (if performed) or reconstituted by linear interpolation (if not). No attempt was made, at this stage, to take individual variations into account, the aim of the study being to characterise the DCC parameters (i.e. the average parameters of each DCC).

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(\*) Amniotic fluid composition can be considered as well as a "causative factor" (for further effects) as a "measurement of health status" (if taken as a consequence of a polluted environment).

(\*\*) Not perfectly consecutive, according to the local possibilities in HAGUENEAU.

(\*\*\*) Effet des avortements spontanés sur la fréquence des naissances gémellaires. Philippe LAZAR, C.R. Acad. Sc., Paris, 282, 1976.

Results

1. Spontaneous abortion study

Table 1 gives the mean rates of twin births in the 34 districts fed with a water from unique origin.

Table 1

water origin	Deep waters		Superficial waters	
	AVRE	VANNE	MARNE	SEINE
Mean $\pm$ se	1.01% $\pm$ 0.33	1.21% $\pm$ 0.95	1.07% $\pm$ 0.22	1.62% $\pm$ 0.00
Number of districts	3	21	9	1

No significant difference appears between the four types of districts. There is then no evidence of any increase of the risk of spontaneous abortion. Indeed, this statement cannot be interpreted too strongly since the absence of significance might be simply due to a lack of power.

2. Chemical exposure in utero

Table 2 show the proportion of amniotic fluids containing the various chemical families looked for.

Table 2

Presence of OMP in amniotic fluids (131 fluids)

More than 20 % of the samples	% of positive	Less than 20 % of the samples	% of positive
Azocycles	55 %	Aromatic amines	18 %
Phtalates	32 %	Organometals	14 %
Polycyclic hydrocarbons	24 %	N-Nitrosamines	13 %
		Chloro-phenols	11 %
		Chlorinated hydrocarbons	4 %

This table clearly shows that the problem of amniotic fluid contamination is an actual one. The question of the specific contribution of water micro-

pollution to this contamination is not easy to answer. Obviously water cannot be the unique cause of contamination, since many other sources, among which air pollution, and, moreover, food contamination can be incriminated. Hagueneau's geographical study was aimed to try to go a little further, since water quality could be measured in various areas together with amniotic fluid composition. However, it was not possible to establish a correlation between amniotic fluid contamination and water quality in the areas in which the mothers lived.

### 3. Growth modifications

For reasons which will be explained in the detailed report, the main analysis was conducted on boys weights. As a major influence of water cannot be expected during the first months of life, when main food is milk (often reconstituted from powder and mineral water), it was decided to compute two growth indexes: the mean weight increments before (V<sub>0</sub>) one year and after (V<sub>1</sub>) one year of age, the latter only being expected to vary with water quality. Table 3 gives the main results of OMP detection and Table 4 the mean indexes V<sub>0</sub> and V<sub>1</sub> according to the sources of water feeding the DCCs.

Generally speaking, table 3 allows to confirm the presence of OMP at a higher rate in superficial waters (MARNE and SEINE) than in deeper ones (AVRE and VANNE), but no major qualitative difference for the various chemical families looked for.

By and large, table 4 shows no major difference in growth rates according to water quality. In particular V<sub>1</sub> does not show any trend which could be associated to water source. And yet the various DCC can be shown not to be homogeneous for weight increment by an overall statistical analysis. In other words, some presumably environmental factors do affect growth in a differential way in the various DCC included in the study, but these factors cannot be directly related to water quality.

### Conclusions

The described coordinated studies did not allow to show any evidence of a specific adverse effect of drinking water on reproduction parameters. However, such a conclusion must be taken with some caution, since the conducted surveys were essentially exploratory ones, not necessarily powerful enough to reveal light effects - if any - of water OMPs on health.

However, these studies allowed to show that a very high proportion of amniotic fluids contain, at birth, quite non-physiological substances: further studies are then necessary both to identify the main sources of such contaminations and their further possible consequences on health..

### Oral Communication

Toxicological and Epidemiological Aspects of Organic Micropollutants in Drinking Waters.  
Invited paper in the Joint European Community - Israel Symposium on Organic Micropollutants in aquatic environment.  
Hertzliah, Israel, April 6-7, 1981.

Table 3 - OMP detection in the DCCs according to water origin (CTE amount and percentages of positive samples for each chemical family)

	CTE mg/l	Aroma. Amines	Bio Amines	Azocy- cles	Chloro- Hydro.	Chlor. Nitro.	N- Nitro.	Organ. metal.	Semi polar phenolates	Phta- Hydro.	Polyc. phosp.	Organ. phosp.	Estro- phenol	Ureins carba
"Pure" sources	Avre n=25	4%	20%	4%	72%	96%	4%	8%	36%	28%	60%	16%	16%	12%
	Vanne n=25	8%	36%	8%	48%	84%	8%	4%	56%	36%	48%	12%	20%	16%
	Marne n=25	12%	28%	16%	68%	80%	4%	0%	48%	44%	52%	16%	36%	20%
	Seine n=26	4%	35%	15%	42%	77%	4%	8%	65%	27%	46%	4%	15%	11%
Mixed sources	Avre +others n=25	4%	40%	0%	56%	96%	0%	4%	48%	32%	60%	12%	12%	8%
	Vanne +others n=24	0%	29%	12%	62%	67%	8%	0%	33%	29%	54%	4%	17%	25%
	Marne +others n=25	8%	44%	8%	48%	76%	4%	8%	52%	28%	56%	8%	20%	20%
Seine +others n=30	7%	53%	13%	47%	73%	13%	16%	50%	37%	60%	13%	27%	23%	



Table 4 - Boys weight increment indexes according to water source

	Water source	DCC number	Vo	V1	Vo (Four rivers)	VI (Four rivers)	VI		
							(deep/superficial)	(superficial)	
"Pure" sources	Deep waters	Avre	354	224	371	212	335	219	
		Vanne	387	200					
	Superficial waters	Marne		294	234	330	225	367	206
				365	216				
		Seine		364	200				
				365	199	361	198		
				354	196				
				352	227				
	Mixed sources	Avre + various others		375	204	372	209	383	208
				390	195				
Vanne + various others			383	208					
			389	199					
			385	198	365	210			
			320	233					
Marne + various others			348	227	348	227	369	210	
			378	208					
			380	205	379	213			
			379	226					
Seine + various others		371	217			371	217		

Contractor : University of Pavia, Italy ( Centro Ricerche  
Nutrizione Umana e Dietetica)  
Contract n° : 201-77-1 ENV I  
Project Leader : Ermanno Lanzola  
Title of project: Mineral characteristics of water and diet and  
Coronary Heart Disease

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## 1.OBJECTIVE OF THE RESEARCH

Following the two-year research project (1977-1978) focused on the mineral characteristics of water and Coronary Heart Disease (CHD), (1) (2) (3) (4) (5), a case-control survey was undertaken in order to evaluate Calcium, Magnesium, Cadmium, Lead, Copper and Zinc in the total diet of the case families and the controls and to see if a relationship exists between the actual intake of those elements and CHD.

The term "case" was defined as a family with at least one member affected by CHD.

The families were chosen from three rural communities in the province of Pavia in which a high mortality rate for CHD, exists (Range  $517-592/10^{-5}$ pop.) calculated for 1970-1976 period.

## 2.MATERIALS AND METHODS

### 2.1 Dietary Survey

The investigation provided for a sample group of 99 families, selected among the three communities, (Filighera, Montescano, Casatisma).

The family was chosen as the epidemiologic unit because it can be considered a homogenous cluster regarding the food consumed. Those families in which a death resulting from CHD had occurred, or in which at least one member suffered from that disease, were defined as a case families.

For the nosographic classification the VIII revision of the WHO (410-414; 400-404) was used. All individuals over 70 years of age were screened out. The cases to be studied were chosen at random from previously compiled lists of families with heart patients. Every case was paired with two control families, residing in the community for at least 10-15 years, according to unit family composition and age and profession of head of

of household. Every family member was asked to complete a questionnaire with the help of specially trained dietitians. In order to establish the weight of the food consumed by each family member, a food atlas presenting various sized food portions was employed and the per capita average daily food consumption weighted for family member body weight, was then calculated.

## 2.2 Sample collection and preparation

Market baskets representing a one-week diet for families in the communities under consideration were collected in local stores and supermarkets. Preference was given to the sampling of locally produced food items. When the market baskets arrived in the laboratory, the food items were processed as for home consumption, homogenized, dried and powdered.

## 2.3 Analytical method

Wet-ashing procedure using  $\text{HNO}_3/\text{HClO}_4$  (1:3) mixture was used for sample dissolution. Lead and Cadmium were extracted using DDDC/Xylene system and measured by Atomic Absorption Spectrophotometry. Calcium, magnesium, zinc and copper were measured in the acid solution after diluting.

The method was validated performing recovery and precision studies. The accuracy was checked measuring the aforesaid elements in NBS. Standard Reference Materials: Oyster (1566), Spinach (1570) and Rice (1568).

## 3. RESULTS

Food consumption profile of case families and controls shows the following:

- a. There are no differences between cases and controls in the food supply sources,
- b. the diets of two groups do not differ qualitatively,
- c. there are no statistically significant differences in the quantity of foods consumed by the two groups.

Dietary intake findings for six elements are showed in Tables 1 and 2. Two-way unbalanced analysis of variance (6) was employed to examine any significant differences in the intake of the six elements between case and control groups within each community and among the three communities.

There was no significant difference between case and control groups. There was, however, a significant difference in the per capita intake of Mg, Zn, Cu, Cd, and Pb among the three communities. Filighera presented the lowest intake of the five elements.

The foods which are the largest contributors to total intake of the six elements are the following: Ca (milk and dairy products); Mg (milk, dairy and cereal prod.); Zn (meat, dairy and cereal prod.); Cu (dairy and cerealprod., fruit); Cd (cereal prod.); Pb (cereal prod., fruit and vegetables, wine).

Drinking water was not included in the calculation owing to the great variability in the concentrations of the elements present as was shown in the previous research work. An approximation of the contribution drinking water makes to the total element intake can be made supposing an average per capita consumption of 1 liter per day. In this case water would supply the following percentages of the total daily mineral intake: Ca, 6-19%; Mg, 5-18%; Cu, <0.1-1.7%; Zn, <0.1-24%; Pb, <1-18%; Cd, <1-3%.

#### 4. CONCLUSIONS

There is no statistical difference between case and control groups both for food consumption and mineral intake.

The significant difference in the mineral intake among the three communities cannot be compared with mortality rates for CHD, since they are very similar in the three communities. Usually, tap water is not an important dietary source of the six elements. Very hard water is responsible for the higher percentage contribution levels of Ca and Mg, while those of the other elements are probably the result of pipeline leakages.

TAB.1-Per capita average daily dietary intake of Ca, Mg, Zn, Cu, Cd, Pb, (case and control groups)\*

Element	C A S A T I S M A			F I L I G H E R A			M O N T E S C A N O		
	Cases (11) <sup>a</sup>	Controls (21)	Total (32)	Cases (13)	Controls (24)	Total (37)	Cases (6)	Controls (11)	Total (17)
Calcium mg/day <sup>b</sup>	577±142 <sup>b</sup>	573±174	575±161	591±198	551±164	565±175	497±210	585±201	554±202
Magnesium mg/day <sup>ooo</sup>	177±26	194±36	188±34	160±37	155±38	156±37	167±27	199±40	187±38
Zinc mg/day <sup>ooo</sup>	8.9±1.0	9.8±1.4	9.5±1.3	8.2±1.9	8.3±1.6	8.3±1.7	10.3±1.8	10.9±2.0	10.6±1.9
Copper mg/day <sup>oo</sup>	1.3±0.3	1.5±0.3	1.5±0.3	1.3±0.3	1.3±0.3	1.3±0.3	1.4±0.3	1.5±0.3	1.5±0.3
Cadmium µg/day <sup>ooo</sup>	46±14	48±12	47±13	32±19	26±13	28±15	54±5	60±14	58±12
Lead µg/day <sup>ooo</sup>	149±41	164±33	159±36	62±19	58±21	59±20	82±20	102±34	95±31

\* Based on average consumption in the three communities over a seven day period

a Numbers in parentheses refer to the number of families

b Each value represents mean ±standard deviation

Probability of F (among communities): °(not significant) °°(P < 0.05) °°°(P < 0.001)

TAB.2 - Per capita average daily content of several elements in the total diet for case and control groups

	Cases (30)	Controls (56)	Total (86)
Calcium mg/day	567±179	566±172	566±175
Magnesium mg/day	167±31	178±42	174±38
Zinc mg/day	8.9±1.7	9.4±1.9	9.2±1.8
Copper mg/day	1.3±0.3	1.4±0.3	1.4±0.3
Cadmium ug/day	42±18	41±19	41±18
Lead ug/day	98±49	107±56	104±53

References

- 1 - E.Lanzola, A.Marinoni, M.Alleggrini, G.Turconi, C.Falcone; Cong. Associazione Italiana di Epidemiologia, Napoli, 31 Maggio 1977
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Contractor: Istituto Superiore di Sanità, Rome, Italy

Contract n° ENV/389 I

Project Leader: Alessandro Menotti

Title of project: CHANGES IN THE CHARACTERISTICS OF DRINKING WATER AND INCIDENCE AND MORTALITY OF CORONARY HEART DISEASE

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#### Background and objective of the research

This study has been conducted exploiting a great deal of information obtained between 1960 and 1975 from the two rural population samples of the Italian section of the Seven Countries Study on coronary heart disease (CHD) and adding supplementary data collected in 1980 in connection with the above mentioned contract.

Two samples of men, aged 40-59 at entry, had been studied for prevalence, incidence and mortality of CHD, and coronary risk factors with direct examinations in 1960, 1965 and 1970 and for general mortality and causes of death until 1975, following the procedures adopted and standardized within the Seven Countries Study. They were made up of 993 men in Crevalcore (region of Emilia) and 719 in Montegiorgio (region of Marche) representing 98.5% of the original chunk samples.

In 1974 following the evidence of a different prevalence of kidney stones with higher rates in Montegiorgio than in Crevalcore, a history of the local water supply was collected from the Municipal Authorities, and samples of water from taps (acqueduct) and from wells and springs were collected and analyzed. In particular in Crevalcore an acqueduct started to operate in 1915 and during the next 20 years the availability of water reached also the extreme areas of the community. Since men considered in the sample were born between 1901 and 1920 only a few of the older men did not use the water from the acqueduct for a maximum of 15 years of their life; whereas all the younger did almost always use acqueduct water. In Montegiorgio, on the other hand, an acqueduct was available since 1901 but serving only the central part of the village whereas a second acqueduct able to reach all the peripheral areas of the community was started in 1960, the year of beginning of the survey. Only along the next 10 years

the water supply has subsequently reached also the most scattered farms of the community and therefore the majority of men belonging to the sample. This means that most people of Montegiorgio - except the fraction living in the centre of the village - have employed for most of their life well waters, and a great majority of them until the age of 40 or more. Similarly well water was used in Crevalcore by the few people not served by the acqueduct.

The chemical analysis made in 1974-75 showed that wells and springs water both in Montegiorgio and Crevalcore was "hard" (about 440 mg/l of  $\text{CaCO}_3$ ) whereas the waters from the acqueduct were definitely softer although not really "soft" (about 130 mg/l of  $\text{CaCO}_3$  in Montegiorgio and 215 in Crevalcore). Similarly high concentration of magnesium was found in the wells waters and high concentration of zinc in the acqueduct waters.

Looking to the cardiovascular conditions in the two areas between 1960 and 1975 the impression was rised that higher prevalence of CHD at entry and higher incidence and mortality later on in Crevalcore were rapidly followed by a catch-up period in Montegiorgio.

The study conducted within the frame of the CEE contract aimed to complete the picture of the water supply in the two rural communities and to study incidence and mortality of CHD for 5 more years, in order to complete a 20 year follow-up period. The hypothesis was made, following the most common theory concerning the relationship between drinking water and CHD, that, along such 20 years period, incidence and mortality of CHD in Crevalcore, initially higher than in Montegiorgio, would have been reached and possibly overtaken by that of Montegiorgio where drastic changes in the characteristics of the water supply had occurred in the direction suggesting and increasing risk of CHD. In fact most data on the subject suggest a higher risk of CHD in population with soft water as compared to areas with hard water. A stable situation in Crevalcore (relatively hard water) contrasted with that of Montegiorgio which initially had a very hard water and later a relatively soft water.

#### Material and Methods

Following the standardized procedures already adopted for many years within the Seven Countries Study, a 20th anniversary examination was offered to all the survivors of the initial cohorts of the two villages, including information on living habits, family history, personal medical



history, special questionnaires on symptoms of cardiovascular and respiratory diseases, physical examination, blood pressure measurement, anthropometric measurements, resting ECG recording, semiquantitative urinalysis and blood sampling for measurement of total serum cholesterol, HDL-c olesterol, and serum triglycerides.

ECGs were read according to the Minnesota Code. Final diagnosis of CHD was made following the rules of the Seven Countries Study which represent an automatic combination of clinical and ECG data. Only hard criteria CHD were considered for analysis.

Standard collection of mortality data for the 4th quinquennium of follow-up was completed following the usual procedure and causes of death were allocated according to standard criteria on the basis of detailed medical informations.

The "water side" of the problem was explored repeating chemical measurements on water samples taken from the same sources considered in 1974-75 and a special questionnaire was developed and administered to the men coming from the medical examination. It aimed to give the best possible picture about the changes in the use of water along the last 30 years. The participation to the examination was similar in two villages and rather high (about 78% of the survivors) in spite of the old age (now ranging 60-79). Indirect medical information were obtained by about an extra 11% of men who could not attend the examination.

Alltogether full medical information (either on cause of death or on diseases occurred during the last 20 years) were available from 928 out of 993 men in Crevalcore (93.4%) and from 665 out of 719 men in Montegiorgio (92.5%). The remaining men were surely alive.

The water questionnaire was available from 484 men in Crevalcore and 389 in Montegiorgio.

The chemical analysis of drinking water covered most of the previously studies sources allowing to confirm a relative stability in the main water characteristics of interest for the study.

### Results

By combining the reported use, along time, of well or aqueduct water, with the measured characteristics of the water from the two sources, the extimated mean hardness to which the two populations have been exposed during a 30 year period has been computed (table 1).

A relative stability in Crevalcore contrasts with a substantial decrease of hardness in Montegiorgio, with an algebraic difference, between the extreme years, of about 160 ppm. Similar changes had occurred for a number of elements among which the most impressive is a drastic drop of Ca, Mg, Na, K and an increase of Zn occurred in Montegiorgio.

Incidence of hard criteria CHD as measured along a 20 year period has been reported by quinquennia of observation excluding from the denominator men already carrier of CHD at the beginning of each 5 year period, and, in quinquennia following the first one, those who had previously died by any cause. Rates per 1000 are always definitely higher in Crevalcore than in Montegiorgio except for a peak in the latter area corresponding to the 3rd quinquennium (table 2). Data on CHD fatal cases only, not reported here in detail, provide the same trend. Altogether the peak at the 15th anniversary in Montegiorgio, already known from previous work, has to be interpreted as casual since later on the higher rates in Crevalcore have been confirmed.

For general reference the overall mortality for all causes has been also reported (table 3) showing a constant excess mortality in Crevalcore then in Montegiorgio.

#### Comments and Conclusions

This study had the purpose to confirm or deny some preliminary indications suggesting that a reduction of water hardness and of the concentration of calcium and magnesium could be accompanied, in the area of Montegiorgio, by a relative increase of CHD incidence, such expected changes having as reference the experience in the community of Crevalcore studied with the same methodology but showing a relative stability of water characteristics. The 20 year follow-up survey did not allow to state that such an expected increase of CHD incidence has occurred, since the early differences between the two communities have been maintained, except for a casual peak of CHD incidence and mortality in Montegiorgio during the 3rd quinquennium of observation.

From the more complete information obtained by the part of the study conducted within this contract, the estimated mean difference of water hardness between the two areas was of about 90 ppm in 1950 (higher levels in Montegiorgio), became about 70 ppm in 1975-80 (higher levels in Crevalcore), the crossing over having occurred between 1965 and 1970. Likely the observed differences are not large enough as to produce measurable

changes of CHD incidence in samples of this size. Calculations reported from Comstock provide a relative risk of 1.20 to 1.25 for a difference in water hardness of about 200 ppm. Moreover the latency between changes in the water characteristics and the eventual effect on CHD incidence is not exactly known.

The substantially stable differences in CHD incidence between the two areas are largely explained by the early differences in blood pressure and body mass index which showed definitely higher means in Crevalcore than in Montegiorgio. The same applied to serum cholesterol at least for levels recorded between the 5th and the 10th year of the study.

The observed data are not in contrast with the hypothesis that water hardness and other water characteristics may represent a risk factor (protective in most instances against CHD), but they are not large enough for providing such a proof.

**Appendix. Oral presentation.**

Colantuoni G., Dima F., Faggioli G., Giuli B., Grisanti S., Marino P., Portoghesi F., Rumi A. and Menotti A.: *Variazioni delle caratteristiche delle acque potabili e patologia coronarica.*

Seduta scientifica GIEP. Congresso Italiano di Cardiologia, Roma 23.5.1981.

Table 1. Estimated change of drinking water hardness to which, on the average, the two populations have been exposed along time (for procedure see text).

Year	water hardness in ppm	
	Montegiorgio	Crevalcore
1950	342	253
1955	330	247
1960	303	236
1965	267	235
1970	207	232
1975	187	244
1980	184	256

Table 2. Coronary heart disease incidence rates (per 1000) in 20 years by quinquennia of follow-up.

Hard cases, including definite myocardial infarction and coronary deaths.

Quinquennium	Montegiorgio		Crevalcore	
	simple	cumulative	simple	cumulative
1st	14	14	16	16
2nd	18	32	28	44
3rd	30	62	25	69
4th	36	98	65	134

Table 3. All-causes death rates (per 1000) in 20 years by quinquennia of follow-up.

Quinquennium	Montegiorgio		Crevalcore	
	simple	cumulative	simple	cumulative
1st	40	60	60	60
2nd	65	105	81	141
3rd	113	218	126	267
4th	131	349	176	441

Contractor : National Institute for Water Supply  
Leidschendam, The Netherlands

Contract No. : 273-77-1 ENV N Project 1

Projectleader : Drs. B.J.A. Haring

Title of project : Epidemiological study on the relationship between  
water quality and health parameters in The Netherlands  
Inorganic water constituents and health parameters

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Objective of the research

In 1971 The Netherlands Water Works Association (VEWIN) proposed central softening of the drinking water in The Netherlands, down to about 2 meq Ca/l (40 mg Ca/l). However, this proposal was criticized by public health experts, because in The Netherlands two studies had shown a negative relationship between water hardness and mortality from cardiac diseases, particularly under women (ref. 1, ref. 2).

The Minister of Health and Environmental Protection asked the Public Health Council (PHC) to consider this matter. In 1975 a commission of the PHC proposed to delay central water softening pending further research.

A Working Group on Health Aspects of Water Softening was established with representatives of various governmental and non-governmental institutes in the fields of water technology and of public health. The studies proposed by the Working Group were supported by grants from the Ministry of Public Health and Environmental Protection, the Dutch Prevention Fund and from the E.C. (Contract no: 273-77-1 ENV N).

During the first study (1977 and 1978) carried out in the framework of contract no. 273-77-1 ENV N data were collected about concentrations of inorganic drinking water constituents and water consumption patterns in The Netherlands. The aim of these studies was to investigate the possible cause of the negative statistical relationship that was found between hardness of drinking water and death rate from cardiovascular diseases.

During the extension period of the E.C. contract (1979 and 1980) recent mortality data (1971-1977) were obtained from the Central Bureau of Statistics. These data were used to calculate statistical correlations with inorganic water constituents like Ca, Mg, Na, pH, Cl<sup>-</sup> and heavy metals. In order to check the consistency of water quality and death rate data during the last three decades, coefficients of intercorrelation for these parameters were determined. Separate investigations during this period were concerned with the determination of changes in the mineral composition of food during

cooking with waters of different hardness. The underlying thought for this type of experiments is that during cooking of food "soft" water may possible extract higher amounts of essential elements (Ca and Mg) from vegetables and potatoes than "hard" water. Finally, the relationship between water hardness, pH and the concentration of heavy metals (Cd, Pb) released from household plumbing systems was investigated.

#### Materials and methods

##### a. *Water sampling and analysis*

The potential exposure to heavy metals released from household plumbing systems is dependant on factors such as water aggressiveness and the consumer behaviour with respect to flushing of the tap before using water for consumption purposes. Consideration of these factors resulted in the development of a proportional water sampling device which can be connected to the tap (see report of 1st phase of the 2nd Environmental Research Programme, EUR 6388 EN).

The analysis of Pb, Cu and Cd in the water samples (50 samples per city) were carried out with anodic stripping voltammetry on a stationary hanging mercury drop electrode using P.A.R. model 374 and 316 instruments. All other metals were determined with atomic absorption spectrometry using Perkin Elmer equipment (model 603 with HGA-500).

##### b. *Correlation studies between mortality and water quality*

In order to study the relation between drinking water quality and death rate from various diseases 30 communities were selected with a number of inhabitants > 40.000 which were served with a reasonable constant quality of drinking water during the last 25 years. Death rates were coded according to the International Classification.

Standardized death rates for 100.000 inhabitants (corrected for age differences) were calculated for men and women older than 30 years for ischemic heart disease, vascular diseases of the central nervous system, malignant tumours of the digestive tract and stomach cancer.

The age-standardized mortalities from these diseases were correlated with the concentration of inorganic water constituents like Ca, Mg, Na, Cl etc. by calculation of Spearman's rank order correlation coefficients.

##### c. *Changes in mineral food composition by cooking in waters of different hardness*

Water for the cooking experiments was sampled at the tap at five different localities in each of six selected water supply areas: The Hague,

Wageningen, Arnhem, Zutphen, Maastricht and Kerkrade. In this way a fairly representative sample of drinking water for each city was obtained.

For the cooking experiments the following foods were chosen: potatoes, cauliflower, carrots and endive. These foods can contribute considerably to the average daily intake of Ca and Mg (ref. 3).

All cooking experiments were carried out in duplicate. 150 g of all examined foodstuffs was cooked in beakers with 250 ml water and 1,25 g NaCl. After draining, the cooked potatoes and vegetables were dry-frozen and ground. Weighed portions of the dry-frozen samples were ashed with  $H_2SO_4$  at  $500^{\circ}C$  and the resulting white ash was dissolved in hydrochloric acid. The water remaining after cooking was adjusted to the original volume (250 ml) with double distilled water to correct for losses by evaporation. After centrifugation the solution was refluxed with  $HNO_3$  and  $H_2O_2$  to destruct organic compounds which might interfere with the elemental analysis by atomic absorption spectroscopy.

A Perkin Elmer model 373 flame atomic absorption spectrometer was used for the determination of Fe, Mn, Ca, Mg, Na, K and Zn in the destructed samples of food and water. Trace elements (Cd, Pb) were determined by anodic stripping voltammetry using a P.A.R. model 374 polarographic analyzer.

## Results

### a. *Consistency of drinking water quality*

Correlation studies between mortality and water quality have been carried out over 1958/1962, 1963/1970 and again over 1971/1977. However, the Working Group doubted whether for 6 communities studied over 1958/1962 and 1963/1970 the water quality data used (1952) were well enough representative because of admixture of water with different quality from other water works. The Group, therefore, selected other communities with a number of inhabitants  $\geq 40.000$ , served by one water works and with no indication of change in water quality over about 20 years. The Group selected 30 communities for the final study; 17 communities of these had been studied as well over 1958/1962 and 1963/1970.

The National Institute for Public Health (RIV) examines water quality at pumping stations on a routine basis. The water quality data from 1952 and from 1977 for these 17 communities showed a high Spearman ranking order coefficient of intercorrelation, except for pH :

pH	: r = 0.41	rMg	: r = 0.83	rHCO <sub>3</sub>	: r = 0.84
K <sub>2</sub> O	: r = 0.93	rCl	: r = 0.78		
rCa	: r = 0.86	rSO <sub>4</sub>	: r = 0.68		

The reasonable consistency in ranking order of the parameters of water hardness, in addition to the fact that the average concentrations also did not differ considerably, allow to correlate water hardness with mortality over the whole period 1958/1977.

There was no consistency in the pH. The pH was calculated on the basis of CO<sub>2</sub>- and HCO<sub>3</sub>-concentrations. This may have been the reason for the low coefficients of correlation. Therefore, in 1979 the National Institute for Water Supply carried out accurate measurement of pH in 28 water supply areas; in addition a new calculation of the Langelier index of water aggressivity was carried out. This study showed a negative correlation between pH and Ca-content:  $r = - 0.70$  (n=28). Although in general natural soft waters are expected to be more aggressive than natural hard waters, various measures taken by the Dutch water production companies to condition (de-acidification) the water in the last decennia have led to an increase of the pH of soft waters. It was not possible to establish fixed data for most water works with respect to the time of introduction of these conditioning measures, because many measures to improve water quality were taken over a period of several years.

b. *Consistency of standardized death rate data*

The mortality from ischaemic heart disease and cerebrovascular disease under males and females  $\geq 30$  yr of age was compared over the periods 1958/1962, 1963/1970 and 1971/1977, age-standardized on the population-1968 (table 1 and table 2); 17 communities.

Over the period 1958/1977 in 17 communities the consistency of mortality from ischaemic heart disease under females and from cerebrovascular disease under males was reasonably good. In a correlation study with water quality data over the period 1958/1977 particular attention had to be paid to these two causes of mortality.

Table 1.

*Ranking order correlation of ischaemic heart disease (IHD) mortality in 1958/1962, 1963/1970 and 1971/1977 (17 communities)*

<u>Males &gt; 30 yr of age</u>	1963/1970	1971/1977
1958/1962	0.42 <sup>x</sup>	0.39 <sup>o</sup>
1963/1970		0.74 <sup>xx</sup>
<u>Females &gt; 30 yr of age</u>		
1958/1962	0.78 <sup>xx</sup>	0.66 <sup>xx</sup>
1963/1970		0.83 <sup>xx</sup>
legenda: o P < 0.10		
x P < 0.05		
xx P < 0.01		



Table 2.

Ranking order correlation of cerebrovascular (CNS) mortality in 1958/1962, 1963/1970 and 1971/1977 (17 communities)

<u>Males &gt; 30 yr of age</u>	1963/1970	1971/1977
1958/1962	0.80 <sup>xxx</sup>	0.57 <sup>xxx</sup>
1963/1970		0.79 <sup>xxx</sup>
<u>Females &gt; 30 yr of age</u>	1963/1970	1971/1977
1958/1962	0.35 <sup>o</sup>	- 0.00
1963/1970		0.66 <sup>xxx</sup>
legenda:	o < P 0.10	
	x < P 0.05	
	xxx < P 0.01	

c. Correlation between mortality and water hardness

In contrast with earlier statistical investigations no significant correlations were found over the period 1970/1977 between IHD mortality and hardness of drinking water in 30 municipalities (table 3). The disappearance of the statistical relation could not be attributed to changes in water hardness. However, investigation of a group of 17 municipalities for which mortality and water quality data are known for three periods (1958/1962, 1965/1970 and 1971/1977) showed that the inverse statistical relation between IHD mortality and water hardness still existed but with decreasing significance of correlation coefficients (table

Table 3.

Ranking order correlation between ischaemic heart disease (IHD) and cerebrovascular mortality (CNS) under males and females > 30 yr of age with Ca- and Mg-content of water in 1977 in 30 communities

<u>Males &gt; 30 yr of age</u>	Ca	Mg
IHD	- 0.01	- 0.19
CNS	- 0.14	- 0.02
<u>Females &gt; 30 yr of age</u>		
IHD	- 0.11	- 0.10
CNS	- 0.12	- 0.07

Table 4.

*Ranking order correlation between cardiovascular mortality over 1958/1977 and water hardness in 1952 and 1977 in 17 communities*

<u>Males &gt;30 yr of age</u>	water 1952		water 1977	
	Ca	Mg	Ca	Mg
mortality				
1958/1962	- 0.05	- 0.08	- 0.31	+ 0.12
1963/1970	- 0.48 <sup>x</sup>	- 0.41 <sup>x</sup>	- 0.45 <sup>x</sup>	- 0.46 <sup>x</sup>
1971/1977	- 0.36	- 0.45 <sup>x</sup>	- 0.36 <sup>x</sup>	- 0.33 <sup>o</sup>
<u>Females &gt;30 yr of age</u>				
1958/1962	- 0.38 <sup>o</sup>	- 0.17	- 0.28	- 0.16
1963/1970	- 0.67 <sup>xx</sup>	- 0.42 <sup>x</sup>	- 0.56 <sup>xx</sup>	- 0.54 <sup>x</sup>
1971/1977	- 0.48	- 0.30	- 0.36 <sup>o</sup>	- 0.37 <sup>o</sup>
legenda:	o	P (one side) < 0.10		
	x	P < 0.05		
	xx	P < 0.01		

d. *Changes in mineral food composition by cooking in waters of different hardness*

Tapwater from 6 communities, varying in hardness from Ca=114 mg/l and Mg=19 mg/l to Ca=17 mg/l and Mg=2.2 mg/l, was used to cook potatoes and vegetables. The results (ref.4) indicated that the Ca-level in the food stuffs generally increased when cooked in hard water, whereas a decrease or only a slight increase was found when cooked in soft water; the Mg-content decreased both when cooked in hard and in soft water.

The results of these tests might support the hypothesis than an inverse relation between hardness and ischaemic heart disease mortality is due to a deficiency of Ca and/or Mg in soft water. Such a deficiency will further be increased by the extractions of Ca and Mg from food by soft water. This particularly may have consequences when the nutritional intake of Ca and/of Mg is low.

e. *Corrosiveness of drinking water and cardiovascular disease mortality*

Investigation of the relationship between water hardness, pH and the concentration of heavy metals (Pb, Cu) released from household plumbing systems showed that at present in The Netherlands the relatively hard waters generally have lower pH values and higher levels of lead and/or copper (ref.5). This means that if negative statistical correlations between water hardness and cardiovascular death rate will still be found in The Netherlands on the basis of recent data(1979), it is not likely that these heavy metals are the causative factor.

Conclusions and any additional comments

The studies carried out in The Netherlands since 1975 have made it clear that The Netherlands is not a suitable country to elucidate the "water story" for the following reasons:

- the number of communities with > 40.000 inhabitants served by one water works, and with an expected consistency in water quality is small (n=30);
- the range in water hardness is limited (Ca 16-117 mg/l; Mg 1-15 mg/l) in comparison to e.g. the U.K. (Ca 4 mg/l tot 160 mg/l). In the 30-communities-studies in The Netherlands 46 % had a Ca-level < 60 mg/l, 33 % < 40 mg/l and 3 % < 20 mg/l in 1977;
- there is a considerable mobility within the country; this would severely hamper a longitudinal study;
- some communities have an age-distribution which very much deviates from the country average, because of e.g. institutions for old invalids, and aged subjects; such a deviation cannot be adequately overcome by age-standardization;
- in The Netherlands, community based data on factors which may contribute to cardiovascular mortality do not exist; therefore, it is not possible to adjust the mortality data for e.g. socio-economic conditions as done by Pocock et al. (ref.6).

Studies in the U.K. strongly suggest that particularly soft water with Ca content below 40 to 60 mg/l may contribute to cardiovascular mortality. The EC also recommended (ref.7) that Ca-levels should not be lower than 60 mg/l. The total number of 30 communities was too small to divide these into sub-categories of water hardness, the British suggestion could neither be confirmed nor be rejected.

The provisional conclusion of the Working Group is that other factors than water hardness overrule to a large extent the potential effect on IHD mortality. Central water softening down to 2-3 meq/l Ca probably will have no observable effect on mortality.

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Contractor : National Institute for Water Supply  
Leidschendam, The Netherlands

Contract No. : 273-77-1 ENV N Project 2

Projectleader : Drs. H.J. Kool

Title of project : Epidemiological study on the relationship between  
water quality and health parameters in The Netherlands  
Organic water constituents and health parameters

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Objective of the research

In the last decade, significant advances have been made in the area of the quantitative analysis of organic compounds in drinking water.

Application of these improved analytical techniques in drinking water have led to the conclusion that the presence of these compounds in water may create health problems (ref. 1, ref. 2).

The growing consensus, that many human cancers are caused by environmental agents with special reference to chemicals, also leads to an increased attention for carcinogens and mutagens in drinking water.

The first part of this report shows the results of a survey concerning the presence of organic constituents in drinking water in The Netherlands. Special attention is given to those compounds which may cause irreversible changes of the body tissue viz. the mutagens and carcinogens (ref. 3).

In the second part of this report an epidemiological study has been presented, in which the organic constituents detected in drinking water, the type of raw water source and chlorination have been considered as variables.

The purpose of this study was to test three hypotheses:

- Communities which use highly polluted surface water instead of unpolluted ground water as source for drinking water will show higher cancer mortality rates.
- Communities provided with chlorinated drinking water show higher cancer mortality rates than communities with water, which is not chlorinated.
- An increase in the concentration of the considered organic groups/compounds will result in a higher mortality rate of several cancer sites.

### Materials and methods

Twenty communities were selected for the survey of organic constituents in drinking water and nineteen of these communities for the correlation studies on seven cancer sites. The selection of the communities was based on the type of raw water source (ground water or surface water), the type of storage facilities (reservoir, dune or bankinfiltration) and the oxidative treatment (chlorine). A composite sample for a certain community or group of communities was collected in case more than one pumping station was supplying the area.

Stainless steel vessels of 200 litres content were used for collecting the samples. Polynuclear aromatic hydrocarbons (PAH) were determined using a modified thinlayer chromatography (ref. 4). Volatile halogenated compounds like chloroform and trichloroethene were analysed by headspace analysis. In addition the concentration method of Grob (ref. 5) (closed-loop gas stripping) was also used. To obtain higher boiling and slightly polar compounds, the concentration method developed by Junk (ref. 6) was used. In this method adsorption of organic compounds on macroreticular resins (XAD) is applied and elution of this resin is carried out with diethylether. The organic compounds in this extract were analysed with gaschromatography-massspectrometry.

The epidemiological investigation was based on a population of  $4.6 \times 10^6$  inhabitants of The Netherlands, which is about 33 % of the population of the country. Cancer mortality rates for both sexes were obtained from The Netherlands Central Bureau of Statistics (CBS) and taken over a period of twelve years (1965-1976). Six cancer sites expected to be linked with drinking water were used viz. esophagus, stomach, colon, rectum, liver, bladder, and one (the lung) expected to be less linked to drinking water. The standardized mortality rates of age group 35-64, as suggested by the National Health Council (ref. 3) were correlated with the concentration range of organic groups, viz. trihalomethanes, total alkylbenzenes, total chlorobenzenes, total phthalates and specific organic compounds viz. chloroform, bromodichloromethane and trichloroethene present in the drinking water of nineteen communities as well as drinking water (chlorinated or not) and the raw water source from which drinking water is prepared. For a negative control in the correlation study, total hardness and calcium data were also included.

## Results

### a. *Survey on the presence of organic constituents in drinking water in The Netherlands*

A total number of 280 organic compounds has been detected in the twenty types of drinking water investigated. Nearly 100 compounds of these 280 could not be identified.

The most important results of the survey were:

- In drinking water derived from ground water some halogenated hydrocarbons like tetrachloromethane, trichloroethene and tetrachloroethene were rather frequently detected.
- The largest number of organic compounds was found in tapwater derived from Rhine water after storage in open reservoirs.
- If during water treatment a higher amount of chlorine was used, an increase of the concentration of trihalomethanes was observed.
- Mutagenic and carcinogenic organic compounds could be detected in drinking water at relatively low levels.

### b. *Correlations between water quality and cancer mortality rates*

In this study several relationships were studied by linear regression analysis. The relationship concern on one hand 7 cancer mortality rates for 2 sexes and on the other hand

- the type of raw water source : surface/ground water, table 1;
- oxidative treatment : chlorinated/unchlorinated drinking water, table 2;
- 7 organic compounds : trihalomethanes, total alkylbenzenes, total chlorobenzenes, total phthalates, chloroform, bromodichloromethane and trichloroethene, tables 3 - 7;
- 2 inorganic parameters : calcium and total hardness, table 8 - 9.

The results of this study whereby no confounding factors were included show higher mortality rates for three cancer sites in communities where drinking water was prepared from polluted surface water than in communities where drinking water is prepared from ground water (table 1). This result is in agreement with earlier findings in The Netherlands of Diehl and Tromp (ref. 7). Other results are shown in the tables 2 - 9.

Table 1  
Correlation coefficients (R) between SMR in both sexes versus surface water/  
ground water as drinking water source

Sex	Site or type of malignancy						
	Esophagus	Stomach	Colon	Rectum	Liver +	Bladder	Lung +
Male	0.02	-0.04	0.01	0.23	-0.39 +	-0.39 +	-0.53 +
Female	-0.18	0.13	0.12	-0.02	-0.11	0.13	-0.43 +

Table 2  
Correlation coefficients (R) between SMR in both sexes versus chlorine treated  
drinking water/not chlorine treated drinking water

Sex	Site or type of malignancy						
	Esophagus	Stomach	Colon	Rectum	Liver +	Bladder	Lung +
Male	-0.27	-0.36	0.00	0.08	-0.56 +	-0.17	-0.52 +
Female	-0.19	-0.15	0.19	-0.38	-0.14	0.12	-0.32

Table 3  
Correlation coefficients (R) between SMR in both sexes versus levels of  
THM's in drinking water

Sex	Site or type of malignancy						
	Esophagus +	Stomach +	Colon	Rectum	Liver	Bladder	Lung
Male	0.60	0.46 +	-0.18	-0.04	0.07	-0.03	0.25
Female	0.29	0.27	-0.24	0.22	0.16	0.03	-0.14

Table 4  
Correlation coefficients (R) between SMR in both sexes versus levels of  
chloroform(Ch) and bromodichloromethane(Br) in drinking water

Sex	Site or type of malignancy													
	Esophagus		Stomach		Colon		Rectum		Liver		Bladder		Lung	
	Ch	Br	Ch	Br	Ch	Br	Ch	Br	Ch	Br	Ch	Br	Ch	Br
Male	0.56 +	0.58 +	0.51 +	0.48 +	-0.09	-0.24	0.00	-0.04	-0.04	0.04	0.10	0.00	0.30	0.19
Female	0.21	0.30	0.31	0.24	-0.36	-0.27	0.30	0.18	0.02	0.15	0.00	0.06	-0.11	-0.18

Table 5  
Correlation coefficients (R) between SMR in both sexes versus levels of  
chlorobenzenes in drinking water

Sex	Site or type of malignancy						
	Esophagus	Stomach	Colon	Rectum	Liver +	Bladder	Lung
Male	-0.11	-0.11	0.01	-0.07	0.47 +	-0.18	0.07
Female	0.15	-0.21	0.43 +	0.01	0.25	0.03	0.15

Table 6  
Correlation coefficients (R) between SMR in both sexes versus levels of  
alkylbenzenes in drinking water

Sex	Site or type of malignancy						
	Esophagus +	Stomach	Colon +	Rectum	Liver	Bladder	Lung +
Male	0.43 +	0.28	0.40 +	0.24 +	0.27	0.19	0.48 +
Female	0.17	0.57 +	0.05	0.59 +	0.13	0.23	0.30

+ R significant (p > 0.05 one tailed test)

Table 7  
Correlation coefficients(R)between SMR in both sexes versus levels of phthalates in drinking water

Sex	Site or type of malignancy						
	Esophagus	Stomach	Colon	Rectum	Liver	Bladder	Lung
Male	-0.32	0.18	-0.03	-0.20	0.30	0.39 <sup>+</sup>	0.12
Female	-0.23	-0.06	-0.16	0.03	-0.16	0.28	0.49 <sup>+</sup>

Table 8  
Correlation coefficients(R)between SMR in both sexes versus levels of calcium in drinking water

Sex	Site or type of malignancy						
	Esophagus	Stomach	Colon	Rectum	Liver	Bladder	Lung
Male	-0.04	0.42 <sup>+</sup>	0.09	-0.33	0.19	0.33	0.09
Female	0.03	0.07	-0.25	-0.04	-0.08	-0.16	0.27

Table 9  
Correlation coefficients(R)between SMR in both sexes versus levels of total hardness in drinking water

Sex	Site or type of malignancy						
	Esophagus	Stomach	Colon	Rectum	Liver	Bladder	Lung
Male	0.00	0.32	0.04	-0.23	0.19	0.42 <sup>+</sup>	0.08
Female	0.20	0.02	-0.28	0.13	-0.06	0.08	0.36

+ R significant (p >0.05 one tailed test)

The results in table 2-9 show that:

- a higher cancer mortality rate of liver(male) and lung (male) was observed in chlorinated drinking water compared to unchlorinated drinking water,
- the level of the following organic/inorganic groups/parameters in drinking water show a positive association with the different cancer mortality rates:

<i>Organic group parameters</i>	<i>Cancer mortality rate</i>
THM	- esophagus(male), stomach(male)
chlorobenzenes	- colon(female), liver(male)
alkylbenzenes	- esophagus(male), colon(male), lung(male) stomach(female), rectum(female)
phthalates	- bladder(male), lung(female)
 <i>Inorganic parameters</i>	
calcium	- stomach(male)
total hardness	- bladder(male)



- between the concentration of trichloroethene in drinking water and cancer mortality rates of all cancer sites considered no positive association was found (not shown).

#### Conclusions and additional comments

The survey of organic constituents in drinking water revealed that a number of organic constituents could not be identified. In order to be able to assess whether these compounds constitute a hazard, existing analytical techniques for identification and quantification should be improved.

From this survey and from earlier observations it can be concluded that carcinogenic and mutagenic compounds are present in drinking water although only in relatively low concentrations. The epidemiological investigation revealed that communities where drinking water is prepared from surface water or where drinking water is chlorinated, a higher mortality rate for different cancers is observed than in communities where drinking water is prepared from ground water resp. unchlorinated drinking water.

An increasing level of alkylbenzenes, chlorobenzenes, THM's, phthalates, calcium and total hardness also results in a higher mortality rate for several cancers.

Considering however the number of regressions viz. 28 for the inorganic parameters (calcium, total hardness, 2 sexes and seven cancer sites) and 126 for the organics (7 groups/compounds, plus surface water/ground water, chlorinated/unchlorinated water, 2 sexes and seven cancer sites), some significant correlation coefficients due to random effects may be obtained. For the above mentioned numbers of regression an estimated average of 6 and 1 significant correlation coefficients for the organics resp. inorganic parameters may be expected, at a p-value less than 0.05. In this study however 21 significant correlation coefficients for the organics are obtained. The 2 significant correlation coefficients obtained with inorganics can be explained by random effects.

The question whether the observed *statistical associations are causal or not* should be clarified by further toxicological studies.

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Contractor: Water Research Centre, UK  
Contract No: 246-77-1 ENV UK (Project A)  
Project Leaders: R F Lacey and R F Packham  
Title of Project: WATER QUALITY AND CARDIOVASCULAR DISEASE: CHANGE  
SITUATIONS IN THE UK

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1. OBJECTIVE

The objective of this project was to identify situations in the United Kingdom where the hardness of drinking water has changed by at least 25% and to find out whether the changes in hardness have been accompanied by significant changes in cardiovascular disease mortality rates.

2. METHODS

Of the many investigations into the link between cardiovascular disease and the hardness of drinking water, perhaps one of the most convincing has been the 'changes' study by Crawford, Gardner and Morris (CGM) <sup>(1)</sup>. The present work started with a critical review of that study and proceeded to the collection and analysis of further data relating to more recent hardness changes. The review indicated the need for some tightening of method and this has been achieved, while keeping however to an overall design similar to that of CGM.

In an investigation of this kind it is essential to make the observations on fairly large communities. Before the local government changes of 1974 the then county boroughs were the major centres of urban population outside London and the work has concentrated on this category of 89 towns.

A definition of a 'change' in water hardness has to specify the size of the change and the time period during which it is to have taken place. For size, the contract objective suggested 25%. CGM had earlier used an absolute change in total hardness of 50 mg CaCO<sub>3</sub>/l. In the UK about one third of supplies are below 100 mg CaCO<sub>3</sub>/l and one fifth below 50 mg/l. One tenth are however over 300 mg/l. The two possible criteria just mentioned

operate differently at the extremities of this range. We therefore recommended a compromise, to designate a 'change' if the water hardness has changed by  $(25 + 0.125H)$  mg  $\text{CaCO}_3/\text{l}$  where H is the initial or final hardness whichever is the lower.

How the time limits for a hardness change should be specified is open to much argument. We decided to identify changes which qualified precisely in the time interval over which the changes in mortality rates are assessed. For each of the county boroughs, information was collected from a number of sources to ascertain what the hardness had been in 1951, 1961 and 1971 (using 5-year means where possible) and this information was used to identify hardness changes which took place in the time intervals 1951-1961, 1961-1971 and 1951-1971.

The term 'cardiovascular' was taken to cover all disorders of the heart and circulation. More precisely it was defined by ICD codes 330-334, 410-468 (6th/7th Revisions) and 393-458 (8th Revision). Death-rate estimates for each town were derived for the periods 1948-54, 58-64, 69-73 centred on the National Censuses of 1951, 1961 and 1971. This was done for deaths from cardiovascular disease and deaths from all other causes. Attention has concentrated on four age-sex classes: Males 45-64 years, 65-74 years; Females 45-64 years, 65-74 years.

For each town, the change in cardiovascular death-rate between times  $t_1$  and  $t_2$ , for a specific age-sex class, was measured by the log ratio,

$$y = \log_e (\text{death-rate at } t_2 / \text{death-rate at } t_1).$$

The principal method of analysis was based on the comparison between three groups of towns:

Group A	towns which have decreased their water hardness
Group B	towns which have increased their water hardness
Group C	no change, control group

In particular we investigated the significance of any trend among the group means,  $\bar{y}_A$ ,  $\bar{y}_B$  and  $\bar{y}_C$ , bearing in mind the variability of  $y$  within groups. This analysis was carried out for the four age-sex classes separately and for combinations of classes where consistency allowed.

We checked whether the changes in death-rates might be associated with changes in three socio-economic factors (mean social class score, % manual workers, % unemployed) which had appeared significant in the recent static regional comparison (2)

### 3. RESULTS

#### 3.1. Re-assessment of the paper by Crawford, Gardner and Morris

Crawford, Gardner and Morris warned their readers that their water data was not very good, even though they had made a special survey to collect it. They declared a change in hardness to have taken place if a town had changed its mean total hardness by 50 mg/l in the thirty or so years up to 1960. This definition allowed some changes which were almost complete before the first mortality period, 1948-54, while others had only just qualified by the time of the second period, 1958-64. The result of this is that the CGM study is very unspecific with regard to the hypothesized time-lag between cause and effect.

In our interim report (3) we drew attention to two other criticisms of the CGM study to do with the data for Canterbury and the method of combining results from the different age-sex classes. On further consideration, the overall effects of correcting for these matters appear to be almost equal and opposite. Assuming that both corrections are made, the conclusion of the CGM study would therefore remain about the same, provided that one accepts the assignment of water hardness changes on which their analysis is based.

#### 3.2. Results from more recent data

On the stricter rules of definition the numbers of water hardness changes which qualify between 1951 and 1961 are too small to support a

statistical analysis. For 1961-1971 we have respectively 4 and 10 towns in Groups A and B. For 1951-1971 the corresponding numbers are 5 and 11.

The changes in cardiovascular death-rates between 1961 and 1971 for the age range 45-64 are shown in Fig. 1, where each point represents a town. The analysis of trends for all age-sex classes is summarised in Table 1.

Table 1 Trends in cardiovascular death-rates related to changes in hardness between 1961 and 1971

	Age range	Mean change in death-rate per designated change in hardness	Approximate standard error	Critical probability in two sided-test
MALES	45-64	- 6%	1.7%	< 0.001
	65-74	- 6%	1.8%	0.002
	Ages combined	- 6%	1.4%	< 0.001
FEMALES	45-64	- 0%	2.9%	ns
	65-74	- 2%	2.1%	ns
	Ages combined	- 1%	1.8%	ns

For changes in cardiovascular mortality the trend in the direction predicted by the 'Water Story' is significant for males, in either age group, but not for females. The results appear to be homogeneous enough for the age groups to be averaged, if desired. The contrast between males and females then becomes significant ( $p = 0.011$ ). For non-cardiovascular mortality there were no significant down trends.

Correlations between changes in cardiovascular mortality rates and changes in the three socio-economic factors were negligible. Moreover there was no systematic tendency for the towns which changed their water hardness between 1961 and 1971 to have simultaneously changed their socio-economic profile.

The results for 1951-71 were generally weaker than for 1961-71. This is not surprising in view of the fact that most of the changes in water hardness came after 1961. The marginal increase in number of available towns was outweighed by the increase in the variance of  $y$ , which is as would be expected from theory.

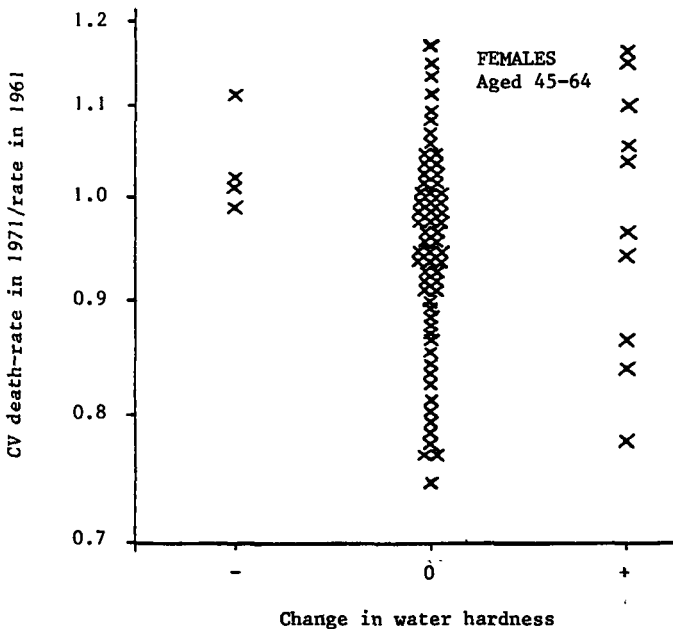
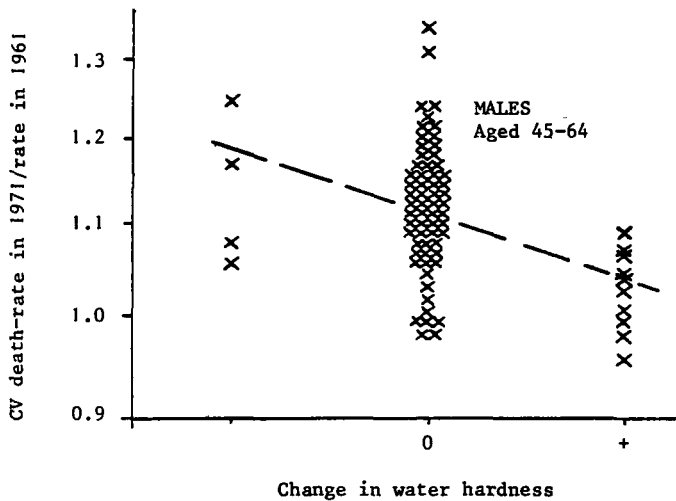
4. CONCLUSIONS

- (1) The important previous study of changes in water hardness and cardiovascular death-rates by Crawford, Gardner and Morris has been reviewed. Its conclusions are upheld, though with stronger reservations about the timing of the changes in water hardness on which it was based.
- (2) In view of (1), tighter criteria for designating a change in water hardness have been adopted for subsequent work.
- (3) Results for the water hardness and cardiovascular death-rate changes between 1961 and 1971 in the county boroughs of England and Wales indicate a significant trend for males, in the direction consistent with the 'Water Story', but no trend for females. The trend in male mortality appears to be specific to cardiovascular disease.
- (4) The findings on the whole add support to the 'Water Story', that the connection between cardiovascular disease and the mineral content of drinking water is real and causal. The size of the effect appears however to be rather small and its nature remains uncertain.
- (5) The results of this project have been reported to the UK Department of the Environment and Department of Health and Social Security, Joint Committee on Medical Aspects of Water Quality<sup>(4)</sup>.

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Figure 1 Changes in cardiovascular death-rates for county boroughs in England and Wales





Contractor: Water Research Centre, UK  
Contract No: 246-77-1 ENV UK (Project B)  
Project Leaders: B T Commins and R F Packham  
Title of Project: POPULATION EXPOSURE TO METALS RELEASED FROM PIPING  
MATERIALS USED FOR WATER DISTRIBUTION IN THE UK

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1. OBJECTIVE

To investigate and evaluate methods of estimating population exposure to metals released from piping materials used for water distribution in the United Kingdom.

2. METHOD

In this project the term 'population exposure' to a given metal is used to denote the quantity of that metal which is present in tap-water and which is ingested by man. An estimate of this may be desired for a particular individual person, for a family living in a particular house, for a community in a given water supply zone or for the whole population of the UK. The level of aggregation at which the exposure estimate is required and the choice of the statistic itself (average, variance or extreme) must of course be borne in mind in evaluating the methods of providing it.

A method of estimating exposure has to combine estimates of the volumes of tap-water consumed with measurements of relevant concentrations of the metal. One difficulty here is that the concentrations of metals released from pipes can vary widely according to the pipe system and the time and manner in which the water is drawn. The actual ingested concentration may further be modified by the type of beverage into which the water is made.

The objective of this project was pursued by three separate but related investigations. The first aimed at investigating the occurrence of a wide range of trace elements in the water supplies sampled at houses in 25 British towns. Information was also gathered about the drinking habits of the inmates. The second and third investigations were more detailed but narrower in scope and smaller in scale. They concentrated on lead and copper and the problems of variability at the individual tap.

### 2.1. Water sampling in the British Regional Heart Study

The British Regional Heart Study is a major epidemiological study of cardiovascular disease in the UK, being undertaken by the Royal Free Hospital School of Medicine, London. The study includes a cross-sectional survey of middle-aged men, some 300 individuals in each of 25 towns. WRC has undertaken tap-water sampling for about 40 of these men in each town. At each house three samples were taken: 'first draw' (after water had stood in the pipes overnight) 'random daytime' and 'fully flushed'. These samples were each analysed for the 26 elements listed in Table 1.

At the same time as the medical survey, the men selected for water sampling were each taken through a questionnaire about their consumption of tap-water based drinks and habits of water use.

### 2.2. Sampling for lead

The within-house variability of lead concentrations in different types of water sample has been studied for a set of 16 random daytime and 16 first draw samples taken from each of 11 houses in Glasgow.

One method of overcoming the problem of variability of individual samples is to use a proportional composite sampling device attached to the consumer's tap. WRC has experimented with the type of proportional sampler designed by RID in the Netherlands. These samplers were installed in eight co-operative households over the period of a week to check whether the households would accept the sampler and use it correctly, and to compare estimates of exposure derived from the proportional sampler with estimates derived from discrete sampling and manual recording of water use.

### 2.3. Stagnation time and flow rate

The effects of stagnation time and of flow rate on the concentrations of lead or copper in water drawn from pipes made of those materials were investigated for seven pipes (3 lead, 4 copper) in different parts of the country.

Mathematical models have been developed which combine the 'stagnation curve' with information on water use to predict the lead concentrations in water drawn for human consumption. Two approaches have been tried. The first was a deterministic calculation which needed extremely detailed information about the water usage of the individuals to whom the results would apply.

That was superceded by a less demanding approach<sup>(1)</sup>, taking a frequency distribution of stagnation times and deriving the frequency distribution of lead concentrations in drawn water. The principle of this is illustrated in Figure 1.

For input data we took a frequency distribution of overnight stagnation times available from the work described in Section 2.1. In the absence of information on stagnation times throughout the day, an informal survey was carried out by placing diaries with households of WRC members of staff.

### 3. RESULTS

#### 3.1. Water sampling in the British Regional Heart Study

The results of analysing the water samples gave estimates of trace element concentrations of 26 elements in 25 towns. Table 1 shows an aggregation of this data for 12 soft water (< 120 mg CaCO<sub>3</sub>/l) and 11 hard water (> 200 mg CaCO<sub>3</sub>/l) towns for two of the sample types.

From the water consumption questionnaire the mean tap-water intake for middle-aged men in the UK was estimated to be 1.25 litres per day. This is divided between: tea 66%, coffee 21%, water 12% and other tap-water based drinks 1%. These results are in very close agreement with those for males aged 31-54 in the separate WRC survey<sup>(2)</sup> not covered by this contract.

Division of the volume of water into the categories 'random daytime', 'fully flushed' and 'first draw' gave proportions 51%, 43% and 6% respectively. The combination of this information with the average concentrations in the three sample types yields the exposure estimates in columns three and six of Table 1.

#### 3.2. Sampling for lead

From the Glasgow data the within-house variation of random daytime samples appeared to be approximately log-Normally distributed, with standard deviation of the logged (base 10) results being about 0.25.

The outcome of the trials of the proportional sampler were technically satisfactory although there were reservations about its inconvenience.

### 3.3 Stagnation time and flow rate

All of the stagnation experiments showed that the concentrations of lead or copper increased sharply in the first few hours of stagnation and levelled off to fairly constant values after 15-30 hours. A typical example of a stagnation curve is shown inset in Figure 1. Stagnation curves were found to be reproducible at the same tap. The effect of flow rate was much less important than that of stagnation time.

The result of the modelling exercise has been to demonstrate the feasibility of the approach. If a standard frequency distribution of stagnation times could be assumed then this would enable the exposure of typical inmates of a house to be estimated from the house's stagnation curve, the main features of which can be determined without extensive sampling.

## 4. CONCLUSIONS

- (1) Information has been gathered about the concentrations of trace metals in water sampled at taps in 25 towns in Britain. This has been combined with volume data to give average estimates of exposure for middle-aged men on a town by town basis.
- (2) Estimation of exposure to plumbing metals is difficult because of the large temporal variability in concentrations, arising mainly from
  - (a) the stagnation performance of the pipe system,
  - (b) the water use patterns of the consumer.
- (3) Discrete water samples are therefore unreliable for estimating the exposure of a particular individual. The effects of (a) and (b) are better combined physically by a proportional composite sampling device. The acceptability of proportional samplers to consumers has not however been tested on a large enough scale in the UK to make a confident recommendation.
- (4) For estimating the exposure of population groups there is the alternative approach of combining separately acquired information about the relevant populations of (a) and (b). This is theoretically feasible and attractive in practice, if the degree of approximation can be tolerated in the context in which the results are to be used.

Table 1 Average trace element concentrations in first-draw (FD) and fully flushed (FF) water and exposures for middle-aged men

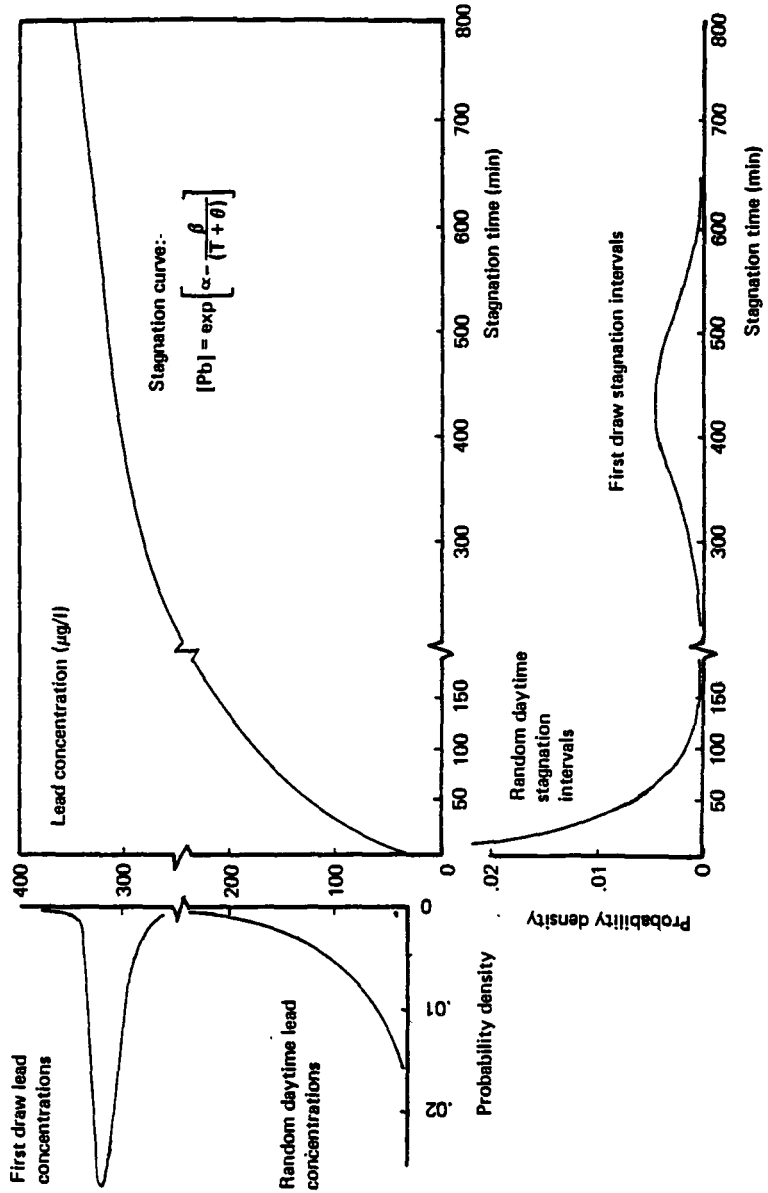
Element	Soft water towns			Hard water towns		
	FD	FF	Intake	FD	FF	Intake
	µg/l	µg/l	µg/day	µg/l	µg/l	µg/day
Ag	3 b	3 b	-	3 b	3 b	-
Al	190	180	230	26 b	27 b	-
B	11	9	13	59	59	74
Ba	45	43	55	88	85	110
Be	0.1 b	0.1 b	-	0.1 b	0.1 b	-
Bi	41 b	48 b	-	42 b	51 b	-
Ca	18,000	17,000	22,000	92,000	90,000	110,000
Cd	2 b	2 b	-	2 b	2 b	-
Co	5 b	5 b	-	5 b	5 b	-
Cr	3 b	3 b	-	5	4	5
Cu	140	24	77	300	14	110
Fe	110	100	130	40	31	44
K	1,000	970	1,200	3,300	3,200	4,100
Li	3 b	3 b	-	7	7	9
Mg	3,300	3,300	4,100	12,000	12,000	15,000
Mn	20	22	27	4	5	6
Mo	37 b	37 b	-	45 b	54 b	-
Na	8,300	8,100	10,000	28,000	28,000	35,000
Ni	20 b	18 b	-	18 b	16 b	-
Pb	47	11	24	14	3	9
Si	2,000	2,000	2,500	4,600	4,500	5,700
Sr	57	56	70	350	340	420
Ti	4	4	5	16	14	18
V	4	4	5	12	12	14
Zn	63	19	39	110	9	42
Zr	4 b	4 b	-	5 b	5 b	-

b means below the analytical limit of detection

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Fig. 1. Modelling the random daytime and first draw probability distributions of tap-water lead concentrations





**TOPIC 17 : WASTE HEAT**



**Contractors:** 1) The Natural Environment Research Council,  
The Dunstaffnage Marine Research Laboratory,  
P.O. Box No. 3, Oban, Argyll. Scotland  
2) Universite Aix-Marseill  II, Station Marine d'Endoume,  
13007 Marseille, France

**Contracts Nos.:** 225-77-1 ENV, UK  
221-77-1 ENV F

**Contract Leaders:** A.D. Ansell.  
H. Mass 

**Title of Project:** Comparisons of the effect on benthic invertebrates from the  
Mediterranean and from northern European waters of  
temperature changes associated with thermal pollution

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Objective of the research

The renewal of these contracts for a further period of two years under the second phase of the Environmental Research Programme allowed a continuation of experimental and field studies of the effects on selected invertebrates of the type of thermal changes usually associated with the discharge of cooling water from electricity generating stations in European coastal waters. The collaboration between Station Marine d'Endoume and the Dunstaffnage Marine Laboratory was continued with the aim of examining how far geographical conditions modify temperature-related functions affecting short and long term survival of invertebrate populations from inshore sandy sediments which may be subjected to discharges of heated water into the coastal zone.

Under the 1st Environmental Programme, emphasis was placed on the determination of thermal tolerance limits for short-term survival of increased temperatures, based on measurements of median lethal temperatures ( $LT_{50}$ ) and median burial temperatures ( $BT_{50}$ ). This emphasis was changed under the 1st phase of the 2nd Environmental Programme to extend observations on the sublethal effects of temperature change, with the aims of defining conditions necessary for long term survival, and of predicting possible effects of temperature increases within the tolerance limits of individual species. Under the 2nd phase of this Programme, that emphasis was continued, with activity centred on 4 main areas, as follows:

- 1) Experimental studies of gastropod molluscs of the families Nassariidae and Naticidae, which occupy a scavenging/predatory role in the inshore sand environment;
- 2) Field studies of bivalve molluscs which are major primary consumers, as well as the main prey of the naticid gastropods;
- 3) Comparative studies of maximum enzyme activities of certain of the bivalve species; and
- 4) Completion of a seasonal study of the meiofaunal copepod populations of two sandy sediments in the Mediterranean, one of which is subject to thermal pollution from an electricity generating station.

A particular interest in this phase was to examine further the inter-relationships of temperature with nutritional and other modifying factors, for both primary consumers and predators in the sandy benthos.

## Results

### 1. Thermal tolerance

In the course of the Programme, the upper temperature tolerance was determined for a total of 20 species from Scottish waters and 25 species from Mediterranean waters, belonging to the following taxonomic groups: Bivalves 14 (14); gastropods 2 (5);

polychaetes 0 (1) and, harpacticoid copepods 4 (5) (Mediterranean species in brackets). 5 of the species of bivalves, 2 gastropods and 1 harpacticoid copepod were common to both geographical areas, and in most cases the representatives included two or more closely-related species from the two geographical areas. For most of the species median burial temperatures were also determined. The results indicate the range of temperature within which lethal effects on at least some species would be expected, for both macrofaunal and meiofaunal species. The results also allow some generalisations to be made on the relationships of temperature tolerance within species, to exposure time, acclimation temperature, or age, size or stage of development; and, between species, to such factors as taxonomic position, vertical distribution in relation to water depth, relative metabolic rate, and geographical distribution.

Results obtained in the present phase of the contract for two species of Ruditapes, R. decussatus and R. philipinarum, from the same biotope in the Mediterranean, illustrate one such relationship; between temperature tolerance and relative metabolic rate. In general, when two species occur together in the same biotope, the species having the higher metabolic rate has a lower thermal tolerance. For the Ruditapes species, measurements of rate of oxygen consumption show that R. philipinarum ( $LT_{50}$ ,  $33^{\circ}C$ ) has a higher metabolic rate at all temperatures in the range  $10-30^{\circ}C$  than R. decussatus ( $LT_{50}$ ,  $35^{\circ}C$ ).

## 2. Experimental studies of the gastropod/bivalve predator/prey relationship.

During this phase of the programme, observations were continued on the predator/prey relationships between gastropods of the family Naticidae and their bivalve prey; in particular comparing Polinices alderi from populations from Mediterranean and Scottish waters. Results of long-term cultures at constant temperature, in some cases extended over more than two years, and of cultures at normally-fluctuating ambient temperatures, provide information on rates of individual functions, including somatic

growth, egg collar production, predation, food ingestion, respiration, and excretion. The relationships between these functions expressed in terms of an energy equation, and as assimilation and growth efficiencies were examined in relation to temperature. Conclusions concerning the dependence of temperature responses on cyclical reproductive activities, and on food availability for Polinices species, and on the consequences of that dependence, were also confirmed and extended by experimental observations with a second species, P. catena.

In P. alderi, reproduction was cyclical, even in constant temperature cultures, with an intrinsic annual periodicity. Rates of growth during pre- or non-reproductive periods were temperature dependant, but during the reproductive period growth rates were low at all temperatures and showed no temperature dependence. Under conditions when food supply was not limiting, most variations in rate of predation and of food consumption could be described in terms of temperature and rate of egg collar production. Without food limitation, temperature had little effect on timing or extent of the reproductive period, provided a critical maximum temperature was not exceeded. Respiration rates were temperature dependant. Somatic growth efficiencies declined with increasing age (and size), but overall growth efficiencies (which include egg production) were independent of age or size. Growth and assimilation efficiencies were little affected by temperature except at near lethal temperatures. Food limitation had very marked effects on the relationships shown, and naticid gastropods show a wide range of compensatory responses under such conditions.

Further comparisons between P. alderi from Mediterranean and Scottish populations, confirm that the differences noted in earlier reports, especially in maximum size reached and in rates of egg collar production, are consistent with the responses to nutritional limitation in the Mediterranean animals, within a similar pattern of temperature response.

Overall, the observations made allow a comparison of preferred (or optimum) ranges of temperature for certain activities by 7 species of nassarid and naticid gastropods from

habitats from the European coastline to be defined, and for detailed profiles of temperature related functions to be given for P. alderi, P. catena, and Nassarius reticulatus.

### 3) Field studies of bivalve mollusc populations

Observations on population structure, recruitment, and growth, and on seasonal changes in tissue weight and biochemical composition, were continued during this phase of the Environment Programme. Observations were made on northern populations of Donax vittatus, Tellina tenuis, Spisula subtruncata, and Venus striatula from two Scottish beaches, at Gullane on the east coast, and Barassie on the west coast. In the Mediterranean, similar observations were made on Ruditapes decussatus at They de la Gracieuse, Gulf of Fos, extending earlier studies of Donax trunculus, Spisula subtruncata and Venus gallina. The results confirm and extend previous observations.

Comparison of the Mediterranean and Scottish species shows a trend in change in life history, and in the events of the seasonal cycle, related to latitude. When populations of the same species are compared bivalves from southern populations show a shorter life span, and earlier sexual maturity than those from northern populations. In population terms, productivity to biomass ratios increase, and interactions between the somatic and reproductive elements of production may lead to smaller maximum size at lower latitudes. There may be less synchrony between individuals in southern populations, with a resulting tendency for reproduction to be continuous, when considered in population terms. For example, in a population of Spisula subtruncata, in Scottish waters, the life span was 5-6 years, sexual maturity was reached in the year following settlement, and growth and reproduction showed an annual cycle. In a Mediterranean population, the life span was short, individuals grew rapidly, reached sexual maturity and spawned within one year, and several cohorts could be produced each year. Ruditapes decussatus, from Scottish waters, shows a life span of more than 12 years, with a maximum size

of  $\approx 70$  mms while in the Mediterranean population studied at They de la Gracieuse, the life span was  $\approx 5$  years and the maximum size only 38 mm, although this may have been affected by fishing.

Comparison of species which replace each other with an overlapping distribution (Donax and Venus species) suggest that similar trends occur within the more limited geographical range of each species in relation to life span, rate of growth, and productivity, but with less extreme variation.

During the seasonal cycle, there is a tendency for changes in tissue weight during the seasonal cycle to be less extreme, and for the importance of storage of reserves to be reduced in southern populations.

#### 4. Field studies of harpacticoid copepod populations.

The initial results of analysis of harpacticoid copepod populations from two sand bottoms in the Mediterranean: one living in the warm water effluent of a power station at Martigues-Ponteau, the second in natural conditions, show possible effects of temperature changes on seasonal population cycles, and on population structure. In the thermal effluent, the density fluctuations observed in natural conditions were damped. The reproductive period started earlier and continued for a longer period in the thermal effluent than in natural conditions.

The results provide an opportunity to compare effects of thermal effluents on harpacticoid populations in the Mediterranean with similar observations made on populations near the generating station at Hunterston in Scotland, by Drs P.R.O. Barnett and B. Hardy. In the south, some species may be excluded from the thermal effluent; for example, one species, Halectinosoma herdmanni, common in natural conditions, was very poorly represented at Martigues-Ponteau. In contrast, under cooler northern conditions, this species flourished in sediments close to the Hunterston effluent discharge.

## 5. Enzyme studies.

The studies of maximal enzyme activities for the bivalve Donax vittatus, begun under the previous phase of the Environment Programme, were continued, and the improved analytical techniques developed in that study were applied to a study of seasonal variation in maximal enzyme activities of D. vittatus, and of the Mediterranean species, D. trunculus, in relation to the major physiological events of the seasonal cycles of reproduction, and storage and utilization of metabolic substrates, in each species. The most pronounced changes found affect certain glycolytic enzymes, particularly phosphofructokinase. Comparative studies of maximal enzyme activities were also extended to include two further species of Donax, D. serra and D. sordidus, from warm-temperate sandy shores of the South African coast. Distinctive differences in enzyme activities between the 4 species studied appear to relate to differences in their habits and ecology, particularly mobility. For example, octopine dehydrogenase activity was high in the foot and adductor muscles of D. serra, and is thought to relate to the extremely rapid and active burrowing movements in this species. Other differences, in phosphofructokinase and citrate synthase activities, indicate a higher metabolic capacity for D. trunculus than for other species, and are perhaps related to the higher temperature tolerance of this species.

## Discussion

These experimental and field studies of temperature responses of marine invertebrates made under contracts with the Environment Programme facilitate the formulation of criteria which may be used in setting temperature limits to protect desirable animal life in the areas of existing or projected thermal discharges. They also provide some experimental basis for the prediction of possible effects of temperature changes arising from warm water discharge.

The data on lethal temperatures and median burial temperatures provide a basis for definition of short-term exposure limits for infaunal invertebrates from sandy sediments. The acceptable maximum limit for short-term exposure is time dependant and based on the thermal resistance lines defined for the most resistant species it is considered important to protect in a given situation. Consideration of time-dependant median burial temperatures, further suggests that limits based on lethal temperatures should be reduced, under certain circumstances, to protect infaunal species, especially in areas subject to wave exposure. The comparative results from Mediterranean and Scottish populations indicate that, for invertebrates from inshore sandy sediments, experimental data derived from observations in one area may be reliably applied, with suitable corrections for small and predictable long-term acclimation, to predictions for the same species elsewhere in its geographical range.

The profiles of temperature-related functions for individual species, and information on the seasonal cycle and its relationship to external factors, provide the basis for the definition of longer-term exposure limits, based on weekly average temperatures. Such limits should be such as to protect important sensitive species from sustained exposure to maintained high temperature; from sudden changes in temperature (under certain circumstances either increasing or decreasing); from temperatures which inhibit reproduction, or lead to breakdown in reproductive synchrony with other important environmental factors; and from temperatures which excessively increase metabolic demand during predictable periods of nutritional stress. The comparative results indicate here, that experimentally determined temperature profiles for infaunal invertebrate species have a general application, with minor adjustments, throughout the geographical range of the species, but that local factors, especially related to nutrition, lead to specific regional considerations in relation to seasonally varying criteria.

These results also provide a preliminary framework for the prediction of possible sublethal effects of temperature increase on infaunal invertebrates, particularly with



respect to seasonal factors, Food limitation, whether sustained or seasonally recurring, is a major factor modifying temperature responses and influencing such predictions. Basic differences between the strategies adopted to deal with seasonally varying food supplies in different species, lead to different predictions concerning the effect of sustained temperature increase on seasonal events, and this in turn affects the way in which such increases may be expressed in different latitudes.

The gastropods examined, and possibly the harpacticoids, show responses to food limitation which allow the maximum reproductive effort to be maintained consistent with prevailing nutritional conditions. The size of the ripe gonad is comparatively small but very high values of reproductive output are achieved by the ability to produce repeated spawnings at very short intervals, when conditions allow. Turnover in the gonad can be very rapid, and temporary food glut is quickly and efficiently utilised in immediate reproductive output. Responses to change in nutritional conditions ensure that a proportion of any non-respired energy intake is allocated to reproduction. There is a limited capacity for storage of metabolic reserves and their utilization for maintenance during periods when food intake is limited. In this case sustained temperature increase may result in a variety of responses involving shifts, extensions, or restrictions of the reproductive period depending on the relationship between temperature and nutritional limitations.

In the bivalves studied, there is also a high reproductive output which increases in proportion with age, but this is achieved by a different strategy. The size of the ripe gonad is relatively large and, while there may be more than one spawning, the indications are that the number of spawnings is restricted. Turnover in the gonad is relatively slow and periods of food glut are exploited, not by an immediate increase in spawned gametes, but rather by incorporation of material in stored reserves, which are then available to be utilized later in a variety of functions. These characteristics of the bivalve cycle provide much greater flexibility in efficient utilisation of short, but predictable, periods of high

food availability (like plankton blooms), as well as providing possibilities of modifying the time relationships between *nutritional intake, gamete proliferation, and spawning*. As a consequence, the predicted effects of temperature increases on the reproductive cycle differ from those discussed for the gastropods. The greater flexibility introduced by the greater capacity for storage of metabolic substrates allows greater independence of temperature, and of other external factors, so that nutritional and/or intrinsic factors may predominate in controlling reproductive timing. In this case, the extent and timing of the reproductive cycle may be relatively insensitive to sustained temperature increase.

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**TOPIC 18 : MARINE POLLUTION**



Contractor: Kernforschungsanlage Jülich, D-5170 Jülich  
Bundesrepublik Deutschland

Contract No: 173-77 ENV/D

Project Leader: Prof. Dr. H.W. Nürnberg, Institute of Applied  
Physical Chemistry, Nuclear Research Center (KFA),  
Jülich

Title of Project: Comparative Studies on Heavy Metal Pollution in  
Selected Regions of European Seas

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### Objectives of the Research

Objectives of general significance were the elucidation of the speciation of dissolved trace metals with certain types of ligands. Comparative studies on the trace metal situation in estuaries at the North Sea and the Ligurian coast have been continued. Moreover, methodological developments lead to new reliable procedures for various trace components. For all topics the research was systematically linked with and based on the achievements obtained during the first phase (1976-78) of the project (1).

### Materials and Methods

In water, suspended particulates and partly also organisms after appropriate pretreatment or digestion procedures differential pulse anodic stripping voltammetry (DPASV) was applied at the glassy carbon supported mercury film electrode (MFE) for Cu, Pb and Cd (2-7) and at the gold disc and twin disc electrode for Hg and Cu (8-11). A new procedure for the determination of Ni and Co traces has been developed (12). Interfacial preconcentration at the hanging mercury drop electrode (HMDE) is achieved by adsorption of the dimethylglyoximates at the electrode surface providing determination limits of 1 ng/l for Ni and several ng/l for Co.

Trace metal contents in sediments were determined by atomic absorption spectroscopy (AAS). AAS checked by DPASV has been also extensively applied for the determination of Cu, Pb and Cd in marine organisms (13, 14). The Hg-content in sea water, sediments and organisms was determined by cold vapor AAS (15) and the As measurements were carried out by hydride AAS (16). Sampling of elemental Hg from the atmosphere was performed by accumulation on silver wool as amalgam.

## Results

### *Speciation Studies*

Based on the obtained experimental evidence for the carbonato-, bicarbonato- and chlorocomplexes of Pb and Cd (17) and literature data for their hydroxy species the general speciation pattern in the sea, due to inorganic complexes, could be established for both toxic metals. Comparative evaluations with and without ion pairing corrections for the interactions between the major salinity components emphasized the particular significance of this ion pairing correction for the speciation of Pb (18). Although it can be concluded, that the elucidated speciation pattern corresponds as a rule fully or predominantly to the situation in the open sea, where the levels of dissolved organic matter (DOM) and correspondingly of organic chelators remains very low, effects on the speciation pattern are to be expected for those estuaries and coastal waters containing elevated DOM levels.

Therefore the previous voltammetric studies on the chelation of Cd, Pb and Zn by the organic model chelators NTA and EDTA had been extended. The results emphasized the particular influences of Ca and Mg, competing for the organic ligands, on the speciation of heavy metals by organic chelators in sea water. Due to the high sensitivity of the applied method of differential pulse stripping voltammetry (DPASV) the experiments could be carried out at sufficiently realistic low overall concentrations ( $10^{-9}$  to  $10^{-8}$  M) of the studied heavy metals (19-21). A result of general significance is that for the formation of heavy metal chelates a ligand exchange mechanism with alkaline earth chelates prevails (22, 23). The required concentrations of these rather strong chelators to achieve noticeable chelation degrees (>10 %) of the studied trace metals (Cd, Pb, Zn) provided prognostic conclusions on the required concentrations of chelating DOM-components being potential candidates for heavy metal chelation in sea water (22-23, 28, 29). In this context no speciation contributions by dissolved amino acids are to be expected, as with respect to their stability constants their levels in sea water are too low. Measurements with L-aspartic

acid (24) and glycine (25, 26) have experimentally confirmed this prognosis for Cd and Zn. A similar prognosis has been made for the chelation of Cd, Pb and Zn by dissolved humic material (22, 23, 28, 29). Results of electrophoretic measurements supported this prognosis (27-29). Meanwhile it has been confirmed directly by voltammetric experiments with humic material of marine sediment origin as ligand that up to 1 mg/l the chelation of Cd and Pb remains negligible while for Zn above 0.7 mg/l the onset of chelation becomes just noticeable and reaches about 15 % at 1 mg/l. Even in coastal waters dissolved humate levels of this magnitude are not to be expected at many locations. These findings are in agreement with the moderate conditional stability constant determined for the Cd-humate. This investigation has also revealed that this polymeric chelator has three types of chelation sites of decreasing stability. However, at the trace level of Cd in sea water only one type of chelator sites, i.e. that with the relatively highest stability constant, is operative (30).

#### *Studies in German Bight*

Within the project part devoted to field studies on the levels of the toxic trace metals Cu, Pb, Cd in the surface zone of coastal waters the investigations at the German coast of East Friesland (seawards of the islands and in the Wadden Sea) and in the German Bight have been completed (31, 32). With 15-20 mg/l for dissolved Cd and an average of 53 ng/l for dissolved Pb rather stationary levels were obtained for the coastal waters. Only around Heligoland the dissolved Cd-level is increased to 28 ng/l. However, in the Wadden Sea a wide range of dissolved Pb (40 to 630 ng/l) is measured while for dissolved Cd the range remains with 25 to 50 ng/l rather similar to that in the other coastal waters of the German Bight. Also the total level, i.e. the sum of trace metal present in the dissolved state and bound to suspended particulate matter stated as concentration per kg sea water, remains with 33 to 45 ng/kg for total Cd in a rather narrow range. For Pb the situation is rather diversified between certain regions. The lowest average value for total Pb exists with 150 ng/kg around Heligoland. Off the coast of Schleswig-Holstein the average level increases to 440 ng/kg and off the

East Frisian Islands the average value is only 220 mg/kg while in the Wadden Sea a wide range from 400 to 3560 ng/kg is measured.

In general the data reflect that the majority of the total Pb concentration per 1 kg sea water is bound to suspended particulate matter, provided there is enough present, as it is the case in the German Bight. Of the total Cd, however, 50 % or more prevail in the dissolved state. Only in the Wadden Sea with its high content of suspended matter over 60 % of Cd are bound to particulates.

At 20 stations from the Dollart to the Isle of Amrum the level of dissolved Hg has been determined by subtractive DPASV at the twin gold electrode (10). The average concentrations are usually between 5 and 15 ng/l. Only at two stations, one in the estuary of the Ems and one in the outer Weser it increases to 25 to 30 ng/l and an exceptionally high value of 65 ng/l was found off Scharhörn.

#### *Comparative Studies on Estuaries*

A field study in the inner Elbe estuary has demonstrated that the developed analytical procedure with simultaneous voltammetric determination of Cu, Pb and Cd can be performed satisfactory on board of a small motor launch and is thus very useful for toxic metal monitoring in rivers and estuaries.

In September 1978 a detailed study on the seasonal behaviour of Cu, Pb and Cd was started in collaboration with the Delta Institute of the Royal Dutch Academy, Yerseke, in the *Ooster- and Wester Scheldt Estuary*. Samples were taken during different seasons from 1978 to 1980 between Zieriksee and Volkerak lock in the Ooster Scheldt under comparable tidal conditions and for comparison in the Wester Scheldt off Hansweert. Chlorinity and the levels of phosphate, nitrate and silica and of chlorophyll and organic carbon were followed as well (33).

Pb is overwhelmingly bound to suspended particulate matter and shows no pronounced seasonal dependence. Also for Cu no clear seasonal correlations are observed probably due to interferences by ship traffic. In the Ooster Scheldt in general the trace metal level was lower in September 79 compared with

September 78 and March 79. For Cd a significant seasonal dependence exists which is related to the phytoplankton content reflected by the chlorophyll level. During the phytoplankton blossom in March Cd has its peak value and is mainly found in suspended particulates while it exists in September preferentially in the dissolved state.

The pollution with toxic metals is substantially higher in the Wester Scheldt and can be correlated to the input degree of polluted fluvial waters from measurements at high water (input of sea water into the estuary) and low water (excess of fluvial water).

Applying atomic absorption spectroscopy the uptake of toxic trace metals by salt marsh plants from the Scheldt Estuary has been subjected to an intensive study. While the accumulation of Hg, Pb and Cu remains rather small a particular accumulation is observed for Cd.

Based on the station of the institute in Fiascherino/La Spezia the inventory of the toxic trace metals Cu, Pb, Cd and Hg and their seasonal fluctuations have been investigated by analysing samples of water, particulates, sediments, mussels, crustaceans and fish from the estuaries of the rivers Magra, Serchio, Arno, Cecina, Ombrone, Albegna and Fiora along the Ligurian and Tyrrhenian coast (33, 34). Preliminary investigations were also started for the Tiber Estuary. Besides the clarification of the trace metal situation in the studied estuaries the obtained results will provide the data basis for the fluvial trace metal input into the Mediterranean Sea from these Italian rivers. The dissolved content of Cu, Pb and Cd is low and corresponds to the average levels (mostly category "low") determined previously (31) for Ligurian and Tyrrhenian shore waters while the surface layers of the sediments have a 3 to 4 orders of magnitude higher content and can act therefore as an efficient and long lasting reservoir. The total Hg-content per 1 l sea water (dissolved plus bound to particulates) ranges from 0.02 to  $> 0.1 \mu\text{g/l}$  in the sequence Serchio  $<$  Cecina  $<$  Magra, Albegna, Ombrone  $<$  Fiora. In the Arno Estuary it alters from winter 79 to spring 1980 from 0.150 to  $0.025 \mu\text{g/l}$ . The substantially

higher Hg-content in the sediments is mostly in the mg/kg range and follows only broadly the foregoing sequence with 0.16 Serchio; 0.3-0.5 Arno, Magra; 1.0 Cecina, Ombrone; 5.4 Albegna, Fiora. These investigations have been carried out partly in collaboration with the CNEN-Laboratory for Marine Contamination in Fiascherino. They are also like the subsequently reported activities contributions to the UNEP/FAO-program "Pollution in the Mediterranean".

#### *Studies in Coastal Waters*

A comparative study was performed on the total As-content in sea water and 167 specimens of 26 different marine organisms from the Mediterranean and other seas (16, 35). While the average total As-level in sea water is 2  $\mu\text{g/l}$  a wide range has been found in marine organisms from 10 to 630 mg/kg dry weight. The highest concentrations had bottom feeding fishes from the North Sea off the Norwegian coast. In contrast to the elevated Hg-levels in Mediterranean fish (15, 36) no elevated As-levels occur compared with fish from other seas (35).

In September 1980 the participation in the British research mission "Operation Drake" led to investigations in the coastal zone of the Gulf of Genoa. Usually the dissolved levels in the surface water are rather low (Cd 5-12; Pb 110-210; Cu 200-730; Hg 7-18 ng/l). Measurements of the complexation capacity with Cu(II) indicated that only negligible amounts of chelating material are present. The availability of the airship "Europa" of Goodyear Company permitted to determine the level of elemental Hg in the atmosphere (150 to 550 m) over the coast line from Diano Marina to Genoa. With the exception of point emission sources in Vado Ligure and Genoa the concentrations are very low (average 2.45 ng Hg/m<sup>3</sup>). A significant Hg-input from the atmosphere into coastal waters of this region is therefore not to be expected.

### Conclusions

The situation concerning the heavy metal pollution in the studied coastal areas and estuaries has become much clearer due to the assembled data. Future research is to be directed primarily to the following items:

- Elucidation of natural DOM-components responsible for non-labile trace metal speciation as reflected by complexation capacity of the water.
- Interaction of trace metals with small size virtually continuously suspended particulates.
- Elucidation of pathways of Hg-input into Mediterranean Sea in view of the elevated Hg-content in many of its organisms.
- Continuation of the observation of the seasonal influences on the toxic metal levels in the studied estuaries and extension of the comparative investigations to further estuaries of European Seas. Supporting measurements on the significance of atmospheric input by rain and dry deposition.

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Contract no. : ENV/391 I  
Project leaders : Giovan Giacomo Giordano, Giovanni Pagano  
Title of project : The gametes and embryos of sea urchins in testing environmental pollution. A screening of some heavy metals in sea urchin development.  
Contributors : P. Bove, M. de Angelis, A. Esposito, A. Rota

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#### Objective of the research

The evaluation of the environmental hazards due to heavy metal pollution requires a complement of information. A compulsive requirement in this evaluation implies the extensive knowledge of some key biological events, such as fertilisation, mitotic activity and embryonic differentiation.

A single test system usually provides a single kind of data, like e.g. mutagenesis or embryotoxicity test systems.

The sea urchin test system may, on the contrary, provide a multiple set of information, concerning different developmental events, from fertilisation to cleavage and to larval morphogenesis. A toxicological screening in the sea urchin test system, therefore, may lead to integrated information on reproductive balance, embryo- and genotoxicity of a given agent, in the same biological material and in any individual experiment. Owing to this peculiarity of the sea urchin system, a screening of pollutants like heavy metals may result in fruitful information which can be rationally extended in the control of environmental hazards. This implies that a most relevant aim of our study is to provide additional and complementary information, aside from those obtained in other test systems, like vertebrate and microbial systems.

#### Material and methods

Sea urchin were collected by the staff of the Stazione Zoologica from the Gulf of Naples. The experiments were carried out on the two species :

### Parental and Sperm Chromosome Abnormalities.

Treatments schedules included the exposure of: (a) zygotes; (b) embryos at different developmental stages; (c) sperm; and (d) eggs. Following the pre-treatment of gametes, development ensued in the virtual absence of the test substance(s).

Observations were performed on living material, except for total observations of fertilized eggs. These were fixed in Carnoy, transferred into absolute ethanol and stained with acetocarmum.

Fertilization rate was determined according to Hagstam and Hagstam (1959). Sperm motility parameters were carried out according to our recent method (Ragano et al., 1980).

Statistical evaluation was performed by means of 2x2 tables ( $\chi^2$ ) and Student's t test.

### Results

When previously reported the action of  $Co^{2+}$ ,  $Cr^{6+}$  and  $Cr^{3+}$  on sea urchin fertilization and development.

$Co^{2+}$ , at concentrations ranging from  $55 \times 10^{-5} M$  to  $55 \times 10^{-4} M$ , exerted a severe embryotoxicity on sea urchin, if present throughout development, while the hatchlings of zygotes or of gametes never resulted in any developmental defects, even by raising  $Co^{2+}$  levels to  $10^{-3} M - 10^{-2} M$ . However, acute sperm exposure resulted in a depression of fertilization rate.

$Cr^{6+}$  displayed some severe abnormalities in gut and skeletal development, and its adverse effects were observed in all treatment schedules, through re-quickening of cytochrome levels ( $10^{-4} M$  for short-term exposures). Moreover,  $Cr^{6+}$  displayed a severe depressive action on mitotic activity during cleavage of both the exposure of either gametes or on the fertilization.  $Cr^{3+}$  compounds, on the contrary, displayed no such embryonic and genotoxic action, only resulting in an impairment of hatching and of larval motility for prolonged exposures in  $10^{-4} M$   $Cr^{3+}$ .  $Cr^{3+}$  exposure of sperm induced a loss of fertilizing capacity, at the same  $Cr^{3+}$  level.

In this study, further investigations were performed with  $\text{Cd}^{2+}$ , and the action of inorganic oxidants was examined by investigating the effects of an energetic oxidising agent, such as  $\text{KMnO}_4$ . Moreover, other organic compounds were tested, i.e. :  $\text{HgCl}_2$ ,  $\text{NiCl}_2$ ,  $\text{NaAsO}_2$ , and  $\text{NaHAsO}_4$ .

Comprehensive data on the action of tested compounds for the different treatment schedules are reported in Tables 1-3. Embryotoxicity was exerted by all of tested compounds, with the exception of  $\text{Cr}^{3+}$ . Evidence of genotoxicity was obtained for  $\text{CrO}_4^{2-}$ ,  $\text{MnO}_4^-$ ,  $\text{Hg}^{2+}$ ,  $\text{AsO}_2^-$ , and  $\text{HAsO}_4^{2-}$ .  $\text{Ni}^{2+}$  displayed a moderate genotoxic action, which is subjected to further study. No such action was detectable for  $\text{Cd}^{2+}$  and  $\text{Cr}^{3+}$ , in wide ranges of concentrations and exposure times. The genetic action of  $\text{Hg}^{2+}$  was expressed by developmental defects following the pretreatment of gametes, for  $\text{Hg}^{2+}$  levels ranging from  $5 \times 10^{-8}\text{M}$  to  $5 \times 10^{-7}\text{M}$ . Moreover, a short (30 min) and early exposure of zygotes resulted in a number of mitotic abnormalities, like hyperchromic metaphase and bridges. These morphological features were associated with an increase in Mitotic Index, which was highest at  $10^{-7}\text{M}$ , as shown in Fig. 1. At this  $\text{Hg}^{2+}$  level, the increase in Mitotic Index was consistently observed throughout cleavage, as shown in Fig. 2. An action of  $\text{Hg}^{2+}$  on spindle apparatus had been previously demonstrated by Ramel (1972) in other systems. That  $\text{Hg}^{2+}$  genotoxicity should not be confined to spindle inhibition, is however suggested by the effectiveness of  $\text{Hg}^{2+}$  sperm pretreatment, which also leads to several developmental defects, including abnormal cleavage.

The comparison of the embryotoxicity and genotoxicity of  $\text{As}^{3+}$  versus  $\text{As}^{5+}$  is suggestive of common features in their action mechanisms, due to the very similar morphological patterns observed. A peculiar defect, following  $\text{As}^{3+}$  and  $\text{As}^{5+}$  sperm pretreatment ( $10^{-5}\text{M}$  to  $10^{-4}\text{M}$ ) was observed in gut differentiation, resulting in the lack of liming and cell clumping. The prolonged exposure of zygotes and the pretreatment of the eggs ( $10^{-4}\text{M}$  to  $10^{-5}\text{M}$ ) resulted in most severe injuries to differentiation, leading to larval populations of cell-filled blastulae in *S. granularris*. The same treatment schedules at the same As levels permitted gastrulation and early skeletal differentiation in *P. lividus*, but pluteus stage was not reached or severe skeletal inhibition was observed. All of the described effects,



however, were more pronounced in  $As^{5+}$ , as compared with  $As^{3+}$ , and appeared to differ of about one order of magnitude in As levels.

The action of  $Ni^{2+}$  resulted in dramatic and peculiar embryotoxicity in both species, though S. granularis appeared to be somewhat more resistant than P. lividus. Skeleton was severely affected at  $10^{-5}M$   $Ni^{2+}$  and cell separation was observed. The eggs of both species were affected by short (5 min) exposures prior to fertilisation in  $10^{-4}M$   $Ni^{2+}$ , undergoing several larval abnormalities in 20% of embryos, reaching 100% in  $10^{-3}M$   $Ni^{2+}$  egg pretreatment. The exposure of sperm to  $Ni^{2+}$  resulted in a slight, but significant increase in developmental defects, by increasing  $Ni^{2+}$  levels from  $10^{-5}M$  to  $10^{-3}M$ . This effect suggests a weak mutagenicity of  $Ni^{2+}$ , which is to be further investigated.  $Ni_2S_3$  was tested in few experiments, suggesting that a direct contact of  $Ni_2S_3$  particles is required in order to result in biological effects, as only the pretreatment of eggs resulted developmental defects (100% for nominal  $10^{-5}M$   $Ni_2S_3$ ).

$KMnO_4$  exposure resulted in a number of severe abnormalities in a narrow range of concentrations around  $10^{-5}M$ , as  $10^{-6}M$   $KMnO_4$  appeared to be ineffective and  $10^{-4}M$   $KMnO_4$  resulted in the fixation of material. However, all of the treatment schedules in both species gave rise to severe developmental defects. The effectiveness of  $Mn_4^-$  on sperm pretreatment is strongly suggestive of a genetic action, which had been ruled out by Tsuda and Kato (1977) in embryonic hamster cells, and recognised as weak by Umeda and Nishimura (1979) in transformed mammalian cells. Our data are to be complemented with the morphological observation of mitotic figures. However, the defects observed in  $MnO_4^-$  exposure of gametes and zygotes appeared at  $MnO_4^-$  levels which were about one order of magnitude below the active levels required for  $CrO_4^{2-}$ . Our investigation on the comparative effects of inorganic oxidants is presently in progress in a dosimetric approach.

Further experiments were performed on  $Cd^{2+}$  action of the fertilising capacity of sperm, for  $Cd^{2+}$  levels which were closer to those observed in polluted bodies of water. As shown in Fig. 3, a surprising inversion of  $Cd^{2+}$  spermotoxicity was observed by decreasing  $Cd^{2+}$  levels below  $10^{-6}M$ . The interpretation of the promoting action of  $Cd^{2+}$  on fertilisation, for low

$Cd^{2+}$  levels, is open. However, these data appear worth testing in the fertilisation of other test organisms. Moreover, bioaccumulation in adults should be taken in account, so that relatively low  $Cd^{2+}$  environmental levels might be raised to spermiotoxic concentrations at the tissue level. These suggestions should possibly lead to further studies on adult sea urchins.

### Conclusions

The embryotoxic and genotoxic affects of the inorganics tested in the sea urchin test system widely confirmed available knowledge, as obtained in other test organisms. Particularly, the well-known genotoxicity of some agents, such  $Cr^{6+}$ ,  $As^{3+}$ ,  $As^{5+}$ , and  $Hg^{2+}$  resulted in evident damage to differentiation and cleavage. The latter was expressed both by changes in the morphology of blastomeres and of mitotic figures. In the case of  $Cd^{2+}$ , such damage could never be observed, its action being confined to embryotoxicity and to changes in fertilisation rate, following the exposure of sperm. These changes consisted of a depression of fertilisation rate at high to moderate  $Cd^{2+}$  levels ( $10^{-6}$  to  $10^{-4}M$ ), while lower Cd levels (such as  $10^{-8}M$ ) resulted in a significant promotion of fertilisation rate. Ni, both as divalent cation and in its water-insoluble subsulphide, appeared to inflict damage to differentiation.  $Ni^{2+}$ , moreover, displayed a weak, but evident increase in developmental defects following the exposure of sperm.

The results obtained in this study point out the need to further investigate the action of inorganic substances characterised by carcinogenic, teratogenic and genotoxic action, in order to better define the potential risks of these chemicals in environmental and human health.

Table 1

Exposure of embryos throughout development

Active concentrations and types of developmental defects observed in screening inorganics in the sea urchin test system. Eventual crystallization water was omitted in listing chemicals.

Substance	Active concentration   M	Affected cleavage	Affected hatching	Affected differentiation		other
				skeleton	gut	
$\text{Na}_2\text{CrO}_4$	$10^{-4}$	-	-	++	++	+
$\text{KCr}(\text{SO}_4)_2$	$10^{-4}$	-	+	-	-	motility
$\text{CdCl}_2$	$10^{-4}$	-	-	++	-	-
$\text{KMnO}_4$	$5 \times 10^{-6}$	+	-	++	++	-
$\text{HgCl}_2$	$10^{-7}$	+	-	++	++	cell separation
$\text{NiCl}_2$	$10^{-5}$	+	-	++	++	cell separation
$\text{Ni}_2\text{S}_3$	inactive ( $10^{-5}$ )	-	-	-	-	-
$\text{NaAsO}_2$	$10^{-5}$	-	-	+	+	epithelium
$\text{Na}_2\text{HASO}_4$	$10^{-5}$	-	-	++	++	cell separation ( <u>S. granularis</u> )

Table 2

Sperm pretreatment

Active concentrations and types of developmental defects observed in screening inorganics in the sea urchin test system. Eventual crystallization water was omitted in listing chemicals.

Substance	Active concentration [M]	Exposure time (min)	Affected fertilization	Affected cleavage	Affected skeleton	Affected gut	Affected differentiation other
$\text{Na}_2\text{Cr}_2\text{O}_7$	$10^{-3}$	10-30	+/-	+	+	+	-
$\text{KCr}(\text{SO}_4)_2$	$10^{-4}$	2	++	-	-	-	-
$\text{CdCl}_2$	inactive ( $10^{-8}$ to $10^{-2}$ )	inactive (2 to 60)	+/- <sup>§</sup>	-	-	-	-
$\text{KMnO}_4$	$10^{-6}$	2	+	++	++	++	++ cytolysis
$\text{HgCl}_2$	$10^{-7}$	1-2	+	+	++	++	-
$\text{NiCl}_2$	$10^{-4}$	5	+/-	-	+	+	-
$\text{NaAsO}_2$	$10^{-4}$	5	-	+	+	++	-
$\text{Na}_2\text{HASO}_4$	$10^{-4}$	5	-	+	++	++	++ cytolysis

<sup>§</sup> depression  $\geq 10^{-6}\text{M}$ , promotion at  $10^{-8}$  to  $10^{-7}\text{M}$

Table 3

Egg pretreatment

Active concentration and types of developmental defects observed in screening inorganics in the sea urchin test system. Eventual crystallization water was omitted in listing chemicals.

Substance	Active concentration [M]	Exposure time (min)	Affected cleavage	Affected skeleton	Affected gut	Affected other
Na <sub>2</sub> CrO <sub>4</sub>	10 <sup>-3</sup>	30-60	+	+	+	-
KCr(SO <sub>4</sub> )	inactive (10 <sup>-3</sup> )	inactive (60)	-	-	-	-
CdCl <sub>2</sub>	inactive (10 <sup>-2</sup> )	inactive (60)	-	-	-	-
KMnO <sub>4</sub>	10 <sup>-6</sup>	10	+	++	++	-
HgCl <sub>2</sub>	10 <sup>-7</sup>	10	+	++	++	cell separation
NiCl <sub>2</sub>	10 <sup>-3</sup> 10 <sup>-3</sup>	10	+	++	++	-
NaAsO <sub>2</sub>	5x10 <sup>-5</sup>	10	-	+	-	-
Na <sub>2</sub> HAsO <sub>4</sub>	5x10 <sup>-5</sup>	10	-	++	++	-

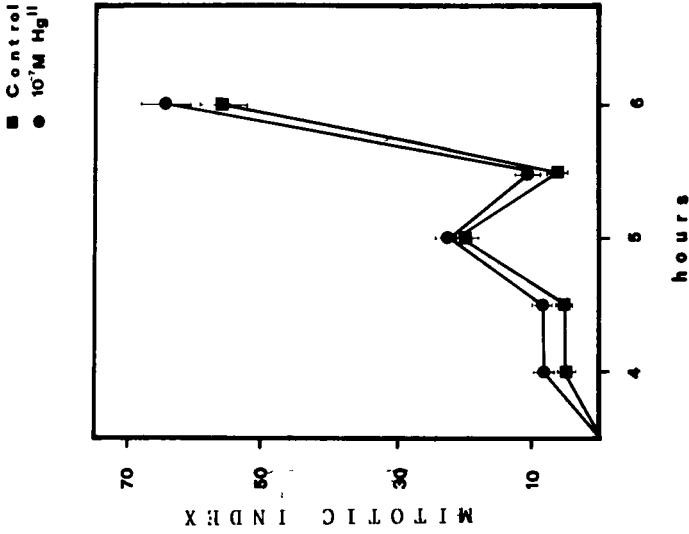


Figure 2. Zygote exposure (30 min, starting 30 min after fertilization) was followed by 2 thorough washes. Fixation was subsequently performed at 30 min intervals starting from the 4th hr after fertilization. Hg<sup>2+</sup>-treated zygotes displayed a significant increase in Mitotic Index, as determined by |t| test.

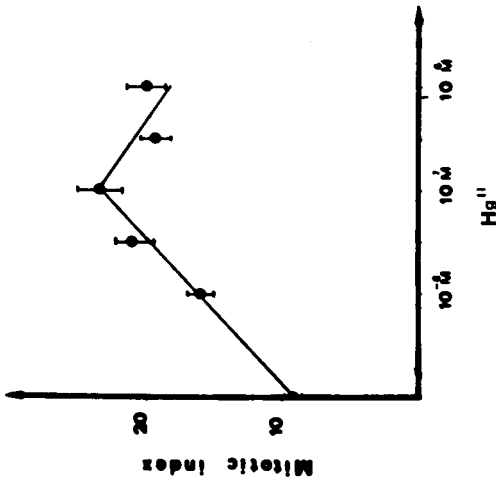


Figure 1. Zygotes were exposed to Hg<sup>2+</sup> 30 min, thereafter washed 2x and fixed 5 hrs after fertilization. Mitotic Index in control zygotes was conventionally given 0 in Hg<sup>2+</sup> level.

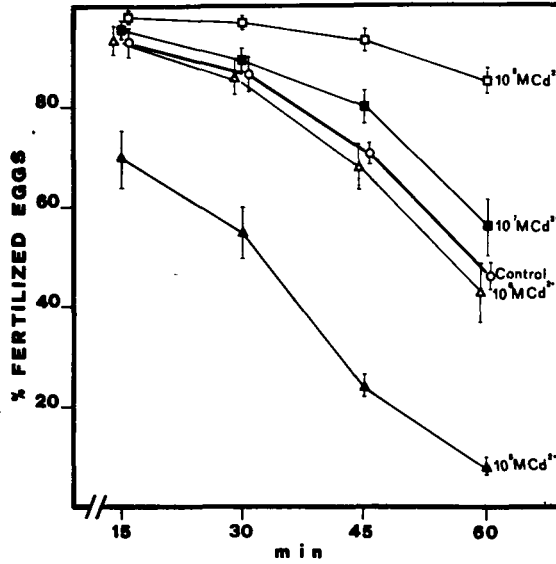


Figure 3. Sperm inactivation experiment (*P. lividus*), mean of six individual experiments. Cd<sup>2+</sup> levels in control seawater were in the range 10<sup>-10</sup>M to 10<sup>-9</sup>M.

Contractor : Istituto G. Donegani S.p.A.  
Novara  
Contract no. : ENV/438 I  
Project leaders : V. DAMIANI and R. DE SIMONE  
Title of project : Identification and analysis of metabolites in  
aquatic environments

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#### Objective of the research

The industrial success of PCB's (thanks to the exceptional chemical and physical characteristics of the various mixtures of the more than 210 isomers deriving from biphenyl) caused their world production to exceed  $10^3$  k tons. Before their use came to be restricted to within closed systems only, the dispersal of much of them in the environment had already created numerous pollution problems.

Research carried out so far on the environmental fate of PCB's has ascertained little biodegradation of components having more than five atoms of chlorine, and an environmental persistence at DDT level even for the less halogenated components.

It has also been demonstrated that environmental degradation of components produces molecules that are more toxic, and that photolysis lowers chlorine content of higher chlorinated PCB's so that metabolisation becomes possible. It is therefore of fundamental importance to understand the mechanisms of environmental degradation, in order to predict the possible evolution of products of higher chlorine content that have accumulated in the environment.

Environmental transport of PCB's is such that photochemical reaction is possible while they can reach sediments with different qualitative/quantitative content in micro-organisms. Accordingly, a possible hypothesis as to the environmental fate of a PCB with high chlorine content is that illustrated in Fig. 1 .



### Material and methods

To verify the above hypothesis, metabolism speed and extent must be evaluated as a function of illumination in two model micro-systems, by analysing sediments as well as biological (detritivorous and water-filtering) indicators.

To this end, the following four tanks were prepared :

<u>Tank_1</u>	8/24 sunlight coastal sediments biomass *	<u>Tank_2</u>	8/24 sunlight deep-sea sediments biomass *
<u>Tank_3</u>	continuous darkness coastal sediments biomass *	<u>Tank_4</u>	continuous darkness deep-sea sediments biomass *

The PCB concentrations must approximate those found in nature (in sediments, a few parts per million). Therefore, more information may be expected from the analysis of biological indicators: Clams (being detritivorous) should serve as amplifiers of sediment processes, while mussels should have a similar role for water processes (essentially photodechlorination and solubilisation of polar metabolites coming from enzymic activities in sediments).

To avoid insoluble analytic problems, as would arise in searching for traces of an unknown metabolite,  $C^{14}$  products has to be used.

#### a) Experimental plant

Our plant - but for the appropriate modifications, practically the same as that used by other workers - consists of an aquarium with a sand bed raising a few centimetres above the bottom of a glass tank, water circulation being regulated to achieve slow but steady percolation through the sand bed. Literature data indicate that micro-biological equilibrium is reached after 1-2 months operation.

Particular care was taken of the water-circulation characteristics (homo-

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\* mussels + clams + zooplankton + phytoplankton

geneity and speed of the whole water mass through the sand bed), by making several tests with Rhodamine B. This coloured tracer is an easily quantifiable compound, exhibiting a well-defined absorption peak at 554 nm.

The tracer distribution in the sediments was quantified by colorimetric measurements of the aethanolic extract from sand samples taken in ideal-sampling squared. A homogeneous distribution was obtained by increasing the aspiration surface by means of a U-pipe having a lateral aperture 100-cm long. Pump movement was regulated to achieve a flow rate of 2.5 litre per minute, which ensured complete recycling every hour.

Furthermore, using Rhodamine, we were able to calculate that the maximum downward speed of water was 1.3 mm per second. Considering Hjulstrom's experiment, one sees that such a speed does not in practice affect sedimentation of 0.004-mm particles.

#### b) Analytical methods

The first object of chemical analysis for this project was to establish the extent of transformation undergone by marked PCB's.

Accordingly, we first searched for experimental conditions in thin-layer chromatography that would ensure absolutely clear separation of polar and apolar products. Best results were achieved using Merck silica-gel 60 plates with n-hexane as the eluant (fig. 3).

The other major analytic problem concerns the extraction of marked products (whether from sediments or bio-indicators), which must be complete for both PCB's and transformed products. To this end, in the literature, different procedures are suggested according to the material to be treated.

Using Soxhlet extraction for 8 hours with a mixture of methanol and n-hexane (30% and 70% respectively), we achieved recovery levels of more than 90% of both Aroclor and Chlorophenol with both types of samples (Table 1).

The overall scheme of the analytical procedure is that illustrated in the flow diagram.

Results and conclusion

Because of electrical damage to the plant, the experiments had to be interrupted. They are now again in progress, and first results, yet to be confirmed, seem to indicate high toxicity of the metabolisation products.

Oral communication

R. DE SIMONE, V. DAMIANI, and G. IZZO : Identification and analysis of PCB metabolites in aquatic environments.

Report to the Marine Pollution Contact Group (Ischia), June 1-2 1981.

T A B L E 1

RECOVERY OF AROCLOR 1254 AND CHLOROPHENOL FROM SEDIMENT AND MUSSEL HOMOGENATE

	S E D I M E N T S			MUSSELS HOMOGENATE		
	ADDED ppb	FOUND ppb	RECOVERY %	ADDED ppb	FOUND ppb	RECOVERY %
AROCOR 1254	500	470	94	100	97	97
	1000	950	95	500	480	96
	1500	1410	93	1000	985	98
	2000	1930	96			
CHLORO- PHENOL	100	95	95	100	90	90
	300	280	93	200	180	90
	700	650	93	500	450	90
	1000	1000	100			

FIG. 1

FATE OF PCB<sub>S</sub> IN THE ENVIRONMENT

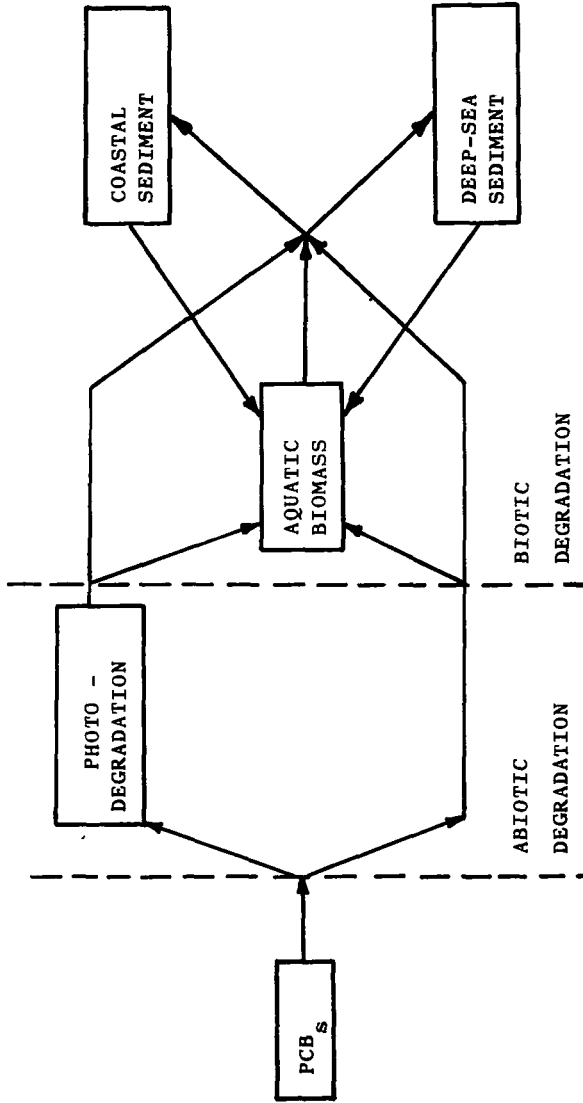


FIG. 2 - DRAWING OF MODEL ECOSYSTEM EMPLOYED (100 x 50 x 150 cm)

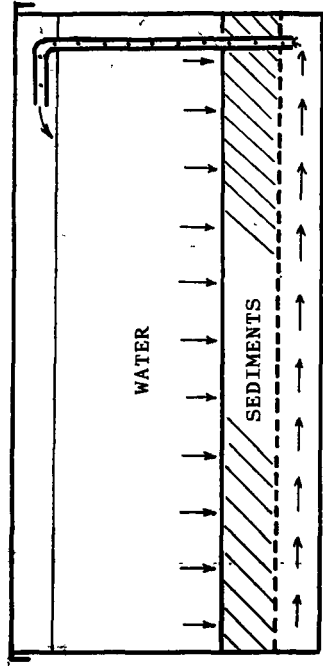
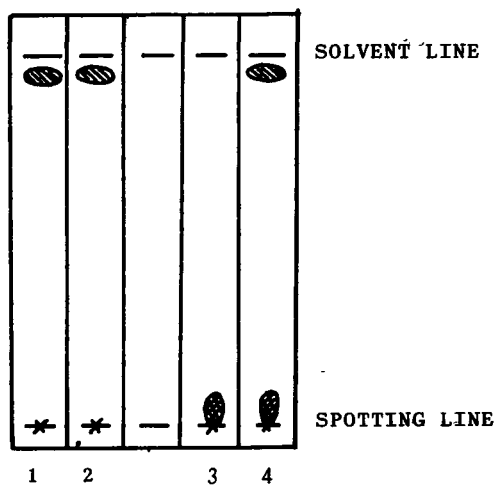


FIG. 3 - CHOMATOGRAPHIC SEPARATION OF AROCLOR 1254 FROM CHLOROPHENOL



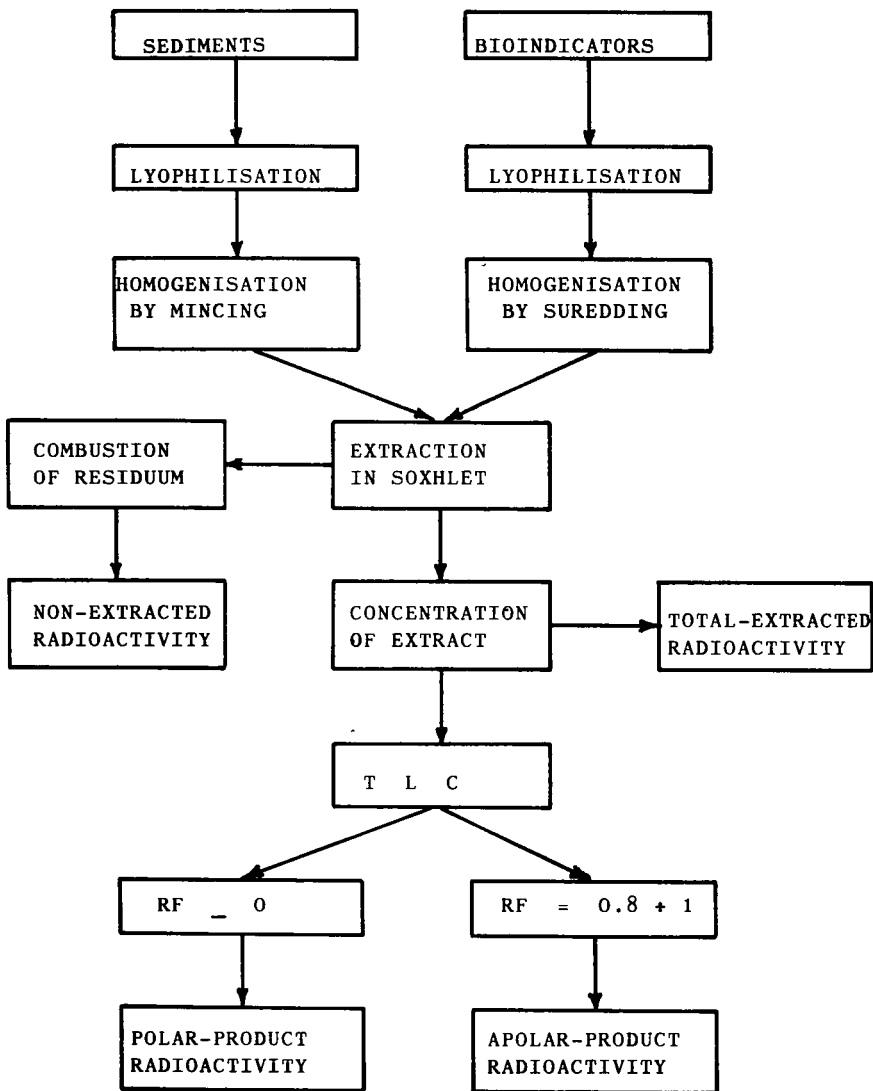
1 : AROCLOR 1254

2 : SEDIMENTS EXTRACT + AROCLOR 1254

3 : CHLOROPHENOL

4 : SEDIMENTS EXTRACT + AROCLOR 1254 + CHLOROPHENOL

# ANALYTIC PROCEDURE



Contractor : Organization of Industrial Research TNO, Div. of Technology  
for Society  
Contract no. : ENV/461 N  
Project leader : Jan Kuiper  
Title of project: Fate and effects on experimental marine plankton  
communities of selected organic pollutants

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#### Objective of research

Extrapolation of the results of laboratory toxicity and biodegradation experiments to field conditions is difficult, if not impossible. In order to assess the value of experiments in the laboratory, experiments are needed with more complex systems which can be regarded as approximating field conditions more closely. To this end many types of model ecosystems have been developed in the past decade.

To bridge the gap between the laboratory and the natural aquatic environment, many investigators have used large plastic bags, enclosing natural plankton communities and suspended in natural waters. Our laboratory started this type of research with Dutch coastal water plankton communities in 1974. After development of the method and investigation of the fate and effects on structure and function of the enclosed communities of mercury and cadmium during the first part of the 2nd Environmental Programme, the impact of selected organic compounds on marine plankton ecosystems housed in large enclosures was studied. Fate and effects of phenol, 4-chlorophenol (4CP), 2,4-dichlorophenol (DCP) and 3,4-dichloroaniline (DCA) were studied in three experiments.

#### Material and methods

Table 1 shows the general set up of the experiments. At the start of each experiment 6 to 8 enclosures were simultaneously filled with 1.5 m<sup>3</sup> of natural sea water each (Figure 1). The different model pollutants, which were chosen because laboratory experiments had shown that they differ widely in biodegradability, were added to the systems in a single dose a few days after filling the bags. A single dose was added since single additions most closely approximate the "normal" field situation if the source of a pollutant is an outfall, a river or a dumping event. During the experiments, which lasted 4-7 weeks, the development of the bacteria, phytoplankton (species composition, biomass, cell numbers, chlorophyll, phaeopigments, primary production (C-14 method)) and zooplankton (species composition, biomass, development from nauplius to adult) was monitored. In addition various abiotic parameters, such as global



**TABLE 1:** Some general data about the set-up of the three enclosure experiments with Dutch coastal water in large plastic bags.

experiment	model pollutant	initial concentrations	number of bags	duration in days
1	control	-	2	35
	DCA	2 $\mu\text{g.l}^{-1}$	1	
		10 "	2	
		25 "	1	
2	control	-	2	42
	4CP	0,1 $\text{mg.l}^{-1}$	1	
		1,0 "	1	
	DCP	0,1 "	1	
		1,0 "	1	
	DCA	0,1 "	1	
	1,0 "	1		
3	control	-	1	28
	phenol	3,8 $\mu\text{g.l}^{-1}$	1	
	4CP	0,3	1	
		1,0	1	
	DCP	0,3	1	
	1,0	1		



*Fig. 1: Plastic enclosures (contents 1.5 m<sup>3</sup>) anchored in the harbour of Den Helder, the Netherlands.*

radiation, concentrations of oxygen, phosphate, nitrite, ammonia, silicates and pH, temperature, salinity, concentration of the pollutant in water and in the sediment, were measured, since these factors influence the development of the organisms on the various trophic levels in the enclosed system. An experiment which focusses directly on a comparison between the results of laboratory toxicity- and biodegradation-tests will be performed in the spring of 1981; in this experiment 4CP, tetrapropylene benzene sulphonate (TPBS) and a polychloro biphenyl (PCB) mixture (Aroclor 1226) will be used as model pollutants.

## Results

### General development of the enclosed ecosystem

The results of the experiments have been reported in detail by Kuiper (1980, 1981). Although no additional nutrients were added during the experiments, phytoplankton production was sufficient throughout the experiments, which lasted 4-7 weeks. The regeneration of nutrients by heterotrophs was obviously large enough to promote phytoplankton growth throughout the experiments. In most experiments a succession of 2 or 3 blooms of phytoplankton occurred. In most of the controls, succession of a first bloom of diatoms was followed by blooms of flagellates. At the end of the experiments  $\mu$ -flagellates were the most important phytoplankton in the controls.

In all experiments calanoid copepods were the most important zooplankton, in both biomass and numbers. Numbers of copepods increased considerably during the experiment. This large increase was probably caused by the absence of suitable predators. In the enclosures the copepods developed from nauplii (partly produced in the bags) to adults with rates which were comparable to those found in the open sea.

As in former experiments, the development of the system in duplicate controls was very similar; the larger differences between the bags could therefore be attributed to the addition of the model pollutants.

### Fate of the added compounds

DCA concentrations in the bags decreased slowly during the experiments, but the patterns of decrease did not point to biological degradation. Results of model experiments in the laboratory showed that the decrease of DCA in the

bags could be explained by absorption and diffusion through the walls of the bags (approximately 5% per week).

4CP, DCP and phenol were removed from the water by biodegradation. The results showed that the rates of biodegradation can differ considerably from those found in laboratory tests. The relative rates could also differ; in one experiment 4CP was degraded more slowly than DCP, which was contrary to the expectations from laboratory experiments.

In many cases the degradation of 4CP, DCP or phenol was also much slower than in the laboratory. Lack of a suitable inoculum at the start of the experiment, or competition with other microorganisms, or high concentrations of readily degradable organic matter (e.g. after a phytoplankton bloom) are possible reasons for these slower degradation rates under more natural conditions. In two experiments the linearly decreasing concentrations of phenol and 4CP indicated that a factor other than the substrate concentrations was limiting the degradation rate. In these cases strong indications were obtained that low phosphate and nitrate concentrations limited the growth of bacteria, due to competition for these nutrients with other microorganisms and phytoplankton.

Another interesting observation during one experiment was that 4CP and DCP had strong effects on the phytoplankton in the contaminated systems a few weeks after the addition of these compounds, at a time at which 4CP or DCP could no longer be detected in the water. This finding confirmed laboratory results, which showed that these phenolics could be rapidly degraded to a compound which was in turn very slowly degraded. Obviously this intermediate was much more toxic than the original compounds.

#### Effects of the added compounds

No effects could be detected on the enclosed ecosystem after addition of 2, 10 or 25  $\mu\text{g DCA.l}^{-1}$ . In fact this experiment served to demonstrate that the plankton communities in different bags develop in the same way, if they are exposed to approximately the same environmental conditions. Addition of 0.1 mg  $\text{DCA.l}^{-1}$  resulted in inhibition of the phytoplankton and in lower numbers of bacteria than in the controls. Addition of 1.0 mg  $\text{DCA.l}^{-1}$  resulted in a high mortality of the copepod populations, lower numbers of bacteria and lower phytoplankton populations than in the controls. It was

also found that increased zooplankton mortality lead to a shift in the phytoplankton species composition, the proportion of larger cells was higher than in the controls. These are ecologically important food chain effects.

Addition of 0.1 mg 4CP or DCP.l<sup>-1</sup> had no important effects on the enclosed phytoplankton or zooplankton, but lead to lower numbers of bacteria just after the addition of the pollutants as compared with the controls. Addition of 0.3 mg 4CP or DCP.l<sup>-1</sup> inhibited the phytoplankton growth rate for a few days following the additions. Initial high mortality and inhibition of the development of the zooplankton resulted from the addition of 1.0 mg 4CP or DCP.l<sup>-1</sup> in one experiment. In the same experiment the phytoplankton was inhibited for a few weeks after the addition of 1 mg 4CP or DCP.l<sup>-1</sup>. In another experiment, in which 4CP and DCP were degraded more quickly, no mortality of zooplankton was detected. In all experiments relatively lower zooplankton densities, and subsequently lower grazing pressure, caused a shift in the phytoplankton community structure in favour of larger phytoplankton species.

#### Conclusions and comments

As with the heavy metals tested previously, concentrations of 4CP and DCP causing effects on the enclosed ecosystem were relatively low, compared with the results of LC50 tests or other laboratory experiments. DCA has produced effects in some laboratory tests at concentrations lower than 0.1 mg DCA.l<sup>-1</sup>. The general conclusion can be drawn that enclosed plankton communities are a sensitive system for toxicity testing. Apart from the fact that the method provides a sensitive tool, it is important in that the enclosed system resembles the natural system in a number of relevant aspects (species composition, growth rates, etc.) for the period of the experiment. This finding provides a basis for the extrapolation of results to the field situation; the method can also be used to validate laboratory tests. Important in this respect is that typical food chain effects could be shown (phytoplankton-zooplankton interaction).

The finding that the degradation rates of degradable organic substances can be limited under more natural conditions by low concentrations of inorganic nutrients is very important for the extrapolation of results of laboratory tests to the field. Laboratory degradation tests are often performed in the

dark, so that competition for nutrients between bacteria and phytoplankton is generally absent. For this reason laboratory tests will easily overestimate the degradation rate in oligotrophic environments, such as most seas and oceans during larger parts of the year.

The usefulness of performing a biodegradation experiment and a toxicity experiment in one experimental system is shown by the detection of the formation of toxic intermediates during the degradation of 4CP and DCP in one experiment.

The results of these and former experiments lead to the conclusion that the use of natural plankton communities enclosed in large plastic bags could be one method to bridge the apparent gap between laboratory and field conditions. Experiments with enclosed marine plankton communities yield useful ecotoxicological and biodegradation information. Moreover, this information seems to be applicable to field situations, since the development of the plankton inside the enclosures seems to be in reasonable agreement with that in the water from which the contents of the bags had been taken. On the other hand it is clear that this simple method is just a first step. The enclosed system in a bag behaves similarly to the much more complex ecosystem in some aspects, but it is not identical.

At the moment a method is available which can be applied to at least two types of problems: the assessment of the impact of specific dumping events and other environmental problems, and the validation of laboratory toxicity and biodegradation tests.

#### Publications and oral communications

- J. Kuiper: The use of large enclosures in ecotoxicology- oral presentation to the EEC contact group meeting, Texel, 1980. (Abstract TNO no. P 81/28).
- J. Kuiper: Ecotoxicological experiments with marine plankton communities in plastic bags. Proceedings Symposium on enclosed marine experimental ecosystems, Sidney, B.C. Canada, August 1980. Springer, New York (in press)
- J. Kuiper: Continued investigations into pelagic microcosms subject to environmental stress by pollutants. IV. 3,4-dichloroaniline. Report MT-TNO no. CL 80/114. 58 pp. (1980)
- J. Kuiper: Continued investigations into pelagic microcosms subject to environmental stress by pollutants. V. phenol, 4-chlorophenol and 2,4-dichlorophenol. Report MT-TNO CL 81/17 67 pp. (1981).

Contractor: Secretary of State for Scotland, represented by the  
Marine Laboratory, Aberdeen

Contract No: 226 - 77 - 1 ENV UK

Project Leader: Mr R Jones

Title of Project: Controlled Ecosystem Studies on Marine Communities

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#### Objective of Research

The main objectives of the research were

- a) An investigation of the factors controlling the mortality and growth of herring larvae.
- b) An assessment of the ways in which an enclosed marine ecosystem of four trophic levels (microorganisms, phytoplankton, zooplankton and larval fish) would be effected by low levels of hydrocarbons in a North Sea production water.

#### Materials and Methods

The approach was to carry out a series of controlled ecosystem experiments (CEEs). The basic experiments were done in large plastic bags suspended in the sea in water of 30m depth. The bags were constructed of reinforced PVC and were 5m in diameter and 20m in length, enclosing about 300m<sup>3</sup> of sea water. They were suspended by individual flotation collars designed in the laboratory's engineering department and power to run the monitoring equipment was provided by 600m of armoured cable run from the shore to two rafts moored near the enclosures. A specially constructed wave break was designed to protect the installations from excessive wave action and this was positioned between the equipment and the open sea.

The deployment and servicing of the enclosures was done by a team of divers, and the sampling and analytical support was provided by a group of chemists and biologists allocated to the project.

In the 1979/80 experiments, bags were dosed with production water from a North Sea production platform at an oil concentration of 5-15 $\mu$ g/l to study the effect of the effluent on the planktonic ecosystem over an extended period, and to follow the vertical transport rate of oil distributed through the water column to the bottom sediment. During the experiment the parameters measured in the water column on a routine basis included chlorophyll, organic carbon and nitrogen, temperature, salinity, primary production, microbial rates of hydrocarbon mineralisation, phytoplankton size spectra, hydrocarbon concentrations, and the amount of material settling out from the bottom of the bags. In addition, the structure of the zooplankton population was determined and changes in it followed by means of net hauls. Fertilised herring eggs were placed in the bag and allowed to hatch in "clean" water conditions before one replicate pair (from the four bags used) was dosed with a North Sea production water to a final hydrocarbon concentration of about 5-15 $\mu$ g/l.

### Results

In 1980 for the first time the experimental work concerning growth and mortality of herring larvae was carried out on a spring spawning herring population from the Firth of Clyde. There were several reasons for changing from autumn spawning to spring spawning fish. Firstly, that autumn experiments could not be run for a long enough period to allow the fish to metamorphose and secondly that in the autumn there are large numbers of '0' group fish (whiting, cod etc.) in the loch and it is difficult to keep them out of the bags when they are being filled. Four bags were used and approximately 40,000 fertilised eggs on glass plates were incubated in each bag. As in

1977 and 1978 larval populations in three of the bags suffered considerable mortality on hatching but one bag was significantly different and maintained a higher larval population throughout. This produced an interesting contrast in that in three of the bags there was apparently unlimited food for the surviving larvae whilst in the fourth bag the larval growth was food limited and towards the end of the experiment there was so little food in this bag that the larvae were surviving under conditions of acute food shortage. The differences in food availability produced differences in the condition factor of the fish and the age at which they metamorphosed from larvae to juvenile fish. In the bags with ample food the larvae metamorphosed at about 60 days whereas in the food limited bag this period was extended. The metamorphosis from larvae to juvenile fish was accompanied by a change in the behaviour of the fish and in their feeding. The juvenile fish formed shoals and fed so voraciously that by the end of the experiment the zooplankton populations were being severely reduced by the high level of predation.

In 1980 a further experiment to investigate the effects of North Sea production water (from the Auk field) on an enclosed pelagic ecosystem was carried out. Four bags were used, two as replicate controls and two as replicate treatment bags. All four contained very similar enclosed water columns and in each bag approximately 40,000 fertilised herring eggs were incubated. Once the herring larvae had hatched the effluent from Auk platform was added to the two treatment bags at a dilution of about 1200x. A further addition of oil was made 10 days later at a dilution of about 700x when the larvae would have been changing from yolk sac feeding to natural feeding. The "total oil" added was in the range 5-20 $\mu$ g/l and great care had been taken in collecting the effluent water out on the platform to ensure that the volatile components of the effluent were not lost. The effects of the effluent water were investigated on each of the four trophic levels contained in the bag (microorganisms, phytoplankton,



zooplankton and primary carnivores, jellyfish and larval herring).

In general there is an immediate response of the microflora and the potential degradation rates for some oil fractions (benzenes, naphthalenes) are greatly increased. However, little increase in rate is observed for 3-5 ring compounds or for the straight chain compounds. Primary production was apparently unaffected by oil or effluent water of these concentrations, but significant responses were detected in the zooplankton. Surprisingly the larval fish appeared to be more robust than the copepod zooplankton although it may be difficult to identify an effect of oil in a larval fish population which is declining rapidly even in the control water columns. The copepods proved very sensitive to both a water soluble fraction of oil containing about 100 $\mu$ g/l oil (Davies et al., 1980) and to Auk effluent water containing about 5-15 $\mu$ g/l of oil (Fig. 1). All components of the zooplankton sampled, bottom invertebrates, Oithona and other copepods and their naupliar stages seem to be sensitive to the effluent water at this dilution (about 1:700) which is about the dilution which might be expected to have occurred 500m-1000m from a North Sea platform (Read and Blackman, 1980).

#### Conclusions and comments

Although all of the detailed data from the experiments carried out in spring-summer 1980 are not yet available, the main objectives of the experiments have been met. Differences in food density between experimental enclosures have been shown to produce different growth rates and to effect the timing of metamorphosis in larval herring. The effects of a North Sea production water have been investigated on a food web of four trophic levels and found to cause a serious decline in the zooplankton populations, whilst having no apparent effect on the larval fish mortality. A more detailed

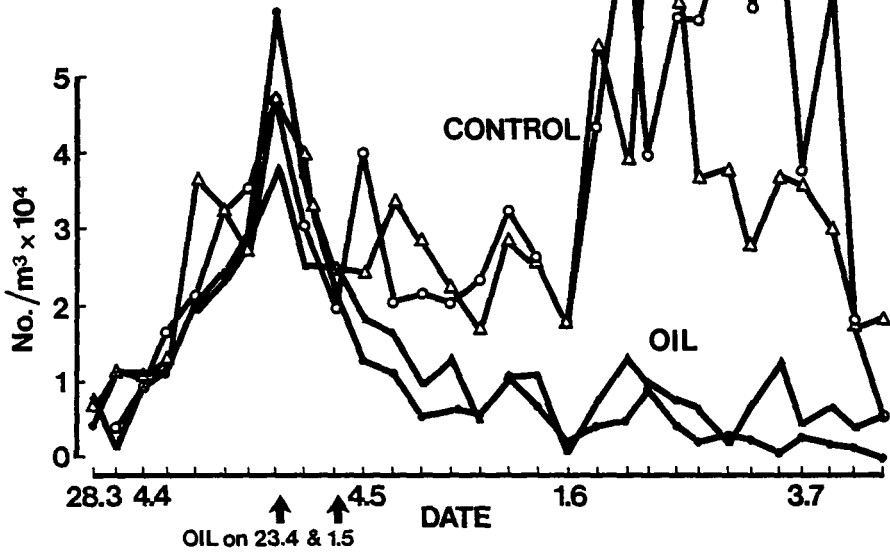
analysis of the zooplankton population structure and the larval growth rates and condition factors may allow an explanation of the mechanism of the observed effect.

Two questions have emerged from the work which could form the basis of future work. Firstly, experiments ought to be conducted to investigate the initial larval mortality upon hatching and to confirm whether this is a natural phenomenon or an artefact of handling the fertilised eggs. Secondly, there are sound reasons for including the pelagic eggs of demersal fish in the toxicity experiments since they are more likely to come in contact with oily water in the initial, post fertilisation, pre hatching phase of their life cycle.

#### Publications and Oral Communications

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2. J M Davies, I E Baird, L C Massie, S J Hay and A P Ward, 1980. Some effects of oil derived hydrocarbons on a pelagic food web from observations in an enclosed ecosystem and a consideration of their implications for monitoring. Rapp. P.-v. Reun. Cons. int. Explor. Mer, 179: 201-211.

# NAUPLII 1980



# CALANOIDS 1980

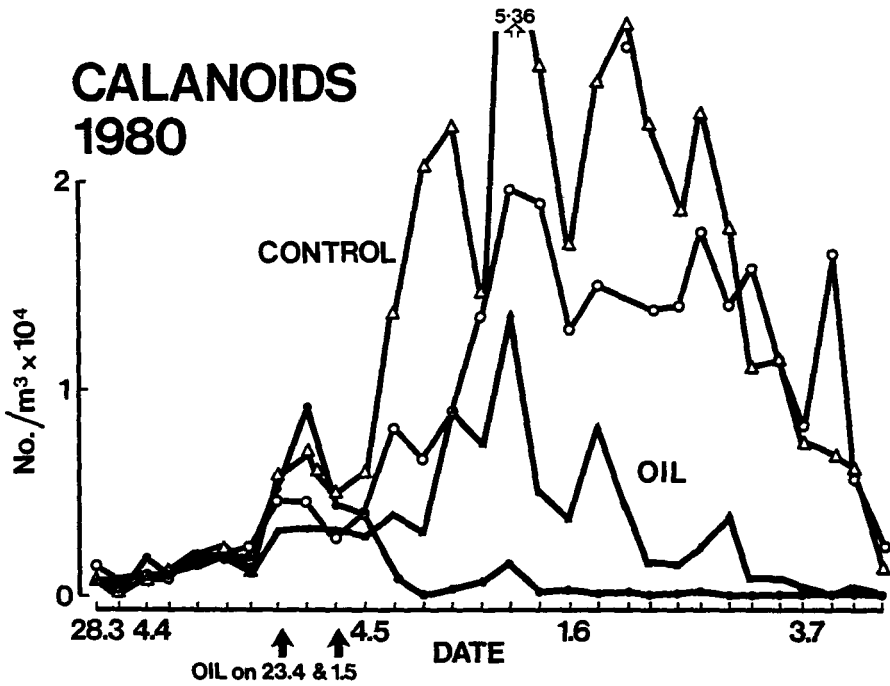


Figure 1. Effect of Effluent Water on Calanoid Copepods and Nauplii.

Contractor : Natural Environment Research Council  
Contract no. : ENV/401 UK  
Project leader : Dr. J. Widdows  
Title of project : Biological effects of sublethal stress and pollution in  
estuarine invertebrates

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### Objective of the Research

The research programme at the Institute for Marine Environmental Research aims to derive practical techniques that will serve as operational and research indicators of environmental quality. Physiological, cytological and biochemical stress indices have been developed and used in the field to assess the health and condition of populations of mussels (Mytilus edulis) exposed to natural and anthropogenic stressors in different environments. During the first phase of the field programme mussel populations from a wide range of environments were studied. A suite of stress indices demonstrated marked differences in the condition and performance of mussels from various sites in southern England and Wales, and generally these differences could be related to the environmental conditions.

In the second phase of the field programme, however, it was necessary to establish whether the recorded differences were indeed environmentally induced rather than genetically inherited characteristics of the populations. This was examined by means of two reciprocal transplant experiments, between two morphologically and physiologically distinct mussel populations.

Research has also been concerned with the deleterious effects of environmental contaminants, many of which are known to have mutagenic and carcinogenic properties, on the genetic material of marine organisms. Techniques for assessing chromosomal aberrations and embryological abnormalities are being developed and these will serve as indices of cytogenetic damage. Laboratory experiments have investigated the effects of aromatic hydrocarbons on embryogenesis and a field study has compared the incidence of genetic damage in embryos originating from polluted and unpolluted sites.

## Material and Methods

The field transplant experiment compared the health and condition of native and transplanted mussels at two sites. One site was the 'relatively clean' Tamar estuary in S.W. England and the other was a potentially polluted site in Swansea Docks, S. Wales. Mussels were transplanted between sites on two occasions. (January to August; August to March) and the physiological, biochemical and cytological stress responses of the native and transplanted animals were determined at two-month intervals using a mobile laboratory. Physiological measurements included rates of respiration, feeding, excretion, food absorption efficiency and scope for growth. Animals were also sampled for the assessment of reproductive condition, taurine : glycine ratio, and latency of the lysosomal enzyme B-hexosaminidase in the digestive cells. Environmental contaminants such as petroleum hydrocarbons and metals (Cu, Zn, Cd, Fe and Pb) in the various body tissues of Mytilus were also measured.

Cytogenetic techniques for assessing genetic and embryological abnormalities in Mytilus (a bivalve) and Pomatoceros (a polychaete) have been developed and subsequently used in laboratory and field studies. Chromosomes were made visible by first inhibiting cell division at metaphase using colchicine, spreading them by treatment with hypotonic saline and then selectively staining with toluidine blue or aceto-orcein. Abnormalities in meiotic and mitotic cell divisions and embryogenesis were examined, following fertilization of the eggs, incubation of the embryos and staining with aceto-orcein. A laboratory study has investigated the effects of aromatic hydrocarbons (benzene and toluene) on mitotic cell divisions and chromosomal abnormalities during early embryogenesis of Pomatoceros; and a field study has compared the frequency of chromosomal abnormalities in the embryos derived from mussels sampled from a clean and a polluted site.

## Results and Discussion

A parallel monitoring of chemical and biological effects was provided by the reciprocal transplant of mussels between two different environments, Tamar estuary and Swansea Docks. Analysis of tissues for hydrocarbons showed that mussels in Swansea Dock contained  $2130 \mu\text{g g}^{-1}$  wet weight (total hydrocarbons) compared with  $596 \mu\text{g g}^{-1}$  wet weight for the Tamar animals, and that there was depuration of hydrocarbons by mussels transplanted from Swansea to Tamar and accumulation by animals transferred from Tamar to Swansea. Mussel tissues were also analysed for metals (Pb, Zn, Cd, Fe, Cu) in order to examine their rates of uptake and depuration in animals chronically exposed to different environmental metal concentrations. The results indicated that some metals were more labile than others. For example Pb showed a rapid accumulation and depuration in the kidney of mussels transplanted to Tamar and Swansea respectively. The concentration of Fe in the kidney was 3-fold higher in the Tamar animals and showed a slow and partial accumulation or depuration following transplantation; whereas the 2-fold population differences in the tissue

concentrations of Cd were maintained throughout the experiments (Swansea > Tamar). The concentrations of Cu and Zn in the tissues of Swansea mussels were found to be two and four times respectively, the concentrations in the Tamar mussels.

The reproductive cycles of the native mussel populations at the two sites were slightly different and this was reflected in the rates of respiration and excretion. The Tamar population had a distinct spring (May) and autumn (September - October) spawning period and there was a concomitant increase in respiration and excretion; whereas the Swansea population had an extended spawning cycle throughout the summer and consequently showed higher rates of metabolism and excretion during August. Although alterations in the reproductive and nutritive storage cycles in response to environmental change are likely to be long-term responses (months), there was an indication of an effect on the respiration rate of the Swansea → Tamar transplant in August, when the responses of the native populations were most different. Feeding rates and food absorption efficiencies were markedly different in the two native populations and within two months of transplanting the mussels adapted to their new environments and were similar to the native animals. The energy budget serves to integrate these physiological responses and provide an estimate of the energy available for growth, termed the 'scope for growth'. This stress index not only demonstrated that the transplanted mussels adapted to their new environmental conditions but also reflected the temporal variations in food availability at the two sites. Native and transplanted mussels in Swansea Docks had a lower scope for growth than the native and transplanted mussels in the Tamar estuary, except during May when there was a higher scope for growth recorded in mussels at Swansea Docks due to a marked phytoplankton bloom.

A biochemical stress index, based on the ratio of two amino acids and termed the taurine · glycine ratio, indicated adaptation; and similarly a cytochemical stress index (lysosomal stability) showed adaptation to altered environmental conditions. At both sites lysosomal stability declined to minimum values in October, probably due to a combination of post-spawning stress and the occurrence of dinoflagellate blooms.

Preliminary cytogenetic studies used Pomatoceros embryos to obtain background information on the cell division rates and the differential sensitivity of dividing and non-dividing cells to environmental perturbations. At present three indices of cellular stress have been identified,

1) cyto-toxicity 2) cell cycle index (the number of cells at a specific stage of mitosis at a given time), and 3) chromosomal aberrations. Laboratory studies investigated the effects of aromatic hydrocarbons (e.g. benzene and toluene) on mitotic cell divisions and chromosomal abnormalities during early embryogenesis. An increase in both exposure concentration (20 to 200 ppm) and exposure time resulted in a reduction in the development rate (mitotic inhibition) and an increase in the frequency of chromosomal aberrations. Toluene was found to be more toxic than benzene.

The field study examined whether there was a measurable difference in the frequency of chromosomal aberrations, or genetic damage, occurring in offspring from mussels sampled from a polluted and a non-polluted environment. Fig. 1 shows the high incidence (25%) of aneuploidy recorded in mussel embryos from King Dock (polluted site), compared with the frequency (8%) in the Whitsand mussels (clean site). Aneuploidy is generally a lethal condition due to the nucleus having more or less than the normal number of chromosomes, and the low frequency recorded in the Whitsand animals is comparable with the 'spontaneous' level reported for other animals at a corresponding stage in development. The higher incidence of aneuploidy in the Kings Dock mussels appears to reflect the higher concentration of contaminants accumulated within the mussel tissues (Table 1).

### Conclusions

The results of the reciprocal transplant experiments have confirmed that the differences between populations, recorded in terms of stress indices, are environmentally induced rather than genetically inherited characteristics of the populations, and largely reflect differences in the degree of environmental contamination. Cytogenetic studies have demonstrated that environmental contaminants can have a damaging effect on genetic material and the viability of offspring from stressed adults. Therefore cytogenetic stress indices will complement our existing suite of physiological, cytological and biochemical stress indices, which together can provide a means of assessing biological aspects of environmental quality.

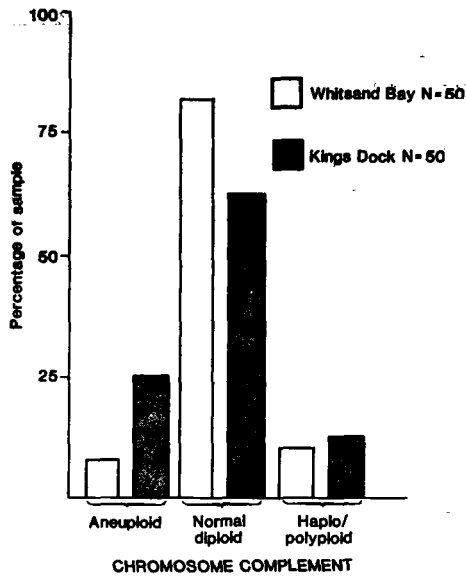


Fig 1. Percentage-frequency of abnormal chromosome complements amongst mussel embryos originating from a clean ( Whitsand ) and a polluted ( Kings Dock ) environment

Table 1. Tissue Levels of Total Hydrocarbons and Heavy Metals in Mussels from King's Dock and Whitsand ( $\mu\text{g g}^{-1}$  dry mass).

	Total Hydrocarbons	Cd	Cu	Fe	Mn	Pb	Zn
King's Dock	7952	7.5	11.2	118	23.7	17.9	291
Whitsand	284	1.8	9.4	226	4.1	12.9	153
Ratio	28	4.2	1.2	0.5	5.8	1.4	1.9



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IN PREPARATION

- DIXON, D.R. Aneuploidy in mussel embryos (Mytilus edulis) originating from a polluted dock.

Contractor: Field Studies Council, Oil Pollution Research Unit, 200  
Contract No: ENV-403-UK  
Project leader: J.P.Hartley  
Title of Project: Organic carbon and macrofaunal zonation in sediments

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#### OBJECTIVES

- 1) To investigate the relationship between organic carbon, sediment particle size and macrofauna in terms of vertical distribution in marine sublittoral sediments.
- 2) To put forward recommendations for a standard sampling technique to be used for examination of organic carbon in sediments in relation to macrofauna.

#### MATERIALS AND METHODS

The study was carried out in Chapel Bay, Milford Haven, a well sheltered area close to the mouth of the Haven. At the selected site the seabed was gently sloping and composed of fine muddy sand suitable for sampling.

Divers obtained a series of 10 replicate cores 30 cm deep and 10 cm in diameter from a small area of the bay for macrofaunal analysis. A further 10 cores 30 cm deep and 5 cm in diameter were obtained for organic carbon (7 cores) and sediment particle size (3 cores) analyses.

The 10 cm diameter cores for the investigation of the benthic macrofauna were sub-divided to 3 cm vertical sections. Each section was sieved using a 0.5 mm mesh and the material retained in the sieve preserved in 5% formalin in sea-water buffered with hexamine. The samples were stored in jars for subsequent laboratory analysis. In the laboratory organisms present in the samples were picked out, identified, counted and analysed for biomass. Biomass estimations were carried out using all organisms present in a section. The animals were washed, oven-dried at 90°C to constant weight, weighed and ashed in a muffle furnace at 600°C. The samples were cooled in a dessicator before re-weighing. Crustaceans, molluscs and echinoderms were de-calcified before drying.

Cores for organic carbon estimations were also divided to 3 cm vertical sections and stored. The organic carbon content of each section was analysed using a combination of CHN analysis (for total organic carbon) and loss of weight on ignition (for total organic matter). Samples for loss on ignition were dried to a constant weight at 90°C, weighed and heated to 475°C for 6 hours in a muffle furnace. The samples were cooled in a dessicator before re-weighing. Heating at 475°C largely avoids problems caused by loss of sediment carbonates at higher temperatures. The scheme outlined above follows the suggestion of Byers et al(1978) for organic carbon analysis of marine sediments.

The cores for granulometric analysis of the sediments were also divided to 3 cm vertical sections. Each section was oven-dried, weighed and subjected to chemical dissociation with sodium hexametaphosphate for 12 hours. The samples were then wet-sieved on a 63 um mesh to remove the silt-clay fraction. The remainder were re-dried and sieved on a series of sieves of different apertures. The fractions retained by each sieve were weighed and the results tabulated. The dry particles were microscopically examined for coal particles which have been shown to significantly affect organic carbon estimates (Southward, 1952; Buchanan and Longbottom, 1970).

## RESULTS

The fauna from the cores corresponds to an Abra alba/Amphiura filiformis community and is fairly rich in both qualitative and quantitative terms. A typical example of the vertical distribution of the fauna is shown in fig. 1. which shows the majority of individuals to be present in the top 6 cm. Although very few individual animals were recorded from the deeper sections of the cores, posterior fragments of maldanid and capitellid polychaetes were occasionally found which show up in the biomass results. As with the number of individuals the majority of the biomass was found in the top 6 cm of sediment.

To date the organic carbon content of the sediments has been estimated by loss on ignition, and a typical example of the results obtained is illustrated in fig. 1. No pattern of vertical distribution

has been found. The average value of percentage combustible material was about 2%. The CHN analysis is still in progress.

The sediment was largely a very fine sand with about 40% silt and clay, and was comparatively easy to core deeply. Visual observation of the cores showed that the upper 4 - 5 cm of sediment was looser than the deeper sediments and contained few shell fragments. This was borne out by the particle size analysis.

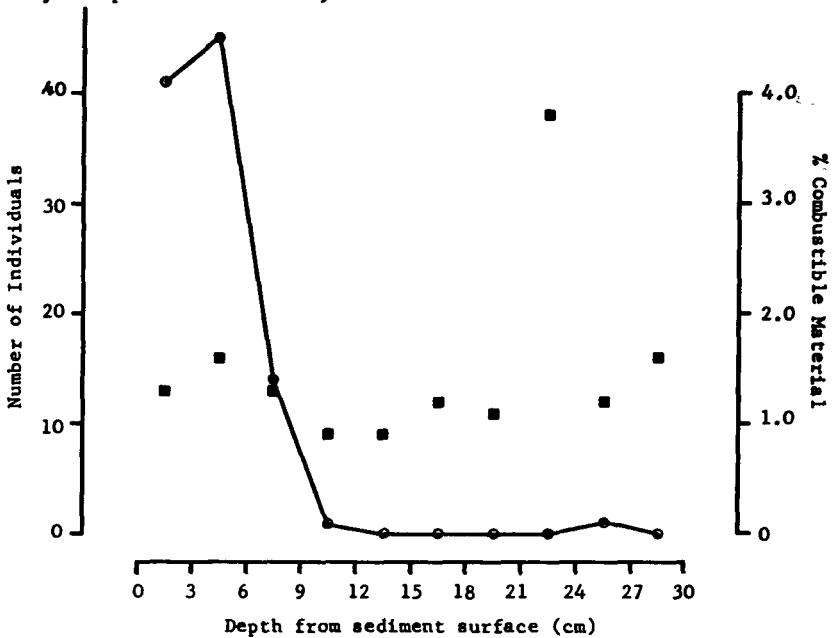


Fig. 1. Example of vertical distribution of macrofauna and combustible material in sediments.

#### CONCLUSIONS

The results and conclusions summarised here are of a preliminary nature as a number of analyses remain to be completed and the data subjected to statistical tests. The results reveal a consistent pattern with the majority of the macrofauna inhabiting the upper 6 cm of sediment. This finding is in agreement with the results of similar studies in fine sediments and vindicates the use of grab samplers and shallow corers in these sediments. There does not appear to be a relationship

between the number of macrofauna individuals and the organic carbon content of the sediment, although as indicated above the relationship has yet to be tested statistically. Final conclusions and recommendations for a sampling protocol are in preparation.

#### ACKNOWLEDGEMENTS

The financial support of the European Economic Community and the Oil Companies Panel of the Institute of Petroleum is gratefully acknowledged. I would like to thank the various members of the Field Studies Council for their generous assistance.

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Contractor: Field Studies Council

Contract no.: ENV-404-UK

Project Leader: Dr. J.M. Baker

Title of Project: Comparison of the fate and ecological effects  
of dispersed and non-dispersed crude oil in a  
variety of marine habitats

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#### Objectives of the Research

- 1) To carry out field experiments (with associated laboratory test), simulating conditions after oil spills and designed to compare the fate and ecological effects of chemically dispersed compared with non-dispersed crude oil.
- 2) To compare a variety of marine habitats in the above way.

#### Materials and Methods

The basic experimental design was a series of treatments applied to experimental plots in each of several different marine communities; semi-exposed and sheltered rocky shores; salt marshes; sea-grass (Zostera) beds; sand/mud flats; and subtidal kelp (Laminaria) forest.

The treatments were:

- (1) Crude oil (Forties or Nigerian)
- (2) Dispersant (BP1100WD or Corexit 7664)
- (3) Crude oil + Dispersant, applied as appropriate - either oil followed by a dispersant spray (simulating beach cleaning) or pre-mixed (to simulate a treated slick near the shore).

Each experiment also contained untreated (control) plots. All treatments and controls were duplicated and were allocated at random (randomized block experimental design).

Oil was sprayed on to intertidal plots immediately after the ebbing tide had left them exposed, thus simulating the stranding of a slick. Dispersant was normally sprayed on to the plots as the tide was rising, so that dilution took place with a minimum of delay (this follows the recommendation of most clean-up manuals). The amounts of pollutants

used varied between the different types of intertidal community because the aim was to cover each experimental area with an oil film that it could 'hold' without run-off. A salt marsh plot can hold a greater volume of oil than a mud flat plot because of the larger surface area offered by the vegetation. This approach meant that oil-treated plots were visually similar to areas of shore oiled with relatively fresh crude following tanker accidents.

Underwater experiments were conducted using hosepipe siphons extending from cliff-top treatment tanks containing diluted pollutants to marked sea-bed areas.

Quantitative biological data, with water and sediment samples for hydrocarbon analysis, were taken as appropriate before experimental treatments and at intervals afterwards.

## Results

### 1. Semi-exposed rocky shore

Limpets (Patella spp.) and small winkles living in and between empty barnacle shells were the most obviously affected organisms. The oil followed by B.P. 1100 WD dispersant treatment had the most severe effect, followed by the oil only treatment. The effect of dispersant on its own as applied in this experiment was relatively slight.

### 2. Sheltered rocky shore

The main change in all marked plots including controls was an increase of furoid algae to 100% cover. Photographic records show that this continues a long-term trend of increase, starting from a low point of less than 10% cover following the hot summers of 1975 and 1976.

### 3. Salt marshes

Oil or oil followed by BP 1100 WD dispersant treatments significantly reduced the density of perennial Spartina anglica and annual Salicornia spp. Low Spartina densities subsequently persisted for two years. Size and survival of Salicornia plants in the year following treatments was greater in the oiled plots.

Dispersant alone had relatively little effect on the vegetation. GC analysis indicated little long-term retention of the applied crude oil in the sediments of any of the treated plots.

4. Sea-grass beds

All treatments reduced the % cover of Zostera noltii. There were no obvious distinctions between the different treatment types, though some ambiguities arose because of different results from replicate plots.

5. Water-logged mud flats

Lugworm (Arenicola marina) cast production was not significantly affected by any of the treatments. Glc and c-gc-ms analyses indicated that most of the applied oil was removed from the plots by tidal action within a few weeks.

6. Well-drained fine sand flats

Treatments slightly reduced lugworm (Arenicola marina) cast production, but no obvious distinctions between the different treatment types have emerged so far. C-gc-ms analysis showed that some dispersant treatments enhanced penetration of oil into the sediments, where it was retained at greater concentrations than untreated oil.

7. Laboratory sediment column experiments

Oil followed by dispersant on a simulated ebbing tide penetrated and was retained below the surface to a greater extent than untreated oil or oil treated on a flooding tide. Pre-mixed oil and dispersant were resuspended or else passed through the column completely.

8. Sub-tidal kelp forest

No effects on the kelp (Laminaria) or on the red alga (Palmaria) were observed. The response to treatment exhibited by the red algae Cryptopleura and Delesseri was an acceleration of natural degeneration. Treatments differentially affected these algae in a consistent manner; dispersant being the most damaging, oil and dispersant mixture having an intermediate effect, and oil only having a marginal effect.

Conclusions

When comparing the results from the different treatments and communities, it must be stressed that one is comparing simulated post-spill



events. Direct toxicity comparisons are not being made; this would involve submitting test organisms to comparable ranges of pollutant concentrations for comparable periods of time. Simulations of shore cleaning may, in contrast, involve comparing the effects of various quantities of oil remaining on the shore for relatively long periods of time with the effects of different quantities of dispersant remaining on the shore for relatively short periods of time before dilution by the incoming tide.

The following points may be drawn out from the data obtained so far, but it must be stressed that monitoring of the experimental plots is continuing. These conclusions must therefore be regarded as preliminary.

1. On semi-exposed rocky shore and saltmarsh, oil or oil followed by dispersant cleaning was more damaging than dispersant cleaning alone. Underwater, prolonged exposure to dilute dispersant was more damaging than prolonged exposure to dilute dispersant plus oil, or water-equilibrated oil.
2. In waterlogged intertidal mud, tidal action quickly removed both untreated and dispersant-treated oil from experimental plots, and little effect was observed on Arenicola cast production.
3. In sandy freely draining intertidal sediments, some dispersant treatments enhanced the penetration of oil into the sediments, where it was retained at greater concentrations than untreated oil.
4. Factors affecting the fate of dispersed oil in sediments appear to include:
  - a) time of dispersant treatment in relation to time of stranding and tidal cycle;
  - b) behaviour of water table in sediments;
  - c) sediment particle size.

#### Publications and oral communications

The following papers have all been presented orally at the conferences concerned, and subsequently published in the conference proceedings:-

Baker, J.M., Crothers, J.H., Mullett, J.A.J. and Wilson, C.M. (1980). Ecological effects of dispersed and non-dispersed crude oil: a progress report. Proceedings of the Institute of Petroleum Conference on Petroleum Development and the Environment, London 20-21 November 1979.

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Little, D., Baker, J.M., Abbiss, T.P., Rowland, S.J. and Tibbetts, P.J.C. (1980) The fate and effects of dispersant-treated compared with untreated crude oil, with particular reference to sheltered intertidal sediments.

1) Chemical dispersion of oil spills: an International Research Symposium Proceedings. Toronto, 17-19 November 1980. 117-151  
Institute for Environmental Studies, University of Toronto, Canada.

2) Proceedings of the 1981 Oil Spill Conference (Prevention, Behavior, Control, Cleanup), Atlanta, Georgia, 2-5 March 1981. 283-293.  
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#### Acknowledgements

The experimental programme started in 1979 and is continuing until 1983. Contract ENV-404-UK covers a period from mid 1980 - mid 1981. We would also like to acknowledge the support of the Advisory Committee on Pollution of the Sea, the U.K. Department of the Environment (Contract DGR/480/510) and the Esso Petroleum Co. Ltd. (an affiliate of Exxon Corporation). The kelp forest experiments were carried out by Mr. J. Mullett of Liverpool University, in co-operation with the Field Studies Council.

**Contractor:** The Natural Environment Research Council,  
The Dunstaffnage Marine Research Laboratory,  
P.O. Box No. 3, Oban, Argyll, Scotland.

**Contract No:** ENV 456-80 UK

**Project Leader:** J. Blackstock

**Title of Project:** The sub-lethal effects of solid waste disposal on  
selected marine benthic invertebrates

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#### Objective of the research

Research under this contract was commenced on 1 May 1980 with the primary aim of assessment of changes in biochemical constituents and population densities of selected species of macrobenthic invertebrates in relation to environmental conditions in the sediments in areas of inshore marine environments affected by inputs of organically-rich waste from domestic and industrial sources. The areas selected for the study were in the vicinity of Garroch Head Sludge Dumping Ground in the estuary of the River Clyde and the Loch Linnhe - Loch Eil system of sea lochs at the inner end of the Firth of Lorne where conditions in the sediments have been affected by a discharge of organically-rich effluent from a wood pulp and paper mill. Further aims of the research were to relate changes in biochemical characteristics of the chosen species to fluctuations in environmental conditions and to incipient changes in the overall population structure of the benthos in each area, and to ascertain whether such factors were consistently interrelated.

In addition, progress in development of further biochemical techniques which could subsequently be applied to assessment of effects of environmental perturbations on selected species of macrobenthic invertebrates was reported.

#### Materials and Methods

At Garroch Head Sludge Dumping Ground a transect of 13 sampling stations extending from 2 km east to 6 km west of the centre of the dumping area was utilised to provide information on a spatial gradient of organic pollution extending

from the considerably affected centre of the dumping area to an area which was apparently little affected by the inputs of sewage sludge.

In the Loch Eil - Loch Linnhe system of sea lochs a transect of 7 sampling stations extending from the vicinity of the head of Loch Eil, some 10 km from the source of the effluent discharge from the wood pulp and paper mill, to Loch Linnhe at a distance of 14 km in the opposite direction from the effluent discharge point was utilised.

Measurements were made of the redox potential (Eh) and pH in the sediments from each transect. Note was taken of the type of sediment present and the oxygen content of the water immediately above the sediment surface was measured. Carbon and nitrogen contents of sediments from Garroch Head and sulphide content of sediments from the Loch Eil - Loch Linnhe system were also measured.

Van Veen grab samples ( $0.1 \text{ m}^2$ ) were taken at each sampling station. The grab samples were sieved on a  $1 \text{ mm}^2$  mesh screen, the residues preserved in 4% formaldehyde and returned to the laboratory for examination. In the laboratory the samples were stained with 0.1% (w/v) Rose Bengal to facilitate the extraction of the animals which were then picked by eye from the residues, identified and enumerated.

The macrobenthic invertebrate populations characteristically include species of polychaete worms which are only found at less affected stations in each transect and may therefore be considered to be sensitive to effects of the inputs of organically rich material. For biochemical studies the predatory polychaete Glycera alba (Müller) was selected as a representative of this group. Capitella capitata (Fabricius) was present in large numbers in the vicinity of the centre of Garroch Head Sludge Dumping Ground and was selected for study as an example of an opportunistic "pollution-resistant" species of polychaete. The selection of these species was also influenced by prior knowledge of their relatively wide distribution in European waters and the consequent relevance of these investigations to studies of effects of organic pollution in other EEC member states. In addition, a certain amount of development of methodology for biochemical analyses of these species has been done in association with research programmes in our laboratory (Blackstock, 1980).

Up to 10 individual G. alba were collected from at least 5 sampling stations in each transect and extracts of quick frozen (in liquid nitrogen) specimens were prepared as described by Blackstock (1978). Activities of

phosphofructokinase, pyruvate kinase,  $\alpha$ -glycerophosphate dehydrogenase, lactate dehydrogenase, malate dehydrogenase, glutamate dehydrogenase, aspartate aminotransferase and citrate synthase in the freshly prepared crude extracts were estimated in duplicate by essentially standard spectrophotometric procedures.

C. capitata from 5 sampling stations between 1 km east and 1 km west of the centre of Garroch Head Sludge Dumping Ground were assessed for stages of sexual development. Up to 12 individuals from each sampling station and at the same stage of sexual development were used to provide pooled samples which were quick-frozen and used for enzymatic analyses. The analytical methods were essentially similar to those used for analyses of the individual G. alba.

In addition, freeze dried G. alba and C. capitata were retained for preliminary investigations of isotachophoretic separation of adenine nucleotides and other metabolites. The method used was based on that described by Gower & Wolege (1977).

## Results

At Garroch Head Sludge Dumping Ground a well defined characteristic spatial gradient of effects of organic pollution was found to exist. High positive values of sedimentary redox potential, at 4 cm depth in the sediment, were found at the western end of a transect of 13 sampling stations, i.e., at a distance of some 3-6 km from the centre of the dumping area. In this area sediment carbon contents were relatively low ( $\approx$  3%) and a diversity of species of macrobenthic fauna was observed. In the vicinity of the centre of the dumping area low positive values of redox potential, high sedimentary carbon contents (7-15%) and high biomass of relatively few species of macrobenthic fauna, including the polychaete Capitella capitata (Fabricius), were found.

The polychaete Glycera alba (Müller) was not found at sampling stations within 1 km of the centre of the dumping area and is therefore considered to be relatively sensitive to the effects of the organic enrichment. In this species, which was collected at 7 sampling stations between 1 and 6 km west of the centre of the dumping area, the activity of the glycolytic enzyme, pyruvate kinase, was lowest in the group collected nearest to the centre of the dumping area. Mean activities of the enzyme malate dehydrogenase were relatively low in groups of individuals collected between 4 and 6 km west of the centre of the dumping area. From 3 km west to 1 km west there were regular increments in malate dehydrogenase activity,

and a significant ( $P < 0.05$ ) negative correlation between malate dehydrogenase activity in crude extracts of G. alba and Eh at 4 cm in the sediments at the sampling stations was observed. Glutamate dehydrogenase activities varied in a similar but less pronounced manner.

The opportunistic polychaete, Capitella capitata, was found in large numbers near the centre of the dumping area and was therefore considered to be relatively resistant to effects of organic enrichment. In this species glycolytic enzyme activities tended to decrease and malate dehydrogenase activities increased with increasing distance between the location of collection of this species and the centre of the dumping area. These enzymatic changes are the converse of the changes observed in enzyme activities in Glycera alba and it is speculated that the different biochemical responses of each species may relate to their very different tolerances of effects of organic pollution.

The Loch Linnhe - Loch Eil system of sea lochs in the west of Scotland receives inputs of organically rich effluent from a wood pulp and paper mill. Examination of a transect of 7 sampling stations extending some 10-14 km on either side of the effluent discharge point has shown that effects of the effluent are pronounced within 3 km of the effluent outfall where low negative Eh values were found and the sediments were black in colour as a consequence of the formation of deposits of sulphide. Eh values rapidly increased, however, with increasing distance from the effluent discharge point and high positive Eh values were recorded in Loch Linnhe at the sampling station situated at a distance of approximately 14 km from the effluent outfall. The pattern of change in abundance and biomass of macrobenthic fauna along the spatial gradient of organic pollution was complex. At all three sampling stations in Loch Eil there was evidence of impoverishment of the macrobenthic fauna. In contrast, biomass and abundance were relatively high in Loch Linnhe and maximal abundance ( $\approx 13,000/m^2$ ) and species richness were found at the sampling station nearest ( $\approx 3$  km) to the effluent discharge point. Biomass and abundance of macrobenthic fauna decreased progressively in Loch Linnhe with increasing distance from the effluent discharge point.

The polychaete Glycera alba (Müller) was obtained from 5 sampling stations at distances of between 6 and 14 km from the discharge point. Mean activities of the glycolytic enzyme, phosphofructokinase, were low in extracts of G. alba from Loch Eil compared with the values found in G. alba collected from Loch Linnhe. Malate dehydrogenase activities were highest in G. alba from Loch Eil and decreased in a regular manner with increasing distance, in Loch Linnhe, from the effluent

discharge point. A significant ( $P < 0.05$ ) negative correlation was found between malate dehydrogenase activities in extracts of G. alba and Eh at 4 cm in the sediments at the sampling stations in Loch Eil and Loch Linnhe.

A preliminary study was commenced using isotachophoretic analyses for simultaneous estimation of nucleotides and other metabolites which may fluctuate in concentration in response to sub-lethal effects of organic pollution on benthic invertebrates. Up to 21 compounds have been detected in extracts of G. alba. These components include the adenine nucleotides (ATP, ADP and AMP), and inorganic phosphate which are known to exert regulatory influence on certain catabolic processes. In addition, lactate and succinate, which are end-products of anaerobic metabolism have been identified in the same analytical runs.

### Conclusions

It is concluded that biochemical changes in Glycera alba collected from Garroch Head and the Loch Linnhe - Loch Eil system are consistent with a response of this species to the different impacts of pollution by organically rich waste in each area. The lowered glycolytic enzyme activities in the Glycera from Loch Eil are considered to represent a biochemical response of the organism to sedimentary conditions which reflect more severe effects of organic enrichment than have been observed in those areas of Loch Linnhe or the Garroch Head Dumping Ground where G. alba were collected. This conclusion is supported by the lower Eh values in the sediments of Loch Eil at the appropriate sampling stations.

In extracts of G. alba from both areas, malate dehydrogenase activities correlated with Eh at 4 cm in the sediments at the appropriate sampling stations, and a consistent quantitative relation between activity of the enzyme and redox potential has been demonstrated.

A pilot study of the usefulness of isotachophoretic analyses for determination of absolute concentrations of metabolites was commenced. The initial results indicate that several nucleotides and metabolic end-products can be separated in a single analytical run. It is considered that the application of this technique to detection of subtle effects of pollution on selected marine invertebrates will subsequently provide information which will considerably facilitate assessment of the physiological significance of the enzymatic changes which have been observed in this investigation.

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Contractor: Dr. J.W. Patching, Department of Microbiology,  
University College, Galway, Ireland.

Contract No: ENV-409-EIR

Project Leader: Dr. J.W. Patching

Title of Project: A study of the biodegradation of oil in  
coastal waters and of the effects of oil on  
microbial activity in the water column

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Objective of the research:

This contract set out to study changes in the activity of marine microbial communities in response to the spillage of oil in coastal waters. It was intended to study two aspects of microbial activity; the direct involvement of microorganisms in the degradation of hydrocarbons, and the effect of hydrocarbons on "normal" microbial activity. Normal activity may be defined as processes such as primary production and the assimilation of dissolved organic material, which would occur in the absence of oil pollution. Disturbance of these processes could have significant effect on food chains and the overall state of the ecosystem. Throughout the study we have aimed to obtain results directly applicable to the natural environment. This has been achieved by the complimentary use of field and laboratory studies. Field studies involved the initiation controlled pollution events in enviromental enclosures floating in situ. Laboratory studies have concentrated on the use of the chemostat for the isolation and growth of microorganisms believed to be significantly involved in the utilisation of hydrocarbons and in "normal" heterotrophic activities, and for studying their behaviour under quasi-natural conditions in the presence and absence of oil.

It was decided to deal only with water soluble components of oils and most studies were carried out using Saudi Arabian

Light Crude Oil (Aramco Crude). This was chosen because it formed one of the major sources of crude oil for the Irish refining industry.

### Materials and methods

After preliminary investigations, a 400 litre floating pelagic unit was devised for field studies, enclosing a column of water extending from the surface to a depth of approximately 1.7m. The walls of the enclosure were formed from nylon reinforced PVC film, the top was covered by a transparent ventilated cover and the base was a fibre glass cone, funnelling sedimenting material into a detachable jar. Multiples of these units were employed, some as controls and some with added hydrocarbons to study pollution events over 3-4 week periods. Standing levels of nutrients, dissolved hydrocarbons and biomass were monitored, the latter by total counts using epifluorescent microscopy, and by chlorophyll measurement.

Radioactive tracers were used to measure the uptake and assimilation of organic material by the community. Labelled Naphthalene and Hexadecane were used to monitor hydrocarbon utilisation, glucose uptake as a measure of microbial heterotrophic activity, and bicarbonate assimilation (under illuminated conditions) as a measure of primary productivity. Changes in oxygen levels in light and dark bottle systems were also employed to provide a measure of net and gross photosynthesis, and of overall heterotrophic activity. Enclosures were deployed in Bell Harbour, an unpolluted sheltered inlet on the south side of Galway Bay, Eire.

Chemostats consisted of glass vessels of 500 ml. working volume, through which a constant flow of medium was maintained by means of a metering pump. Cultures were aerated and agitated and maintained at a temperature of 14°C. Media used were based on artificial seawater with added nitrogen, phosphorous, and a limiting carbon source.

This was either the soluble components of Aramco crude oil, or, for the study of "normal" heterotrophs, glucose. Initially chemostats were inoculated with seawater. Operating the chemostats resulted (in the absence of inter-species interactions in the isolation of the organism most efficient at assimilating the limiting nutrient at the set growth/dilution rate (Jannesch, H.W., Arch. fur Mikrobiol 59, 105-179). By studying steady state biomass and nutrient levels at different dilution rates the relationship between the limiting nutrient and the organisms growth characteristics ( $K_s$   $\mu_{max}$ . and yield) was determined.

### Results

Initial studies with the environmental enclosures showed that they were capable of maintaining a microbial ecosystem discrete from, but (in controls) behaving similarly to that in the surrounding water. To instigate a controlled pollution event, dissolved components of Aramco Crude Oil were added to enclosures so as to achieve initial levels of 0.2 ml. oil/l. This concentration fell to background levels (about 0.003 ml/l) within 8 days.

Activity measurements, however, showed a depression of both photosynthesis and overall heterotrophic activity (dark bottle oxygen uptake) relative to controls over periods of up to 20 days. During this period community uptake rates for naphthalene and hexadecane were enhanced, but biomass levels were similar in both experimental and control enclosures.

A Glucose limited chemostat culture operated at a dilution/growth rate of  $0.05\text{hr}^{-1}$  was used to isolate a "typical" marine heterotrophic bacterium from an unpolluted sample of Galway Bay seawater. The organism was identified as Arthrobacter sp. and was found to possess the strong affinity for substrate (low  $K_s$ ), low non-nutrient limited growth rate ( $\mu_{max}$ ) expected of an autochthonous marine isolate. Use of

glucose limited medium 20% saturated with Aramco Crude depressed  $\mu_{max}$  by 25% and also appeared to decrease the yield with respect to glucose.

Attempts to use chemostat cultures fed with media containing water extracts of Aramco Crude as sole carbon source for isolating hydrocarbon utilisers (both pure and mixed cultures) have proved partially successful. Isolation has occurred but steady states (except of short duration) have not been achieved; formerly due to contamination problems and latterly due to the rapid take over of cultures by surface attaching mutants. Steps have been taken to deal with both problems. Further isolation runs are being carried out and attempts are being made to achieve steady state cultures of isolates from previous runs.

Short term (24 hr.) laboratory experiments have shown that low levels of the water soluble fractions of refined petroleum products may have varying effects on the rate of oxygen uptake by non-illuminated seawater samples. Kerosine caused a marked, and automotive diesel a slight increase in oxygen uptake. Petrol caused a marked decrease possibly due to its lead content. Naphthalene also depressed oxygen uptake but not at such low concentrations as petrol.

### Conclusions

Results from field experiments have shown that relatively low levels of dissolved hydrocarbons in the marine environment may promote microbial activity responsible for their metabolism whilst depressing the normal activities of photosynthesis and general heterotrophic activity. These effects may persist for some time after detectable levels of the pollutant have disappeared. Use of the chemostat proved successful in isolating an organism which was both qualitatively and quantitatively typical of the marine environment, and showing the depressing effect of dissolved

hydrocarbons on its growth related activities. This project was of short duration and attempted to investigate what is an extremely complex phenomenon. The main conclusion is, therefore that the approaches used may be successfully employed for its study. Work is continuing using these approaches.

Oral communication on this research:

Oil Spills and Microbial Activity in Coastal Waters,  
EEC Contractors Meeting - Aberdeen, November 1980.

Contractor: Marine Biological Laboratory, University of  
Copenhagen, Denmark

Contract no: ENV/350 Dk

Project leader: Lars Hagerman, Hans Christensen

Title of project: Sublethal effects of crude oil extract  
on the shrimp Palaemon adspersus Rathke

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#### Objective of the research

The aim of the project is the studying of low level oil pollution on larval development, osmoregulation, ventilation and respiration of an euryhaline crustacean - the shrimp Palaemon adspersus, a species used for human consumption in Denmark. The consequences of oil pollution of the individual animal has been theoretically transferred to the population level. An evaluation of the the methods used for monitoring oil pollution has also been considered.

#### Material and methods

Larvae and adults of Palaemon adspersus were exposed to crude oil extract (North Sea oil from the Piper/Claymore fields), continually supplied via a dosage apparatus constructed by Johannessen (1978). The animals were exposed to 200, 100, 70, 50, 20 and 0 ppb for up to 23-40 days.

Larval development. Larvae in different larval stages were exposed to 200, 100 and 0 ppb oil extract. The number of dead larvae were counted daily as well as the number of larvae alive. All larvae were measured and the larval stage checked at intervals.

Osmoregulation. Adult P. adspersus were exposed to 200, 100, 70, 20 and 0 ppb for 0-34 days. The osmotic concentration of the hemolymph was measured with freezing point technique once weekly and similar samples were taken for 3 weeks when the animals recovered in clean sea water after exposure to the oil extract.

Ventilation. Adult P. adspersus were exposed to 200, 100 and 50 ppb crude oil extract for 14 days. The ventilatory activity was recorded daily with impedance techniques (Hagerman & Uglov, 1979). The ventilatory activity was also

recorded during a six weeks recovery period.

Respiration. Adult P. adspersus were exposed to 200, 100 and 0 ppb for 13 days. During this period and for another 27 days the respiration was measured twice daily.

### Results

An oil pollution with a low concentration of the water soluble fraction influences the physiology of the euryhaline shrimp Palaemon adspersus in several ways. The osmoregulatory ability of the adult is decreased due to a partial destruction of the gill epithelium over which most of the water and ion transport normally takes place. The ventilation of the gills, expressed as scaphognathite activity, is getting irregular with long lasting pauses when exposed to a weak oil pollution. The beating of the scaphognathite is controlled by the suboesophageal ganglion which seems to be affected by the water soluble fraction of the crude oil. This disturbance of the nervous system can also explain the low activity level and low mobility of the exposed shrimps. The respiration is, although influenced by a lot of exo- and endogenous factors, significantly increased in shrimps exposed to crude oil extracts.

Low level oil pollution affects the larval development in several ways: a higher mortality specially during the first larval stages, a decreased activity level and appetite with a resulting increased scavengism/cannibalism. Larvae exposed to oil is smaller, but not delayed in their development compared to normal, unpolluted larvae.

### Discussion

The changes in the physiology and larval development of Palaemon adspersus might have consequences also in the ecology like for example changes in the migratory behaviour of the shrimp so that areas where osmoregulation and ventilation needs less energy are favoured but where other ecological conditions might be non-optimal. To counteract the negative influence of the oil on the organism an increased standard metabolism seems necessary and this energy is taken from energy otherwise available for food search, escape, social behaviour and reproduction.

The measurements of blood osmolarity seem very suitable for field monitoring while ventilation measurements and the study of larval development is suitable for monitoring in the laboratory, even for animals exposed to oil in vivo.

#### References

- Hagerman, L. & R.F. Uglow, 1979. Heart and scaphognathite activity in the shrimp Palaemon adspersus Rathke. *Ophelia* 18, 89-96.
- Johannessen, K. I. 1978. Biotester med oljehydrokarboner. Metodikk for kontinuerlig ekstraksjon og dosering. Økotoksokologi, Forskningsrådenes samarbeidsutvalg, Ås, pp 289-293 (in norwegian).

#### Publications

- The project has resulted in the following manuscripts:
- Baden, S. 1981. Impaired osmoregulation as indicator of stress in Palaemon adspersus Rathke, exposed to sublethal concentrations of crude oil extract. 11 pp.
- Baden, S. & L. Hagerman. 1981. Ventilatory responses of the shrimp Palaemon adspersus Rathke to sublethal concentrations of crude oil extract. 10 pp, submitted to *Marine Biology*.
- Baden, S. 1981. Oxygen consumption rate in the shrimp Palaemon adspersus Rathke, when exposed to the water-soluble fraction of crude oil. 10 pp.
- Baden, S. 1981. Larval development of the shrimp Palaemon adspersus Rathke, when exposed to the water soluble fraction of crude oil. 11 pp.





**TOPIC 19 : NOISE POLLUTION**

Contractor: University of Düsseldorf  
Contract n° ENV - 415 D  
Project Leader: Barbara Griefahn  
Title of project: Effects of noise on sleep and performance

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### Objective of the research

People complain more and more about sleep disturbances. Among those evoked by environmental stimuli road traffic noise became most important.

Sleep disturbances are regarded as a stressor and thereby a risk factor for health, especially for the development of cardiovascular diseases. Thus it becomes necessary to low down noise levels and this implies the knowledge of upper limits above which noise cannot be tolerated any longer.

A working hypothesis basing on an extensive evaluation of the literature postulates a chain from chronic sleep disorders via those with consecutive effects on mood and performance and finally to functional and even organic diseases.

The proof of this hypothesis implies several restrictions:

- it has to be carried out in a field study where people with long-term exposure have to be regarded
- field studies are influenced by many variables whereby - for reliable results - the number of measurements necessarily increases. The full chain of the hypothesis cannot be investigated by only one team or within one single investigation. This requires the cooperation of research teams working in this field; their methods applied should be comparable
- studies in the laboratory are useful to solve particular problems under controlled conditions.

### Material and methods

A pilot study designed to demonstrate the dependency between sleep disorders, subjective assessment and performance was carried out in France, in the Federal Republic of Germany, in The Netherlands and in the United Kingdom. The studies are far-reaching comparable in method an evaluation.

The study in the FRG: (GRIEFAHN, B. & E.GROS)

Subjects: The sleep of 10 couples (25-63,  $38.9 \pm 10.5$ ) living in streets with high traffic load (15-146 months,  $66 \pm 44.7$ ) was recorded during 12 consecutive nights each. The subjects were healthy, had normal hearing, were no shift workers, did not take drugs, and were asked not to drink alcohol during test series. They were elected from 240 subjects who completed an extensive questionnaire sent out by mail.

Experimental design: due to the small number of subjects sleep disturbances cannot be clearly related to noise. Therefore an experimental condition was established. During nights 6-10 five couples (habituated to sleep with closed windows) opened the windows whereas the others then used earplugs.

	Normal condit.					Experimental					Normal	
	1	2	3	4	5	6	7	8	9	10	11	12
earplugs-group	"noisy"					"quiet"					"noisy"	
windows-group	"quiet"					"noisy"					"quiet"	

Noise levels: noise was measured within the sleeping room during sleep time. The microphones were positioned in about 1 m distance to the ear of each subject in the same height.

During normal conditions the average noise levels calculated for each subject separately varied from  $L_{eq} = 35.4$  dB(A) -  $48.2$  dB(A)  $L_1$  from  $41.0$  -  $56.6$  dB(A). For the windows-group the average increase during the experimental phase was  $L_{eq} = 6.8$  dB(A) resp.  $L_1 = 9.3$  dB(A). For the earplugs-group this difference was estimated from measuring hearing thresholds in the morning before and after removing the plugs. Sound attenuation turned out to be at least 9 dB(A) (for lower frequencies).

Recordings: recordings were completed in the town of Essen from May to October 1978.

Before the beginning of each test series several psychological tests were carried out.

During test series the following parameters were continuously recorded throughout the nights:

- noise level dB(A)
- real time
- 1 EEG (electroencephalogram)
- 2 EOG (electrooculogram)
- 1 FPA (fingerpulse amplitude)
- 1 HR (heart rate)
- 1 MOT (body movements)

Regularly recorded were:

- maximum and minimum temperature within each night
- questionnaire for sleep quality, every evening and morning
- four-choice-reaction time for 10 min. every evening and morning

### Evaluation

Noise level: calculation of  $L_{eq}$ ,  $L_1$  for each night

EEG and EOG:

- analyzed off-line using an automatic hardware system
- output then smoothed with a special computer program
- for each night calculation of 114 parameters grouped as follows:
  - number of shifts between sleep stages
  - duration of sleep stages relative to total sleep time
  - falling asleep and 1st sleep cycle
  - indicators of the course and the rhythm of sleep

Statistical procedure: because of preceding calculation both weekends (nights 1, 2, 8 & 9) are disregarded. When comparing noise and quiet for earplugs and window-subjects combined the last 2 nights are disregarded, too.

The effect of noise was investigated for all subjects combined and for the following subgroups

- earplugs-group (10 subjects) - male subjects (10 subjects)
- windows-group (10 subjects) - age  $\leq$  40 years (8 subjects)
- female subjects (10 subjects) - age  $>$  40 years (8 subjects)

### Results (comparison between noise and quiet)

Subjects 1-20: for all subjects combined the time between sleep onset and the 1st epoch of stage 4 was longer in noisy nights, stage REM was reached earlier. The temporal distribution of the awake-stages relative to the center of the night, the barycenter indicates a shift towards the earlier parts of the night. The time estimated for falling asleep was longer. Compared to the evening before, the subjects normally made more errors in the morning during the four-choice-test. This additional number of errors increased when sound pressure was greater during the night between.

Female subjects: sleep latency (measured between lights off and sleep onset) was prolonged for about 5 minutes. The time between sleep onset and stage 4 increased and stage REM occurred earlier.

Compared to quiet nights the amount of stage 4 was less within the 1st sleep cycle, the amount of stage 1 increased. The barycenter of the awake-stage shifted towards sleep onset. The number of awakenings from stage REM and the number of shifts to stage 1 revealed to be greater. The estimated time for falling asleep was 16 min. longer (and thereby 3 times overestimated). The total time assessed for intermittent wakefulness was 19 minutes longer. This probably then leads to the judgement of decreased sleep quality in noisy nights.

Male subjects: a reduced number of awakenings, especially from stage 1 was found. Shifts to stage 1 were less often and the total duration of stages 1 and awake decreased. These results indicate a better rather than a worse sleep during noise. No corresponding increase of any other stage was found meaning that the amount of all the other stages probably became a little bit greater. The effects on EEG- and EOG-data did not affect subjective assessment of sleep quality. The reason may be the length of a single awake state which is in both conditions less than 2 minutes and by this are not remembered in the morning.

Opposite to this the number of errors recorded with the performance test in the morning was greater after noise. Related to the evening before an increase of errors was significant on the 1%-level.

Subjects  $\leq$  40 years: younger subjects seem to be affected only within their 1st sleep cycle. More time was necessary to reach both Delta-stages (3 & 4), less time from onset and the beginning of stages 2 and 3 up to stage REM. The amount of stage 3 decreased whereas the amount of stage REM increased. Subjective assessment of sleep quality as well as performance during the four-choice-test was not affected. Assuming that the EEG and EOG-data are really indicators of "objective" sleep quality, these sleep disorders may be compensated either because they are regarded as unimportant or perhaps by ambition during the test.

Subjects  $\geq$  40 years: within noisy nights the awake-stage are shifted towards the beginning, REM-stages turned out to occur less often. The time judged for falling asleep was longer and significantly more errors were made in the performance test following a noisy night. In accordance to this the assessment of actual per

performance was found to be less compared to the evening before.

#### Subjects sleeping under different conditions

Regarding the conditions Normal (3-5) Experimental (6,7,10), and Normal (11-12) for both the subgroups separately the values of the different parameters were related as follows:

- no changes throughout test series
- successive increase or decrease (regarded as adaptation)
- decrease or increase during the experimental phase only, significant compared to the preceding and/or to the following normal condition. If at the same time the opposite group did show an inverse or at least no effect the result was related to noise.

Earplugs-group: in the normal condition which was noisy for this group the occurrence of stage 4 relative to sleep onset was delayed, the barycenter of stage awake indicates a shift towards the evening but the total time within this stage was shorter. Performance as assessed by the subjects was additionally less compared to the evening before. Performance as objectively measured with the four-choice-test decreased significantly after noisy nights (more errors were counted).

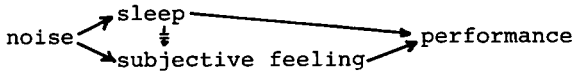
Windows-group: no alterations could be related to the experimental (noisy) condition when considering the data of the EEG and the EOG. The subjective assessment of sleep, however, was altered. The estimated time for falling asleep was longer as well as the total time of intermittent wakefulness. More awakenings were remembered. The subjects felt more tired and sleep quality was judged to be less as already expected in the evening before. Compared to the number of errors counted in the evening performance decreased.

Here again discrepancies revealed between physiological and psychological parameters. It is a common belief that greater noise levels cause poor sleep quality. Thus, when opening the windows these subjects were aware of possible sleep disturbances and already expected to sleep worse when asked for, perhaps only due to consciousness of the greater noise level and the fear not to sleep enough the time for sleep latency, the number of awakenings and the total time spent awake were overestimated. This subjective feeling then may have caused the slight decrease of performance. The earplugs group on the contrary did not expect to sleep worse during noise because they are habituated to this level but, they actually did and made less errors in the morning. Thus it

seems that performance is affected by noise but subjective estimation is at least a very important moderator.

### Conclusion

Alterations possibly related to noise are found within each subgroup. But physiological data, subjective assessment and performance are not necessarily likewise affected. These effects may occur either separately or in connection with the others. In spite of the discrepancies evident between the 3 types of possible indicators for sleep quality the hypothesis postulating the chain:  
noise → sleep → subjective assessment → performance  
cannot be rejected but they introduce the possibility of the following connection:



and by this the most important question for the true parameters of sleep quality with respect to health. Are these the EEG and EOG data or rather psychological parameters. In view of the results found in this study the second connection postulated may be more realistic whereby several moderators are influencing the effects (e g tendency for overestimation, ambition etc.).

### Summary

Effects of sleep quality in a far-reaching sense probably caused by noise were found within the different subgroups.

The alterations of the EEG and EOG occurred mainly in the earlier parts of the night and are described by sleep latency, first occurrence of deeper sleep stages or stage REM and the barycenter of stage awake.

Subjective feeling was affected as indicated by sleep quality, number of awakenings remembered, estimated time of intermittent wakefulness, tiredness and assessment of performance.

During a 10-minutes performance test more errors were made after noise nights.



Contractor : Institut de Recherche des Transports - Centre d'Évaluation  
et de Recherche des Nuisances, Bron, France

Contract n°: 149-77-1 ENV F

Project Leader : Michel VALLET

Title of the project : Joint european study of the effects of noise on  
sleep and psychological performance

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## 1 - OBJECTIVE OF THE RESEARCH

It consists in studying the long term effects on sleep of people living near an urban motorway. In all perception problems, an habituation takes place, generally with a physiological or psychological cost.

If the effects of noise induce modification on the sleep pattern, the study will make possible to evaluate the acoustic level which must not be exceeded to protect the welfare or the health of the population.

## 2 - METHOD

The differents associated teams have decided to carry out an "in-situ" experimentation, and not as people don't sleep in normal conditions when they are in a laboratory.

### - Locality and subjects

Subjects are carefully selected (no sleep trouble, audiogram, psychological tests). Two subjects were recorded in Paris (circular road) 24 have been recorded in Lyon near A42 expressway. 22 subjects are couples and are sleeping together. They have been exposed to traffic noise for 3 or 5 years. 4 ss age < 30 years, 30 < 10 ss < 50 years, 12 ss > 50.

### - Experimental Design

Subjects have been recorded during 12 consecutive nights, after 3 nights of adaptation to electrodes. Recordings start with usual condition i.e noisy condition (N1) :

3 nights. They change of bed-room (with the same bed) to sleep in quiet condition (Q) : light, temperature are controlled.

5 nights. (6 subjects have been recorded during 8 nights in quiet condition as to observe a long recovery period). Subjects came back in noisy condition (N2) at the end of the period : 3 nights. Blocks start on Monday or Tuesday (cf. figure 1).

The sleep of 6 subjects has been recorded after a definitive double glazing of the windows. In this case, the recorded nights are not consecutive.

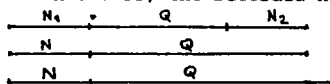


Figure n°1

### - Registration

insulation

2 EEG, 1 EOG, EMG, ECG

Simple reaction time ; subjective sleep quality  
indoor noise level in dB(A) and for 30 nights in dB  
temperature maximum and minimum.

Table 0 Comparison of Noise and Quiet Nights xxx p<.01 xx p<.05 x p<.05 (one tail) T p<.10	Noise	Quiet	DIFFERENCE				NOISE-QUIET (Σ of #)			
			All subjects				Effect of Sex		Effect of Age	
			p	≠	-	+	M	F	Old	Young
									>45	<45
a) Sleep Stages										
Total Sleep Time (min)	455	455		+1,3	12	9	-2	+7	-3	+8
Sleep Latency (min)	18	17		-0,8	10	12	0	-1	-1	-1
REM Latency (min)	95	84	xxx	15	4	16	+13	+18	+11	+21
Sleep Stage 1+2(min)	272	263		9	9	13	-2	+23	+7	+12
% TST	60	58		1,1	6	16				
3+4(min)	63	65		-2	11	11	-1	-2	-2	-1
% TST	14	14,6		-1,5	10	9				
REM(min)	83	99	xxx	-14	15	4	-11	-15	-15	-11
% TST	23	29	xxx	-3	17	4				
Wake Stage min	28	24	x	6	5	15	+7,5	+4,5	+3	+11
% TST	6	4,5	x	2	5	16				
Barycentre of REM	270	268		4	10	11	-1	+4	-1	+4
Barycentre of Stage 4	89	107		-18	12	9	-29	-6	-26	-8
Changes to Wakening	8,2	6,9	T	1,3	7	15	+2	+2	+3	+1
b) Cardiovascular										
Heart rate (b.p.m) <sup>1</sup>	63	61,9		1,1						
Heart rate variability	12,58	11,64		0,9						
c) Performance										
Reaction time (m sec) <sup>2</sup>	308	276	x	15	4	12	+21	+6	+14	+16
d) Sleep questionnaire										
Subjective Sleep Quality	7,2	8,1	xx	-0,9	16	6	-1	-1,5	-1,3	-1,1
No of Wakenings	1,9	1,5		0,4	12	10				
Sleep Latency (min)	23	18	T	6	7	14				
Av Period Awake (min)	14,1	12,9		1	9	9				

1) data for 35 nights

2) data for 16 ss only

Analysis

EEG, Visual sleep scoring. A comparison with automatic systems has been tried (Cambridge and TNO-Amsterdam)

EEG. Mean heart rate by cardi tachometer

Noise : BK 4426 : L1, L10, L50, L90 Leq by 10 minutes periods

reaction time : means of 2 blocks of 5 minutes : simple R.T. of Wilkinson.

3 - RESULTS

A total number of 268 nights has been analyzed. Data are presented by table 0  
The mean noise levels are Leq 42-52 dB(A) in Noisy period, Leq 27-44 dB(A) in quiet condition. The difference between the 2 conditions varies from 2 dB(A) to 14 dB(A). 4ss data are not presented because the noise levels don't vary enough.

Main findings

The average value for noisy nights - the average values for quiet nights give statistically significant ( $p < .05$  for Wilcoxon test) differences for the following parameters :

- 1) psychological quality of sleep increases for quiet nights
- 2) REM Latency is shorter for quiet nights
- 3) Time spent in REM Stage is longer in quiet condition
- 4) Time Spent in Wake is shorter for quiet condition
- 5) The Reaction Time is shorter in quiet condition
- 6) The number of awakenings is smaller in quiet condition (Trend  $p < .10$ )

Physiological data concern three points : sleep pattern disturbance, sleep response to peak noise level and effect of noise on heart rate.

3.1 - Sleep pattern disturbance

After data presentation stage by stage, the first step is to consider the correlative variation of several physiological parameters : total sleep time (TST) , time spent in Wake stage sleep (W) and latencies of the sleep and of REM.

Table 1 shows by subjects (ss) whether TST increases in quiet condition ( $\nearrow$ ) and whether W increases or decreases correlatively.

Table 1 is summarized as follow :

TST increases : 15 subjects out of 25	$\left\{ \begin{array}{l} W \searrow 7ss \\ W = 5 \text{ ss} \\ W \nearrow 3 \text{ ss} \end{array} \right.$
TST decreases : 8 subjects out of 25	$\left\{ \begin{array}{l} W \searrow 5 \\ W = 2 \\ W \nearrow 1 \end{array} \right.$
TST no change : 2 subjects out of 25	$\left\{ \begin{array}{l} W \searrow 1 \\ W \nearrow 1 \end{array} \right.$

Generally speaking some of them have a longer sleep in "quiet" condition and less waking time. In term of stage duration, only 3 ss out of 25 don't show a better sleep in quieter condition. The latency of sleep and REM generally decreases, except for 1 ss.

The analysis of each night shows that people have some sleep due to change of room during one or two nights.

The second step consists on the Evaluation sleep quality improvement related to the noise abatement (in Leq). In this tentative, we built an index of relative physiological quality of sleep (RPQS) based on the physiological parameters, which varies on a 7 points scale. When TST decreases of 10% the index RPQS is 5 points. If TST don't vary RPQS is 1 point, when stages 3+4, 2+1, REM, W change of 10%, when sleep and REM latencies change of 10% RPQS is 1 point.

For a 2 dB(A) abatement the RPQS is low. see figure n° 2  
For an abatement better than 10 dB(A) RPQS varies between low and high rates. The correlation is  $r = 0.44$  ( $p < 0.05$ ). The improvement of the physiological quality of sleep is due to noise abatement.

The first finding of the EEC study show clearly that after several years, the sleep is always disturbed by noise.

In the third step, we consider absolute noise Leq values; we observe that a very low noise abatement at 42 dB(A) provokes, after a five years period of habituation, an improvement in sleep quality. The conclusion is that the acceptable level, after a period of physiological habituation is obviously lower to 40 dB(A) inside the bed-room, during the night.

### 3.2 - Effects of individual noise events on sleep parameters

The EEG effects are analyzed when they occur within 30 seconds of the beginnings of the noise. Temporary EEG effects are : transient effects PAT (duration > 6 sec), Sleep stage changes and awakenings (at least 1 epoch duration).

106 nights from 10 subjects, with 63482 single noise events have been analyzed which provoke 2281 disturbances of the sleep. Each night, in noisy condition we observe 31 disturbances and only 15 in "quiet" conditions. If we take into account the sleep stage changes and awakenings, the numbers of disturbances per night are 21 in noisy conditions and 9 in "quiet" conditions.

After five years of exposure to noise, individual noise events always provoke disturbances. There is no habituation to noise

The analysis of the rate of disturbances by peak noise level is presented figure n° 3.

In the range of noise levels observed, the mean values necessary to provoke a PAT is 47,6 dB(A), a sleep stage change 48,4 dB(A), an awakening 50,3 dB(A).

If the peak noise level did not exceed 40 dB(A) inside the bedroom it would avoid 82% of PAT, 80% of sleep stage changes and 87% of awakenings, in our sample of subjects. We don't mention any difference between men and women in the sleep sensibility. The subjects who are older than 45 years, show a tendency to be less sensitive to peak levels (difference statistically significant for transient effects and sleep stage changes, tendency for the awakenings).

The main finding consists of the difference in noise peak levels necessary to provoke a sleep disturbance, in quiet and noisy conditions.

We consider (table 2) the mean peak levels

	Noisy condition			Quiet condition			T test diffé- rence
	Noise le- vel Peak dB(A)	$\sigma$	N	Noise le- vel Peak dB(A)	$\sigma$	N	
Awakenings	52,56	7,86	333	43,85	5,85	114	p < .01
Sleep stage changes	51	7,37	641	42,38	5,23	299	p < .01
Transient effects	50,57	6,29	584	41,99	5,33	310	p < .01

In quiet condition the mean peak level that provokes an EEG effect is 42,44 dB(A) and 51-53 dB(A) in noisy condition even if there are less disturbances in quiet nights than in noisy ones. The conclusion is that the peak noise level of a single event is not sufficient to access the disturbance probability.

The distribution of the peak noise levels which provoke a sleep reaction shows that a part of them are lower than 40 dB(A). The question is . The sleepers exposed to noise for a long period don't present physiological habituation whether they always react to low noise ? We analyzed the temporal evolution of the arousals and the stage changes frequency among some subjects, already recorded in 1974 in a previous experiment and exposed to noise during this period. The number of temporary reaction slightly increases perhaps in relation to the growing traffic. It rises from 24 reactions per noisy night in 1974 to 35 reactions in 1978 ; the noise peak values are similar for the 2 periods contrary to the results obtained in laboratory on short periods of exposure, we observe an increase of the numbers of EEG reactions with the exposure time (figure n°4). It could happen a limited habituation, but it is far to be complete.

### 3.3 - Heart rate response to noise during sleep

According to the common method used in the four europeans teams, <sup>in 855</sup> heart rate analysis is done on 1 minute period during the whole night. The correlation between the mean heart rate (Hm) and the noise (L<sub>eq</sub>) for every minute is usually positive (see table 3)

Subjects	nights with positive correlation Hm x Leq	nights with positive correlation Vm x Leq	Total number of nights analysed
20	5	3	5
21	2	1	4
22	8	2	9
23	6	2	6
24	9	7	9
25	8	7	9
26	11	3	11
27	7	5	9
Total	56	30	62

Table 3 : Number of nights showing a positive correlation between noise and heart response during the sleep

The correlation between the heart rate variability ( $\sigma/Hm$ ) and the noise (Leq) is positive for about half of the nights.

These data show that the single noise events always provoke heart rate responses after several years of noise exposure.

The linear correlation don't get a peak level threshold. For that, we have applied a partitionning programm (M. MAURIN) to the data of mean heart rate (by minute ) and Leq (minute).

The best threshold of the noise is 37 dB(A). This level in Leq minute approximatively corresponds to 40 dB(A) in peak level, for the traffic generally observed during recorded nights. Above this peak level there is a increasing rate of heart responses.

#### CONCLUSIONS

After the 4 - years common programm, the physiological experiences carried out inrealistic conditions, with people exposed to traffic noise for a long time, show that the sleep remains disturbed by noise at several levels.

- physiological structure of the whole night sleep,
- EEG and cardiac responses to single noise events,
- performance and psychological sleep quality in the morning.

This programm gives elements for the experimental validation of the ideal "World Health Organization" proposal for the night noise regulation : "a level of less than 35 dB(A) Leq is recommenced to preserve the restorative process of sleep".

However this Leq noise level of 35 dB(A) inside the bedrooms don't exclude noise peaks over 40 dB(A) to which the sleep is sensitive

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VALLET M., GAGNEUX J.M., BLANCHET V. Noise and sleep at home Stage changes and arousals. Proceedings of 5th Europ Congr. of Sleep Res. Amsterdam 1980 - Karger Edit.

KUMAR A., CAMPBELL K., HOFMAN W., VALLET M., JURRIENS A., VAN DIEST R. Evaluation and validation of automatic and visual methods of sleep Stage Classification of human Sleep recordings done at home. Proceedings of 5th Europ Congr. of Sleep Res. Amsterdam 1980 Karger Edit.

SS	T.S.T.				Latency	
	↗	durée min	↘	durée min	Sleep	REM
1	↗	28	↘	15	↘	↘
2	↗	13	↘	21	↘	↘
3	↗	25	↘	33	=	↘
4	↗	10	↘	12	↘	↘
5	↗	28	=	10	↘	↘
6	↗	42	↘	7	↘	↘
7	↗	30	↘	21	↘	↘
8	↗	16	↘	10	↘	↘
9	↗	5	↘	7	↘	↘
10	↗	9	↘	21	=	↘
12	↗	8	↘	40	↘	↘
13	↗	30	↘	34	↘	↘
14	↗	12	↘	32	↘	↘
15	↗	15	↘	10	↘	↘
16	↗	69	↘	62	=	↘
17	↗	18	↘	18	↘	↘
18	↗	35	↘	8	↘	↘
19	↗	20	↘	20	=	↘
20	↗	31	↘	12	↘	↘
22	↗	33	↘	8	↘	↘
23	↗	20	↘	8	↘	↘
24	↗	50	↘	41	=	↘
25	↗	12	=	=	=	↘
26	↗	12	=	=	=	↘
27	↗	12	=	=	=	↘

La durée est notée en minutes et la latence en secondes (min)

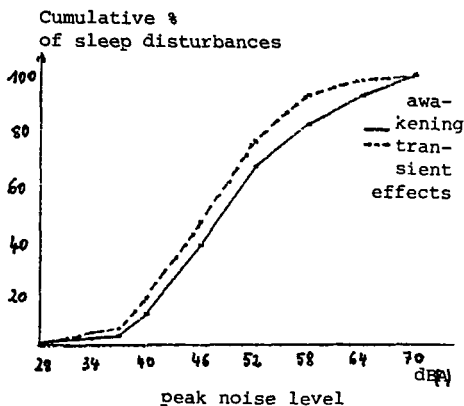


Figure n° 3 : Cumulative distribution of the noise effects on sleep (awakening and transient effects).

Table n° 1 : Correlative variation of Total Sleep Time (TST) and waking period ; variation of latencies

Relative Physiological Quality of Sleep

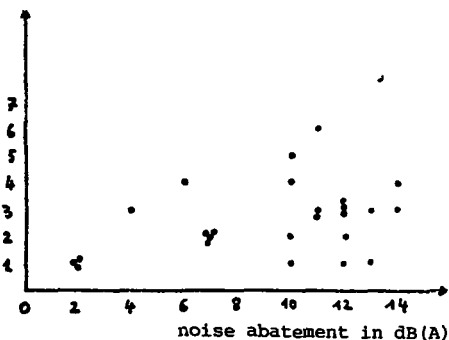


Figure n° 2 : Relationship between Relative Physiological Quality of Sleep and noise abatement

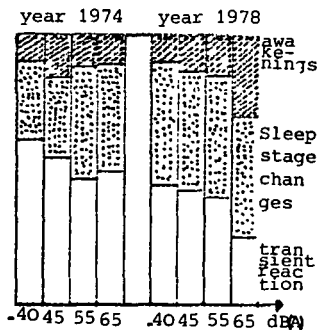


Figure n° 4 : Effects of exposure time to noise on sleep disturbances

Contractor : Centrale Organisatie TNO on behalf of the  
TNO Research Institute for Environmental Hygiene  
Delft, the Netherlands

Contract n<sup>o</sup> : 260-77-1 ENV N

Project leader : A.A. Jurriëns

Title of project : Effects of noise on sleep and psychological  
performance (joint European study)

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## 1. Objective of the research

To study physiological effects and psychological after-effects of traffic noise during sleep with people at home.

## 2. Materials and methods

### 2.1. General

Twelve subjects in different age groups and of both sexes having passed a screening on health aspects were asked to sleep for certain periods in a relatively noisy and in a relatively quiet condition. Each night, acoustical and physiological signals were recorded and each morning and evening, psychological data were registered. From all material registered, certain variables were selected by the joint teams, which variables were generally analysed in terms of differences between condition averages. Besides this joint analysis, other all-night and transient effects were analysed.

In this project the TNO Research Institute for Environmental Hygiene co-operated with the Psychophysiology Laboratory of the University of Amsterdam, especially regarding analyses on noise peaks, temporal characteristics on sleep stage pattern, ECG, respiration and multivariate analysis of psychological variables.

### 2.2. Recordings

The subjects concerned lived along the highway The Hague - Rotterdam, in Rotterdam and Delft. A change in noise condition was realized by fixing double glazing (3 subjects), and closing (1 subject) or opening (8 subjects) windows, where necessary with provisions for thermal insulation.

Because of the expected large variation of the variables within a condition, a scheme with relatively many nights (10) per condition was preferred, divided into blocks of 5 nights. For practical reasons,



incidentally more nights and/or more smaller blocks were employed. Except for one, the subjects started with their normal condition and each block usually started at Sunday night.

Indicating the conditions by noisy (N) and quiet (Q), the distribution of subjects over condition sequences was : N - Q : 5, Q - N - Q : 3, and Q - N : 4. It should be noted that these indications are relative, quiet not meaning silence.

At night the indoor sound level in dB(A) and the physiological signals EEG, EOG, ECG, EMG, motility and respiration were recorded, in the morning reaction times during a 10 minute test and scores on subjective sleep quality and mood, and in the evening scores on well-being by day.

### 2.3. Analyses

The following analyses were carried out :

Sound : statistical distribution and  $L_{eq}$  of sound levels during total sleep time (based on equivalent sound levels per 30 s), statistical distribution levels per 10 minutes,  $L_{eq}$  and the standard deviation of sound levels per minute, and noise peak characteristics.

Noise peaks exceeded  $L_{90}$  (over 10 minutes) by at least 10 dB(A) and for about 15 s before and after such a peak, there were no higher peaks. They were classified in classes of 3 dB(A) relative peak level and of 1 s risetime, the time needed for a 3 dB(A) rise.

EEG (EOG) : automatic sleep stage analysis per 30 s, temporal sleep stage pattern characteristics and the statistical distribution of delta intensity levels per 30 s during total sleep time.

The latter is a logarithmic measure of the RMS value of the filtered out delta activity in the EEG. From this distribution, also a measure for "delta presence" was calculated : the time the level exceeded  $L_{1-5}$ .

ECG : mean heart rate and coefficient of variability of the heart rate per night, mean heart rate, mean R-R interval, coefficient of variability of the heart rate and of the R-R interval per minute, correlated for each night with synchronous sound level variables, and the relation between changes in heart rate and noise peaks.

Respiration : mean respiration rate per minute, correlated with synchronous sound level variables for each night.

Psychological variables : mean reaction time during 2 blocks of 5 minutes and the sum and the difference of these block means, calculation of the subjective scores as percentages of maximum scores, and a multivariate analysis on psychological variables.

### 3. Results

By differentiating between subjects with experimental condition noisy and subjects with experimental condition quiet, normal and experimental condition averages of all-night variables were also compared to examine if trends in the noisy versus quiet comparison were to be attributed to the experiment itself (condition sequence). In the following sections, the main results are presented, mostly in terms of numbers of subjects having a positive or negative difference between condition averages.

#### 3.1. Sound

Typical sound level differences (base :  $L_{10}$  during total sleep time) between noisy and quiet nights were : 5 dB(A) (2 subjects), about 8 dB(A) (4 subjects) and 10 - 16 dB(A) (6 subjects),  $L_{10}$  for different subjects ranging from 45 to 56 dB(A) (N) and from 38 to 46 dB(A) (Q). The analysis of noise peaks showed that the difference in indoor sound level did not influence substantially the total number of peaks during a night, but only their distribution over peak levels. Also the number of peaks with a risetime up to 2 s was about the same in both sound conditions.

#### 3.2. EEG

Sleep stage analysis. During the noisy condition and underlined by an analysis on the influence of changes in total sleep time, most (9 out of 12 or 11) subjects spent more absolute time in stage 4 and in the stages 3+4, more absolute and relative time in stage W (sign distribution significant) and less absolute time in stage REM.

The first two trends could not be explained by condition sequence, as the normal versus experimental comparison showed. The decrease in REM could only be related to noise more certainly after a detailed examination of the results of the 3 subjects with a Q-N-Q sequence.

Temporal characteristics of sleep stage pattern. From the results on four subjects analysed so far, the conclusions were that in the quiet condition the number of sleep cycles was larger than in the noisy condition and that the barycentre of slow wave sleep (stages 3 and 4) was shifted towards the end of the night during the noisy condition.

Delta activity. The general conclusion was that the mutual differences between lower ( $L_{75}$ ,  $L_{90}$ ,  $L_{95}$ ) and higher ( $L_{10}$ ,  $L_{25}$ ,  $L_{50}$ ) statistical delta intensity levels had increased during the noisy condition (7 to 10 out of 11 subjects).

This conclusion - a widening of the histogram at lower and at higher levels - is in agreement with conclusions on sleep stage scoring : more stage W (lower levels) and more slow wave sleep (higher levels).

### 3.3. ECG

Parameters per night. In the noisy condition 9 out of 11 subjects had an increased mean heart rate per night. The average increase over all subjects was 2,9 beats per minute and was significant.

Correlation between noise and ECG parameters per minute. The results showed that, about equally in both conditions (in about 90% of the nights), an increase of both sound level variables per minute ( $L_{eq}$  and  $\sigma$ ) was related to an increase of the number of beats per minute and to an increase of the coefficient of variability of the number of beats per minute (total numbers of positive and negative correlations for all subjects significantly distributed, in all cases).

An across-night analysis showed no decrease of correlation during the experiment, which is to be interpreted as no adaptation.

Furthermore, a sleep stage related analysis of the correlations for four subjects showed that, both in noisy and in quiet condition, the rate of significant positive correlations between  $L_{eq}$  and the number of beats per minute was not lower during slow wave sleep than during other sleep stages.

Noise peaks and beat by beat variation. Though the noise peaks caused subject-dependent changes in the number of beats per minute, generally speaking, the noise peaks disturbed the heart rate in both conditions as follows : Noise peaks with a rise time within 1 s for a 3 dB(A) rise caused an increase of the heart rate by 1 - 6 beats per minute; there were 100 - 400 of such noise peaks in a night. The mean heart rate within a stimulus period was higher for peaks with higher levels. Also these results showed no effects of adaptation to noise.

Respiration. In both conditions, changes in  $L_{eq}$  per minute were in about 75% of the nights significantly correlated with changes in respiration rate per minute. In general there were more positive correlations, though the distribution of positive and negative correlations was not significant. An across-night analysis showed no decrease of correlation during the experiment, which again is to be interpreted as no adaptation.

### 3.5. Psychological variables

Reaction time. The increase of the mean reaction time during the noisy condition (7 out of 11 subjects) was probably a condition sequence effect, due to declining motivation during the second period.

The supposition that looking at differences between the second and the first half of the test would obviate such influences was not confirmed.

Subjective variables. Of these variables, only the subjective sleep quality on a continuous scale showed a clear deteriorating trend during the noisy condition (9 out of 12 subjects) irrespective of sequence.

Multivariate analysis.

For this analysis the following variables were used : mean reaction time, two scores for subjective sleep quality, different mood scores and scores for tiredness and daily functioning derived from a questionnaire concerning daily activities. All variables behaved in accordance with the hypothesis : deterioration during the noisy condition, but only significantly in a few cases.

A multivariate test of the hypothesis showed no significant effect. In the exploration it proved that the inter-correlations between the variables tended to be stronger in the noisy condition, in some cases significantly. This means that during the noisy condition sleep did not have a restorative effect, as is the normal expectation.

Conclusions and additional comments

Summarizing, the following conclusions result from all analyses.

- A change in indoor sound level does not influence substantially the total number of noise peaks during a night, but only changes their distribution over peak level classes.
- Noise tends to increase wakefulness, to reduce REM sleep and to increase slow wave sleep, and possibly disturbs the distribution of the latter over the concerning sleep stages and its nightly pattern.
- During the noisy condition, the mean heart rate per night increases.
- Increases in sound level and in variation in sound level per minute are significantly related to increases in mean heart rate and in variation in heart rate per minute, equally in both sound conditions.
- Noise peaks with high (relative) peak level and sharp rise time are most disturbing.
- Changes in sound level per minute are mostly significantly correlated with changes in respiration rate, in general positively.
- There is no adaptation to stimuli-induced cardiac and respiratory responses.
- The subjective sleep quality tends to deteriorate after noisy nights.
- Noise seems to reduce the restorative effect of sleep.

Age and sex influences and dose - response relations were not apparent.

3 The conclusions on physiological effects can be divided into tonic disturbances (changes in all-night EEG and ECG variables) and phasic disturbances (stimuli-induced cardiac and respiratory responses). Changing the indoor sound level changes tonic aspects, but both sound conditions cause comparable phasic disturbances and appear to have comparable numbers of noise stimuli during a night. This leads to the general conclusion that noise levels seem to be more related to tonic disturbances, whereas the number of single noise events seems to be more related to phasic disturbances. In view of the generally accepted interpretations, with respect to health consequences, of especially increasing wakefulness, reduced REM sleep and increasing cardiovascular load, and in view of the conclusions on the deteriorating subjective sleep quality and the reduced restorative effect of sleep, these disturbances should be interpreted as : noise has an adverse effect on sleep and subsequent well-being. Improving a noise situation to protect people's health should mean a reduction of both tonic and phasic disturbances. This might result in taking measures reducing not only sound levels, but also the number of noise events.

Future research should cover both tonic and phasic disturbances. The presence of phasic effects, also at lower sound levels, is evident, so fundamental studies on disturbing stimuli features should be emphasized. For tonic effects studies with statistical conclusive force seem to be necessary. In view of their known risk for health and the feasibility to record them, cardiovascular variables are the first to be considered. Studies on both aspects should always include the recording of after-effects. The latter may also be studied alone in epidemiological studies.

#### References

On the Fifth European Sleep Congress of the European Sleep Research Society, Amsterdam, September 2 - 5, 1980, the following communications have been presented. They will be published in the Proceedings of this Congress : Sleep 1980, Karger, Basel, 1981.

A.A. Jurriëns

Noise and sleep in the home : effects on sleep stages.

W.F. Hofman, A. Kumar, P.A.M. Poelstra, R. van Diest

Noise and sleep in the home : effect on heart rate.

W.F. Hofman, P.A.M. Poelstra, A. Kumar, A.A. Jurriëns

3 Effects of traffic noise during sleep on the relationship between performance, mood, sleep quality and functioning during wakefulness.

A. Kumar, K. Campbell, W.F. Hofman, M. Vallet, A.A. Jurriëns,

R. van Diest

Evaluation and validation of automatic and visual methods of sleep stage classification of human sleep recordings done at home.

Contractor : Medical Research Council, UK

Contract No : 183 - 77 - 8 ENV UK

Project Leader : R. T. Wilkinson

Title of project : Noise in the Home: Its Effect upon Physiological and

Subjective Assessments of the Quality of Sleep and upon Subsequent Performance

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### Objective of the Research

The aim of this research was to assess the effects of traffic noise above a given level (Leq 60 dBA or L1 70 dBA 1 m from the roadside wall of the house) upon the sleep of people living along the roads concerned. The quality of sleep was assessed in three ways: By physiological measurement (EEG, EOG and ECG), by subjective report of the night's sleep and daytime wellbeing, and by performance the next day. The existing level of noise was compared with a level reduced by means of double glazing the windows of the bedroom concerned. The proposition was that if this improved the quality of sleep then existing noise levels were inconsistent with sleep of a normal standard. In addition it was planned to examine, within the night's record, effects of transient noise peaks upon the measures taken, again to define threshold levels above which normal physiological patterns of sleep could be said to be disturbed. Within the groups of subjects a comparison of individuals on the basis of age and sex was proposed, in order to assess the interaction of these individual characteristics with any effects of noise which were to be found. Within this general framework the aim of Phase II of this research, of which the following constitutes a report, was to analyse in greater depth, the data collected during Phase I with particular reference to EEG frequency, transient effects, individual differences, and correlations between measures of sleep quality.

### Materials and Methods (General)

The general strategy has been to record subjects in their own homes for the first week under normal conditions of noise. Install double glazing and leave them for one week to get used to it. Record them during the third week under these 'quiet' conditions. Remove the double glazing, leave them for a further week to get used to that. Record them for a fifth and final week under normal noise conditions again. Practice recordings at night and practice on the performance tests took place on the Saturday and Sunday of the first week and also on the Sunday preceding the other two weeks of recording. None of these records were used in analysis. Thus, analysis days were confined to the weekdays. Four of the subjects, who possessed double glazing, were given the noise/quiet conditions in reverse, by removing double glazing for the middle week. These were the first subjects tested and, due to a variety of reasons, including relatively low noise levels and the illness of one of the subjects, which affected the others, their data were not included in the final analysis.

Subjects: Thus a total of 272 subject/nights were recorded covering 16 subjects, only 12 of which have been used in the final analysis. From the 17 nights recorded for each subject a maximum of 13 were available for analysis, the rest being practice runs. Of the 12 subjects whose data have been used, 7 were under 45 years of age and 5 over 45; 5 were male and 7 female. Various constraints were placed on acceptance of subjects: 1) No person could be accepted who was on medication of any kind. 2) Hearing had to be within normal limits as assessed by a standard audiometric test. 3) General health needed to be good. The subjects were paid an honorarium in recognition of their co-operation.

Locality: The four rejected subjects were all recorded in Cambridge. The rest of the accepted subjects were recorded in various parts of London along major road ways, for example the North Circular Road and Western Avenue, where the required noise criteria were met. Pairs of subjects were always recorded living in the same house. Five pairs were husband and wife; one pair was mother and daughter.

Noise: Both analogue noise and dBA noise levels were recorded through a Sound Pressure Level Meter located near the subject's head onto a tape-recorder located in or near the bedroom. One-sec samples of noise throughout the night were analysed to give Leq dBA levels in the bedroom for each night.

Physiological Recording: A "portable" technique of recording EEG, EOG and ECG was used, which has been described elsewhere (Campbell et al, 1979, Campbell and Wilkinson, in press). The following channels of recording were obtained: 2 EEG, (C3 to right mastoid; C4 to left mastoid), 1 EOG, 1 ECG (chest electrodes) and 1 signalled awakening channel (a button in the subject's palm which was to be pressed whenever the subject found himself awake during the night; quality of recording of this channel was poor and no results are offered). An automatic sleep analysis system was used to provide  $\frac{1}{4}$ -sec scores of Alpha (8-12 Hz), Spindle (12-15 Hz) and Delta (0.5-2.5 Hz) frequencies in the EEG and of the presence of Rapid Eye Movements (REM).

This information together with a polygraph write-out of the EEG during the night, was used to derive a Sleep Stage analysis on a semi-automatic basis.

EEG Frequency and REM: Although analysis into stages is the most widely used method of assessing sleep in terms of the EEG and EOG, a perhaps more objective and reproducible alternative is simply to consider the amounts of time spent in the presence of Alpha, Spindle or Delta frequency, and in REM.

Heart Rate: This was assessed for the whole night by measuring each interbeat interval, with appropriate rejection of artefacts, taking the average and converting to beats/min. Heart Rate Variability was the Coefficient of Variability of the interbeat intervals within one night.

Subjective Tests: Two questionnaires were employed: 1) a locally designed questionnaire to assess the quality of sleep, quantity of dreaming, awakening during the night, and sleep latency. This was administered shortly after awakening each morning. 2) The Stanford Sleepiness Scale (SSS) in which subjects estimated how alert or drowsy they were. This was administered before subjects went to bed and again immediately upon waking.

Performance Tests: In the morning following each night's sleep four performance tests were administered: 1) Unprepared Simple Reaction Time (USRT), where subjects responded as quickly as possible to the unwarned lighting up of a visual display which counted and displayed their reaction time in msec. 2) Four Choice Serial Reaction Time (Wilkinson and Houghton, 1975) in which subjects pressed one of 4 buttons to any one of 4 lights which came on in random order, response to one bringing on the next. 3) Auditory Short Term Memory, in which lists of 8 digits were presented orally to be written down from short term memory immediately the list ended. 4) Vigilance (Wilkinson, 1970), in which subjects listened to  $\frac{1}{4}$ -sec tones coming one every 2 sec for the occasional tone which was slightly shorter than the rest. The first three tests lasted 10 minutes each, the Vigilance for 1 hour.

Statistical Analysis: Nonparametric statistical tests have been used for most analyses. The tests are described in Siegel (1956).

#### Comparison of Normal Noisy Conditions with "Quiet"(Double-glazing)

Table 1 presents scores averaged over all 12 subjects for the first (noisy), second (quiet), and final (noisy) weeks, indicating the nature of the effect of noise and whether the difference between noise and quiet was significant (Wilcoxon Test) over all subjects. The two final columns

Table 1. Comparison of Noise and Quiet *** P < .01 ** P < .05 * P < .05 (one tail)	Week 1 Noise	Week 2 Noise	Week 3 Noise	Effect of Noise		
				Overall	Greater for: Old(O) or Young (Y)	Men(M) or Women (W)
a) Sleep Stages						
Total Sleep Time(min)	420.1	432.0	426.8	Less	O*	
Sleep Latency(min)	8.2	8.1	7.3			
REM Latency(min)	70.7	85.7	75.7	Less	O	M
Sleep Stage 1 min	13.4	17.7	16.8	Less	O	M
% TST	3.2	4.1	4.6	Less		
2 min	211.7	210.2	211.3			
% TST	50.6	49.2	50.0			W*
3 min	40.8	45.3	44.6	Less	Y	
% TST	9.9	10.7	10.5			
4 min	44.9	50.0	44.2	Less**	Y	W***
% TST	11.0	11.3	10.6			
REM min	94.0	87.7	94.6	More	Y	M
% TST	22.6	20.9	22.2	More		M
Wake Stage min	9.7	12.8	8.9	Less	O	M
% TST	2.2	3.4	2.8	Less	O**	
Barycentre of REM (1)	54.2	59.9	58.0	Earlier		
Barycentre of Stage 4(1)	25.5	24.8	24.7			
Changes to Wakening	1.0	2.0	1.6	Fewer		
b) Sleep Features						
EEG Alpha (min)	31.4	34.0	30.4	Less		W
EEG Spindles (min)	2.5	2.2	2.2			W
EEG Delta (min)	78.7	88.8	83.4	Less**		W
EOG Rap. Eye Mvt. (min)	5.7	6.2	5.9	Less		
c) Cardiovascular						
Heart Rate (b.p.m.)	63.4	66.0	64.7	Lower**		
Heart Rate Variability	17.8	18.1	18.7			
d) Performance						
Reaction Time (USRT)(msec)	265	249	253	Slower**		W
4-Choice Reaction Time(msec)	460	417	400	Slower	O	W
Vigilance - Hits	21.8	26.0	27.0	Fewer		
- False Alarms	8.5	8.4	8.7			
Sh. Term Mem.(lines corr.)	24.2	29.1	33.6			
e) Subjective Questionnaires						
How well did you sleep?(2)	49	58	53	Worse*	Y	
How do you feel now?(2)	52.1	52.3	50.5			
Quantity of Dreams(3)	23.1	21.1	25.1	More	Y	M
Sleep Latency(min)	15.3	18.2	15.1	Less	O	
No. of Wakenings	2.2	1.5	1.4	More**		
Av. Period Awake(min)	6.5	8.1	8.4	Shorter	Y	M
Pre-Sleep SSS (night)(4)	3.6	3.4	3.5			
Post-Sleep SSS(morning)(4)	2.8	2.6	3.1	Drowsier		

Notes: 1. Centre of distribution through night(0 = start, 100 = end of night).  
2: Worst (0), Best (100) I can remember. 3: Not at all (0), More than ever (100).  
4: Alert (1), Almost Asleep (7). 5: No entry in cols.4 to 6 means little effect.



indicate respectively whether this effect of noise was greater for older (O), above 45 years, or for younger (Y) subjects, and, in the last column for men (M) or for women (W). Mann-Whitney U Tests compared the two groups for significance.

EEG/EOG Sleep Stages: Only one of the measures provided a significant result: number of minutes spent in Stage 4 increased when double glazing was installed, and resumed its lower level on return to noisy conditions.

EEG Frequency and REM: Delta sleep increased significantly when double glazing was introduced on the middle week and fell again on the return to noise. A similar pattern occurred with Alpha, but just failed to reach significance. Spindles and REM were unaffected by the noise variable.

Cardiovascular Measurement: Heart Rate was increased significantly under the quieter conditions of double glazing, although the change was small. Heart Rate Variability was unaffected. A correlation was obtained within each night of the minute-by-minute measures of Heart Rate and noise level. For all 12 subjects this correlation was positive. Although the average of all correlations was only +0.11, this result is significant ( $P < .01$ ) on Sign Test. Thus within the night increased noise was associated with higher Heart Rate, yet on an overall basis Heart Rate was lower on noisy nights than on quiet ones. This suggests an overall depressant influence of noise on ECG with an opposite effect superimposed due to transient changes within the night.

Performance Tests: There was a significant speeding up in Unprepared Simple Reaction Time during the week of quiet. This, as with Stage 4 and Delta sleep was reversed again on the return to noisy conditions. Of the other tests, practice effect made it difficult to decide whether 4-Choice Reaction Time and Vigilance were affected by noise. Short Term Memory clearly was not.

Subjective Assessments: Two of these were significant: To the key question "How well did you sleep last night?" the response was "better" during the quiet week than during either of the noisy weeks. Second, the number of awakenings was significantly fewer on the quiet week than for the average of the two noise weeks.

Individual Differences: There was no clear indication that either age or sex greatly influenced the effects of noise on sleep, although the number of subjects was too small to draw any firm conclusions. Both Total Sleep Time and % Wake Time in the EEG were reduced more by noise in the old than the young. Also the tendency for noise to reduce EEG Stage 4 was greater for women than men.

Noise Levels: The normal average noise level for noisy nights was 46.6 (range 42 to 52) Leq dBA. For quiet nights the double glazing reduced this by an average of 5.8 Leq dBA to 40.8 Leq dBA.

#### Correlations between 'Measures of Sleep Quality'

Many measures have been taken in this study. It would be confusing to present correlations between all of them. Instead three have been chosen, all of which were significantly 'improved' in quiet, and which represent three different 'measures' of sleep quality: Delta Activity (physiological), Unprepared Simple Reaction Time (performance), and "How well did you sleep?" (subjective). Pairwise product-moment correlations among these are shown in Table 2, between subjects on the basis of the noise minus quiet effect, and within subjects across individual nights. For the latter Table 2 shows the average correlations over all subjects ( $N = 11$ ) and in brackets, the number of subjects with a correlation in the direction of the average. Subjects with the greatest adverse effect of noise on subjective sleep quality were the ones whose EEG Delta was most reduced by noise, but the other two between-subject correlations, both involving reaction time,

were near zero. Within subjects all correlations were in the expected direction, but none were significant.

Table 2	Reaction Time	Subjective Rep.	EEG Delta	
Reaction Time		+0.04	-0.03	
Subj. Report	-0.18(8)		+0.45	<u>Between</u>
EEG Delta	-0.20(7)	+0.17(7)		<u>Subjects</u>
		<u>Within Subjects</u>		

Transient Effects: Noise Peaks on the EEG

Method: The data were samples, at approximately 1/sec, from the previous analysis of EEG Alpha, Spindle or Delta frequency, and REM, together with parallel samples of the noise level. A computer programme scanned the noise record for peaks within 15 ranges, each 3 dBA wide, ascending from 30 dBA to 75 dBA. A peak was rejected if a comparable (within 3 dBA) or higher peak occurred within a period 32 sec before it or 12 sec after it. This excluded any nearby individual peaks and also any smaller peaks on the slopes of the peak concerned, and ensured that only isolated peaks with sharp rise and fall times were accepted. Having detected a peak in the range the computer programme stored counts of Alpha, Spindle, Delta, or REM for 7 '1-sec' samples before the peak, for the peak itself, and for 8 samples after. These samples were then averaged over all peaks in each range, and then averaged over all subjects separately for noisy and quiet nights. Finally, for this report, these averages are shown collapsed into two ranges of noise level, above and below 54 dBA.

Results: These curves are given in Fig. 1. Delta failed to show the expected reduction after the peak, whatever the level. Instead peaks at all levels, and for both noisy and quiet nights, were associated with a transient increase in Delta at and around the peak point. This may reflect the occurrence of K complexes (brief bursts of Delta-like responses evoked by exogenous or endogenous sensory stimuli). The same phenomena appeared in Alpha frequencies, but Spindles were little affected by noise peaks. REM appeared to be depressed by peaks and to rise substantially after them, again irrespective of peak level or whether the night was a noisy or quiet one.

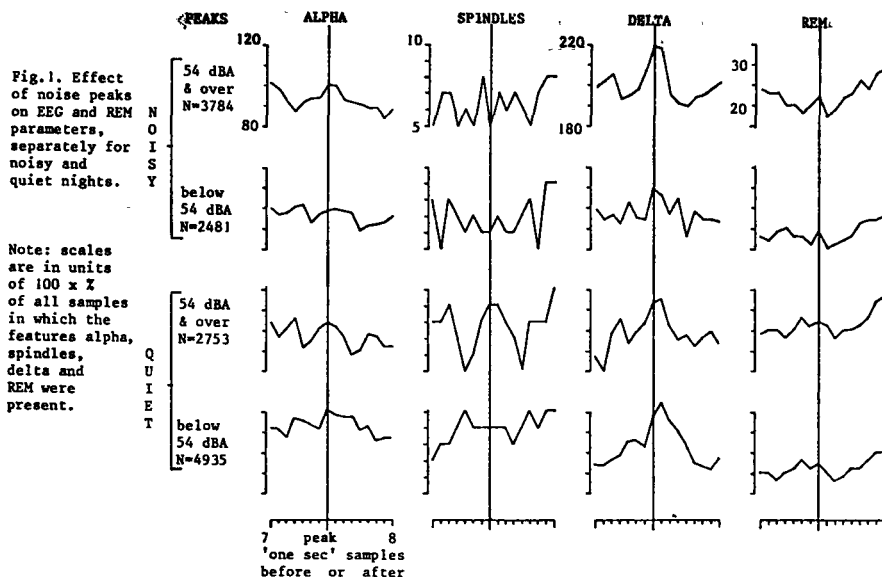
Transient Effects: Noise peaks and Cardiovascular Function(ECG)

A peak analysis similar to that described for the EEG has been carried out on 11 of the subjects covering a total of 8 noisy and 10 quiet nights only. ECG rates for periods 16 sec before and after noise peaks were examined for the ranges of noise peak used before. There were no apparent effects of noise peaks at any level upon the ECG, either with or without double glazing.

Conclusions

In residential areas with very high nocturnal traffic noise (an average of 46.6 Leq dBA at the bedside) the effect of double glazing the bedroom windows (giving an average of 5.8 Leq dBA attenuation) was to move three independent 'measures of sleep quality' moderately but significantly in the direction of better sleep. The measures were Subjective Report of sleep quality, Reaction Time performance, and Delta Activity in the EEG at night. Noise attenuation also increased EEG Stage 4 and Heart Rate, and reduced the Number of Wakenings recalled. The conclusion is that traffic noise at these levels is harmful to sleep.

In marked contrast to this overall evidence, an examination of the effects



of transient noise peaks during the night failed to reveal any changes in either the EEG or the ECG which would suggest that either sleep or cardiovascular function was being disturbed by individual noise peaks, whatever their level. The conclusion, therefore, is that the sleep of these permanent residents had become adapted to the sound of individual vehicles passing in the night, but that over the whole night the almost constant succession of noise peaks prevented these people from reaching levels of sleep as deep or satisfying as they would normally do.

This study has been constrained to consider only those residential areas having the very highest noise levels in the country, amounting almost to continuous traffic noise throughout the night, and also those people who were under no form of medication, that is were quite normal from a cardiovascular point of view, and felt no need to take sleeping pills. The fact that such people had not moved away from the area (83% of the subjects owned their own houses) suggests also that they had become used to the noise. This, therefore, is a 'best choice' selection of subjects. That they can still show an overall improvement of the quality of their sleep with noise attenuation suggests that for a population selected less for immunity the effects of noise might be greater.

One aim of future work, therefore, should be to study locations having fewer, but not necessarily quieter, vehicles passing, and to accept people on sleep, or cardiovascular medication as well as normals. This should increase the chance of seeing physiological effects of transient peaks during the night, and hence of defining, as has not been possible here, an acceptable noise peak level. A second aim should be to increase greatly the numbers of people studied. This may be done by using the present experience to rationalize the methodology, limiting measures to those which provide a good chance of positive results and are less costly and time-consuming. In this way, as well as covering more kinds and levels of residential traffic noise, it should be possible to place more emphasis on the study of individual differences. This should reveal any groups whose health is at risk due to traffic noise and who need to be treated with special consideration, once we have objective ways of identifying them.

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Note: Papers referred to in the text are marked '\*'; papers, communications, etc, deriving from this research are marked '+'.  
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Contractor: Universität Erlangen-Nürnberg (Institut für Physiologie  
und Biokybernetik. Prof.Dr.W.D.Keidel)

Contract Nr.: ENV 334 - 80 D

Project leader: Prof. Dr. M. Spreng

Title of project: Comparison of effects of impulsive noise and continuous noise using objective physiological parameters

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--Objectives of the pilot study

The purpose of the study was the finding of more or less influenced sensorineural and vegetative physiological parameters of human subjects being exposed to continuous and impulsive noise of equal sound energy under laboratory conditions. Results from physiological studies of that kind are necessary to judge and complete subjective statements about annoyance, which, regarded alone, may perhaps induce too large or too small corrections for the rating of impulsive noises.

--Materials and methods

In the pilot study, starting in March 1980, 43 experiments had been carried out with 8 young (17 to 25 years) male subjects having no abnormal hearing loss (less than 15 dB). On five different days the subjects have been exposed (randomized) to continuous white noise [WN], white noise bursts (200 ms) [WNB], silence [S], impulsive noise presented regularly (100 ms) [INR], and impulsive noise presented irregularly [INI]. The noise exposures (10 min) have had an equivalent sound pressure level of 80 dB (WN: 80 dB; WNB: 87 dB; INR and INI: 96 dB).

During the preceding and succeeding periods (quiet) as well as during and immediately after the exposure the following physiological parameters have been recorded on tape

ECG (electro-cardiogram)

Peripheral responses of the auditory system (e.g. brain-stem response)

EEG (electro-encephalogram)

On line and from the recorded signals averaged values for several time periods have been calculated with additional methods. To study the dynamic behaviour of the blood pressure regulatory system a short-time

analysis has also been done.

At the end of the experimental series the subjects have been asked for their subjective experience of the noise exposure.

#### --Results

ECG (long-time analysis): The absolute values of the arrhythmia quotient of the heart rate show a decrease or constancy short after the beginning of the stimulation with the white noise bursts and with the other impulsive noises. They are increased during the stimulation with the continuous white noise. Thus the difference of the average values in the period 1 min after beginning minus 5 min after beginning of the noise is positive or zero for all the impulse noises, but significantly negative in the case of a stimulation with continuous white noise. The effect that the arrhythmia quotient does not increase during impulsive noise stimulation mainly is caused by the enlarged number of polarity changes.

The relative differences with respect to the longer (5 min) preceding period, however, behave slightly opposite, but show no significant result.

#### ECG (short-time analysis)

The recovery of the heart beat intervals from their shortening immediately after the beginning of the noise is reduced or abolished (also the shortening may be suppressed) by the impulsive noises. Therefore the evaluated "recovery values" are significantly reduced compared with that one following continuous white noise stimulation. Additionally the mean differences of the heart beat intervals immediately preceding and succeeding the beginning of the noise show a clear trend of suppressing the normal tendency of increasing heart beat intervals in the progress of the experiment, especially during the stimulation with the impulse noises presented regularly and irregularly.

#### Peripheral responses

The inter-amplitude differences of component V and III of the compound nerve and brain-stem response result in an increase caused by the three impulsive noises. This means a reduction of the amplitude of component III (and/or a slight enhancement of the

amplitude of the component V) as a sign of different influences of the impulsive noises upon the more central parts (brain-stem) of the auditory system.

EEG: The spectral components in the slow range of the alpha-rhythm (8 Hz) as compared with the preceding period of silence show significantly higher activity evoked after the beginning of the impulse noises (mainly INR and INI).

The influences of the impulsive noises (WNB, INR, INI) are summarized as relative values (rectified) in the following table 1 and compared with the results of the questionnaire (table 2)

	WN	WNB	INR	INI
ECG				
Arrhythmia quotient (1 ms discrimination)	100	125	302	195
ECG				
Arrhythmia quotient (10 ms discrimination)	100	203	279	197
HEART RATE (recovery value)	100	160	150	183
HEART BEAT INTERVALS (short-time analysis)	100	78	172	213
PERIPHERAL RESPONSES (inter-amplitude differences)	100	350	184	275
MEAN	100	203	217	213
EEG (8 Hz)	100	220	2800	1120

TABLE 1 Relative changes (rectified) of the physiological parameters caused by the impulsive noises (WNB, INR, INI) with respect to the stimulation with continuous white noise (WN).

	WN	WNB	INR	INI
How annoying ?	100	126	143	152
How annoying in your living room ?	100	118	124	135
% highly annoyed in living room	(75) 100	(75) 100	(100)133	(100)133
MEAN	100	115	133	140

TABLE 2 Relative changes of the subjective ratings of annoyance.

--Conclusions and additional comments

As shown with the table 1 there are effects and trends of the physiological parameters which correlate roughly with the subjective statements.

Because in this pilot study only one measurement could be made per subject and condition, and the age of the subjects had been limited from 17 to 25 years (most stabile regulatory systems) larger variabilities and smaller effects as usual have resulted.

The parameters and values listed in table 1 seem to be valuable for a further study with environmental noises, which are longer lasting and of slightly higher intensity. Older subjects should be included in that study and the dynamic behaviour of the parameters should be more emphasized.



Contractor: Institut für Lärmschutz, Düsseldorf, Germany.  
Contract No: ENV-335-80D  
Project leader: E. Buchta  
Title of project: Effects of Impulse Noise on Human Beings  
Group 3: Annoyance in the Laboratory

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### OBJECTIVE OF THE RESEARCH

A joint project to study annoyance due to impulsive sounds in the laboratory was initiated and partially funded by the Commission of the European Communities, Directorate-General for Research, Science and Education, under the Environment and Raw Materials Research Programme.

The participating laboratories in England (ISVR Southampton), Netherland (TNO Soesterberg), Denmark (TU Lyngby) and Germany (IfL Düsseldorf) carried out the same experiments by the same four tape recordings (copies) of four sounds.

### MATERIALS AND METHODS

In a prepared experimental room 16 volunteers men from 20 to 30 years old were exposed to the following noises:

- a) (T) fluctuating traffic noise: The fluctuations of the sound level correspond to  $\pm 5$  dB (ISVR Southampton).
- b) (P) pile driving noise reproduced from tape No. 12 used for the Round Robin Test for loudness (repetition rate 2 pulses every 3 seconds), University Lyngby.
- c) (G) isolated gun shots at random intervals (Poisson's distribution with a mean interval of 2 seconds between shots (TNO Soesterberg).
- d) (S) synthetic impulses with fast exponential rise time (1 ms) and slow exponential decay time (100 ms) at constant rate of one pulse per second (TNO Soesterberg).

Each of these noises was presented for five minute periods at four different levels to sixteen subjects according to a pre-determined experimental design sequence. The room in which the measurements were carried out was similar to a living - room. The instruments and the operator of the set up were situated in another room outside of the listening room.

The completed test procedure, including a hearing test at the beginning and a coffee break halfway through, took approximately 2 hours for each subject.

Full details of the study are described in the Individual Contract Report 1980/81 which was prepared in accordance with contractual requirements. In this report the principal results only are described.

## RESULTS

### Question 1: Reported annoyance in laboratory

The means for SOUNDS and LEVELS are shown in Table 1. The traffic noise is less annoying at the  $Leq = 49$  dB(A) level. Between 56 and 70 dB(A) traffic noise is the second most annoying noise. At the lowest  $Leq = 49$  dB(A) the gun shots annoyance rating becomes the highest and traffic annoyance rating the lowest (Fig.: 1).

The slope of the grand mean (overall ratings for all four sounds) is 1.2 steps in subjective response for increasing the  $Leq$  level by 7 dB(A).

The regressions for each sound are given in Fig.: 3. The difference between slopes are not significant.

Physical Data are shown in Table 3.

### Question 2: Annoyance projected to home environment

For question 2 the annoyance ratings of synthetic impulses were generally higher than for the other sounds also at the lowest level  $Leq = 49$  dB(A). At the highest level ( $Leq = 70$  dB(A)) piling and gun fire gave the minimum ratings of annoyance (Fig.: 2).

The grand mean of the subjective scale value rises from 5.8 to 7.2 for the  $Leq$  level from 49 dB(A) to 70 dB(A).

The regressions for the four noises are shown in Fig.: 4.

### Other Questions

Questions relating to the percentage of subjects annoyed " a little ", " moderately " or " very much " were also asked, but the results they produced were not definite as those for question 1 and 2.

At the end of the whole test the volunteers have also grade the four types of sounds, heard again successively at the same  $Leq = 60$  dB(A) during 15 seconds on a decreasing scale of annoyance from the most annoying sound to the least annoying sound. After presentation the volunteers could correct their rank order given before the 15 second presentation. The rank ordering showed that traffic noise is least, synthetic impulse

has the highest mean rank and gun fire with pile driving are close together.

## CONCLUSIONS

The result of the laboratory measurements shows that calculated Leq levels correspond with values measured by integrating noise meters BK 2218 or RC 324.

The difference of the " impulse level " and the " true Leq " is 7 - 8 dB(A) for the three impulsive sounds (synthetic, gun fire, pile driving) and 1.2 dB(A) for the traffic noise.

The annoyance ratings on a 10 point linear scale are higher for the synthetic noise at three noise levels (56 - 70 dB(A)); the second most annoying is the traffic noise at high Leq ( 70 dB(A)). At the lowest Leq (49 dB(A)) the gun shots annoyance rating becomes the highest. In general, the impulsive sounds were more annoying than traffic noise at lower levels.

All volunteers were " very much annoyed " by the four noises if the Leq level reaches 63 dB(A). One volunteer did not want to continue the experiment after the third session and he had to be replaced.

## REQUIREMENTS OF THE COMMON RESEARCH

Traffic sound is the yardstick to which all other impulsive sounds will be compared and it should be recorded outside at different noise levels.

The repetition rate and temporal regularity of the impulses could be modified. The impulsive sounds are those registered in the environment like gun shots, pile driving, door slamming (car), dog barking, compressed air hammer, horn of a car, industrial sounds like punch and press (outside).

Questionnaires should be the same type as those used in the pilot phase ( linear scales for annoyance). For a following test 30 - 50 persons in each laboratory could be exposed to the whole series of noises in short term sessions to express relative annoyance ratings.

## REFERENCE

E. Buchta (1980/81) Pilot Phase: Effects of Impulsive Noise on Human Beings  
Subgroup 3: Annoyance Ratings in Laboratory Conditions.  
Individual Contract Report 80/81

Level	Noise	S	P	G	T	Overall
49		4,0 $\pm$ 1,5	4,0 $\pm$ 1,9	4,5 $\pm$ 2,2	3,5 $\pm$ 1,8	4,0 $\pm$ 0,4
56		5,7 $\pm$ 1,6	4,8 $\pm$ 1,8	5,3 $\pm$ 2,0	4,9 $\pm$ 1,8	5,2 $\pm$ 0,4
63		6,8 $\pm$ 1,6	6,1 $\pm$ 1,5	6,2 $\pm$ 1,2	6,4 $\pm$ 1,5	6,4 $\pm$ 0,3
70		8,1 $\pm$ 1,1	6,7 $\pm$ 1,7	7,3 $\pm$ 1,5	7,8 $\pm$ 1,5	7,5 $\pm$ 0,6
Overall		6,2 $\pm$ 1,7	5,4 $\pm$ 1,2	5,8 $\pm$ 1,2	5,7 $\pm$ 1,9	5,8

TAB.1.1 MEDIAN ANNOYANCE RATING SCORES FOR THE QUESTION 1

"How annoying did you find the noise you have just heard, if you heard it all the time?"

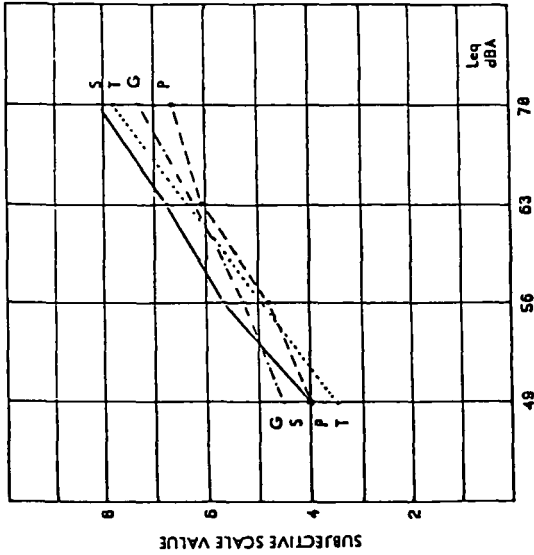


FIG.1.1 MEAN ANNOYANCE SCORES (QUESTION 1)

Level	Noise	S	P	G	T	Overall
49		6,2 $\pm$ 2,1	6,2 $\pm$ 2,3	5,8 $\pm$ 2,0	5,1 $\pm$ 2,4	5,8 $\pm$ 0,5
56		7,1 $\pm$ 1,8	6,8 $\pm$ 1,6	6,6 $\pm$ 1,8	6,4 $\pm$ 1,9	6,7 $\pm$ 0,3
63		8,0 $\pm$ 1,3	7,7 $\pm$ 0,8	7,8 $\pm$ 1,0	7,6 $\pm$ 1,3	7,8 $\pm$ 0,2
70		8,8 $\pm$ 0,4	8,1 $\pm$ 1,0	8,1 $\pm$ 0,8	8,7 $\pm$ 0,9	8,4 $\pm$ 0,4
Overall		7,5 $\pm$ 1,1	7,2 $\pm$ 0,9	7,1 $\pm$ 1,1	7,0 $\pm$ 1,6	7,2

TAB.1.2 MEDIAN ANNOYANCE RATING SCORES FOR THE QUESTION 2

"How annoying would you find this noise if you heard it all the time in your own living room in the evening?"

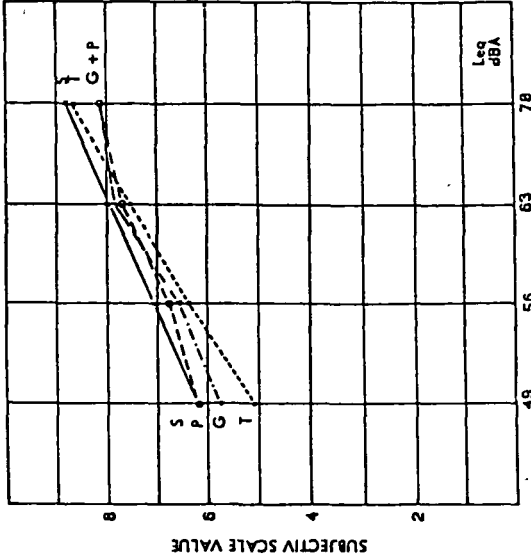


FIG.1.2 MEAN ANNOYANCE SCORES (QUESTION 2)

	TRAFFIC NOISE				GUNFIRE				SYNTHETIK IMPULSES				PILE DRIVING					
	RC 324	4426		4426		4426		4426		4426		4426		4426		4426		
		FAST	SLOW	IMPULSE	FAST	SLOW	IMPULSE	FAST	SLOW	IMPULSE	FAST	SLOW	IMPULSE	FAST	SLOW	IMPULSE	FAST	SLOW
L <sub>eq</sub>	70,2	70,1	71,4	70,1	70,1	77,8	70,1	70,1	77,8	70,1	70,0	77,4	70,0	70,0	70,0	77,9	70,0	77,9
L 1 %	75,8	74,5	77,3	78,5	73,3	80,6	76,5	71,8	79,2	77,7	72,4	80,3	77,7	72,4	80,3	77,7	72,4	80,3
L 5 %	74,3	73,2	75,3	77,8	73,0	80,1	76,2	71,5	79,2	76,7	72,1	79,8	76,7	72,1	79,8	76,7	72,1	79,8
L 10 %	73,3	72,7	74,6	76,8	72,8	79,9	75,5	71,5	78,9	75,7	71,9	79,3	75,7	71,9	79,3	75,7	71,9	79,3
L 50 %	69,3	70,0	70,8	57,3	70,1	77,9	64,7	70,3	77,7	62,2	70,1	78,0	62,2	70,1	78,0	62,2	70,1	78,0
L 90 %	65,3	66,7	66,6	47,3	65,8	74,4	52,2	68,8	75,9	49,9	68,4	76,5	49,9	68,4	76,5	49,9	68,4	76,5
L 95 %	63,8	65,7	65,3	46,5	63,3	71,9	50,7	68,5	75,4	48,9	67,9	76,3	48,9	67,9	76,3	48,9	67,9	76,3
L 99 %	61,1	62,0	61,8	46,0	59,1	69,9	49,5	68,0	74,9	48,2	67,4	75,8	48,2	67,4	75,8	48,2	67,4	75,8

STATISTICAL LEVELS ABOUT THE FOUR TYPES OF NOISES

Type	Peak Hold	Impulse	Fast	Slow
Gunfire	94,0	81,0	80,0	74,0
Synthetic	91,5	79,0	78,0	72,0
Piling	94,0	80,0	78,0	73,0
Traffic	88,0	76,0	75,0	74,0

	7 dB
Gunfire	7 dB
Synthetic	7 dB
Piling	7 dB
Traffic	2 dB

IMPULSIVE CHARACTER

- Notes: 1. All levels at nominal  $L_{eq} = 70$  dB(A)  
 2. All levels A-weighted at subject's head position

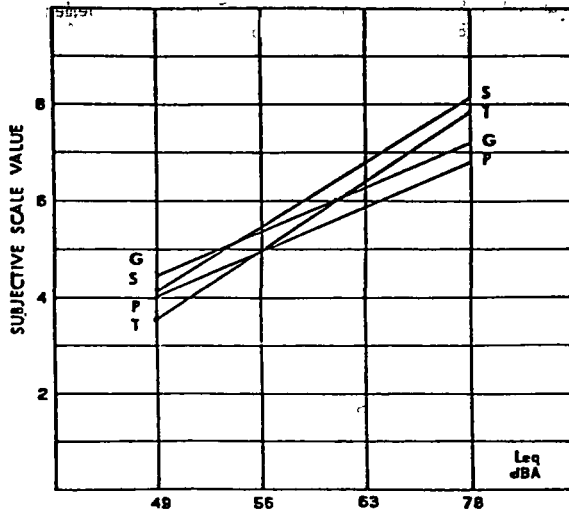
A - WEIGHTED MAXIMUM LEVELS  
 ON 2666 METER ( $L_{eq} = 70$  dB(A))

TABLE 3

Sheet A / Question 1

How annoying did you find the noise you have just heard, if you heard it all the time ?

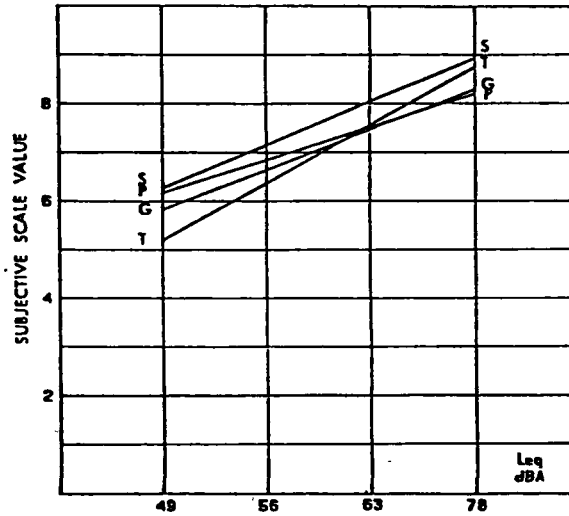
FIG.: 3



Sheet A / Question 2

How annoying would you find this noise, if you heard it all the time in your own living room in the evening ?

FIG.: 4



REGRESSIONS FOR SYNTHETIC, GUNFIRE, PILING AND TRAFFIC

Contractor : Société d'Etudes pour le Développement Economique et Social  
84 Rue de Lille, 75007 Paris.

Contrat n° : ENV/344 F

Project Leader : Michel JOUVENT

Title of Project : 'Effects of impulsive sounds on human beings,  
Pilot Study

---

Objective of the research :

The objective of the main research is to assess the relative importance of "impulsive" sound, defined as composed of short bursts of sound, among the other kinds of noise occurring in the environment of human beings. The pilot study had two main purposes :

- design and test of a questionnaire to be used for the main survey
- test of various ways of measuring "impulsive" sounds and characterizing their impulsiveness.

The research has been carried out by four research teams (England, Ireland, Netherlands, France) which have designed a common questionnaire.

Design and test of the questionnaire :

The questionnaire had two main purposes :

- Know which kind of noise people hear (impulsive or not impulsive), the frequency of occurrence for each noise heard, and what people think about them (annoyed, pleased, disturbed ..) by means of a wide list of various noises, including impulsive noises.
- Give information about the environment of the interviewed persons, including the amount of noise they hear in general, and know what they think about the effects of noise and environment on their health.

The questionnaire has been tested on 50 persons (for each country). The areas chosen in France were in a large city and its suburbs, near construction sites, with a wide range of impulsive noise.

The questionnaire proved suitable for the main study, provided that the average duration of the interview is reduced to 30-40 minutes by shortening the list of specified sounds.

Noise measurements :

Noise measurements have been carried out on the areas when the questionnaire has been tested, and graphic analysis of the noises registered on tape have been made, according to the EEC directive 79 113. This type of measurement is based on the difference between the sound levels on the "slow" and "impulse" modes of the sound level meters. A sound is "impulsive" for the directive when this difference exceeds 4 dB.

This method proved useful to determine which noises are "impulsive" during a short period (less than 10 minutes), but new methods based on statistical analysis should be developed for long-term analysis (24 hours analysis for exemple).

Conclusions :

The pilot study shows that it is possible and worthwhile carrying out a larger survey based on the present questionnaire, reduced in length by modifying the list of noises, the respondents being chosen in the major kinds of noise areas of each country.



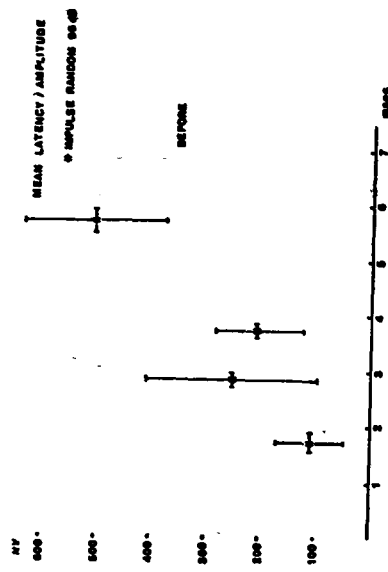


FIG. n. 2

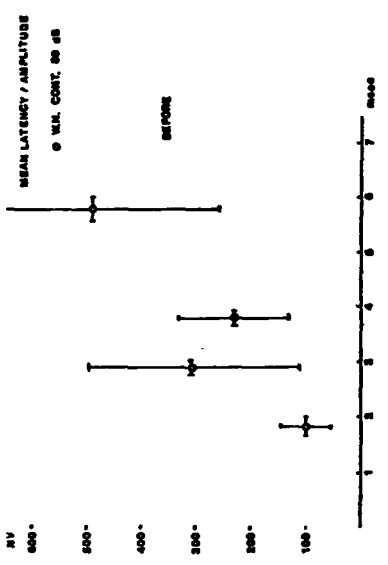
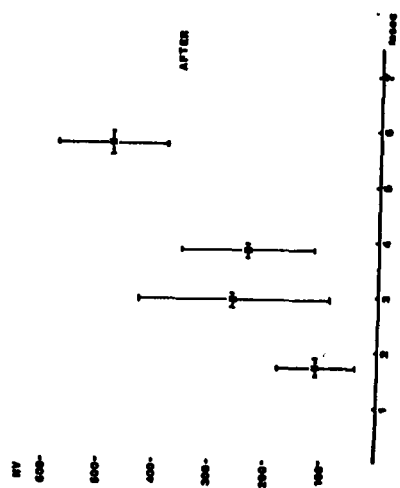
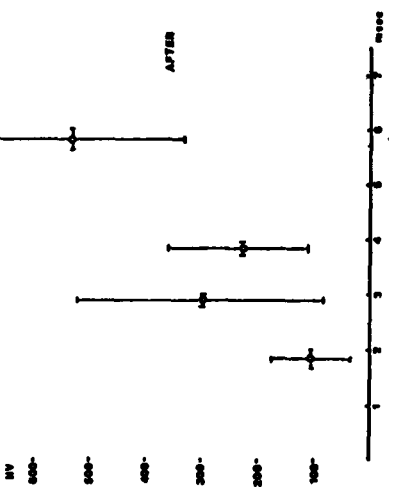


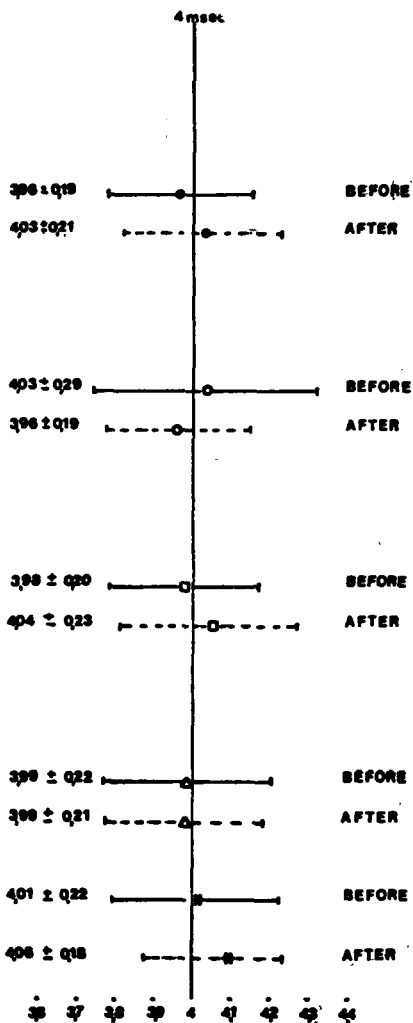
FIG. n. 1



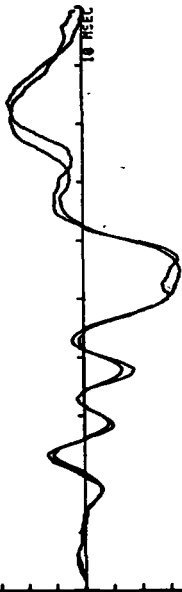
MEAN INTERWAVE LATENCY

[I-(IV-V)]

- SILENCE
- WN CONT 80 dB
- WN BURST 87 dB
- △ IMPULSE 96 dB
- ⊕ IMPULSE RANDOM 96 dB



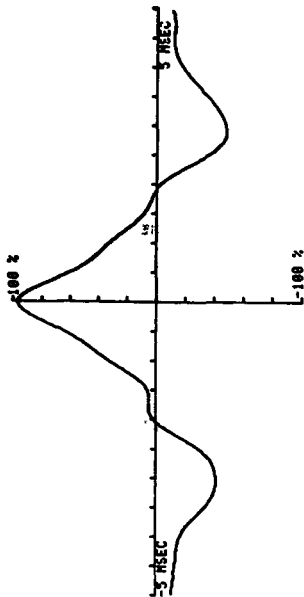
500 NU SI=98 DB PPS=20 CLICK SC=1824 TR 0 CH



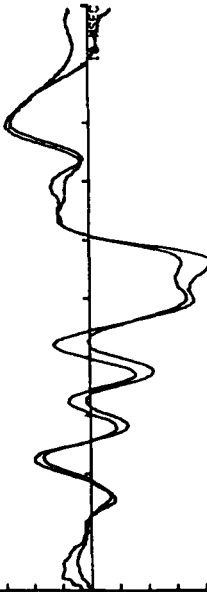
OP / MN CONT 88 DB TEMPLATE FIG 4/A

R(0) (PERCENT) =97  
P MAX (PERCENT) =97  
Z MAX (PERCENT) =289  
D1 (/USSEC) = 512  
D2 (/USSEC) = 762  
DUMP CORRELATION? 0

MN CONT 88 DB CROSS CORR FIG 4/B



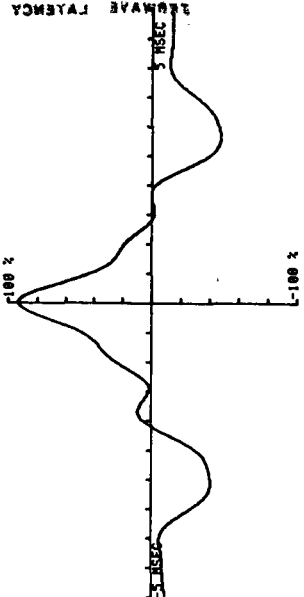
500 NU SI=98 DB PPS=20 CLICK SC=1824 TR 0 CH

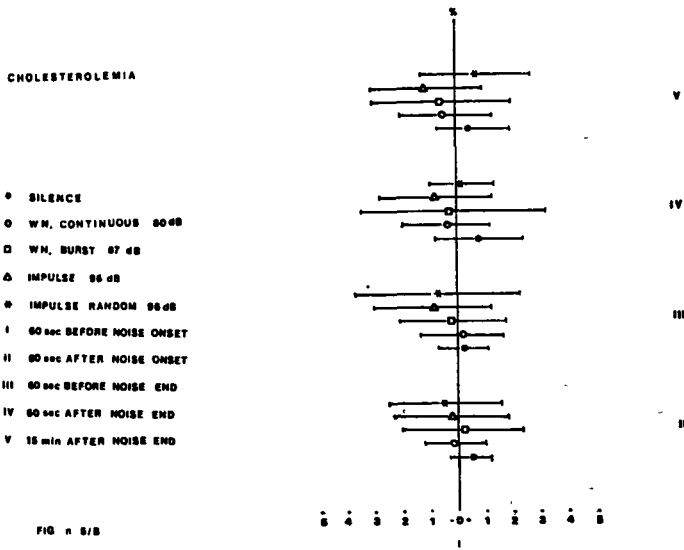
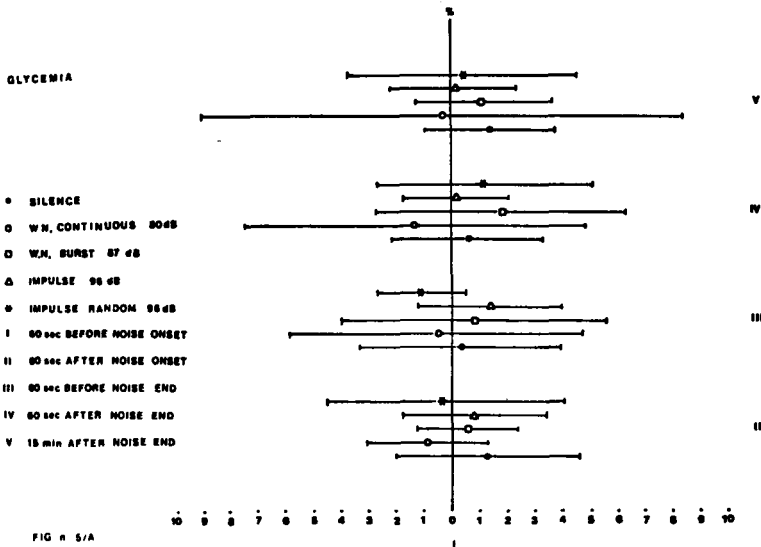


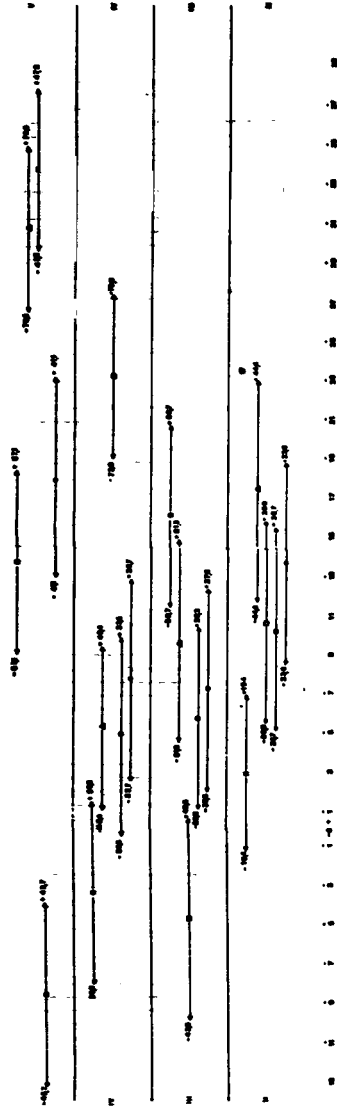
OP / MN IMP RANDOM 96 DB TEMPLATE FIG 4/C

R(0) (PERCENT) =93  
P MAX (PERCENT) =93  
Z MAX (PERCENT) =165  
D1 (/USSEC) = 512  
D2 (/USSEC) = 762  
DUMP CORRELATION? 0

MN IMP RANDOM 96 DB CROSS-CORR FIG 4/D







- BALANCE
- BAL. CONTINUOUS READ
- BAL. UNDER 20 LB
- SAMPLED IN AIR
- SAMPLED IN ROOM
- BALANCE UNDER 10 LB
- BALANCE UNDER 5 LB
- BALANCE UNDER 2 LB
- BALANCE UNDER 1 LB
- BALANCE UNDER 0.5 LB
- BALANCE UNDER 0.2 LB
- BALANCE UNDER 0.1 LB

PL. 1.0

**Contractor: University of Turin (Italy)**

**Contract n°: ENV/349 I**

**Project leader: Prof. G. Rossi, Head, Dpt. of Audiology**

**Title of project: Physiological effects of impulsive noises (Pilot study 1980)**

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As part of the joint European research project on the effects of impulsive noise, the Turin University Audiology Department was commissioned (Contract ENV/349/I-(S)) to carry out a preliminary study of brain stem potentials, certain blood parameters, and heart activity on exposure to continuous and impulsive noise of equal Leq.

The purpose of the study was to see whether fatiguing stimuli with the same energy content produced different effects on these parameters in function of time.

#### Materials and methods

##### a) Study of brain stem potentials

The subjects were 10 males aged 19-23 yr and free from ear disease. Tonal audiometry using an Amplifon 300 was carried out to ensure that each subject's threshold level for frequencies between 125 and 8000 Hz was not more than 15 dB. The fatiguing stimulus was recorded on 1/4" tapes at 7.5 ips, and sent out to each laboratory.

Tape contents:

Tape 1 1) pure tone at 1000 Hz, 80 dB SPL (1 min); 2) silence (5 min); 3) four series of 1024 clicks, each lasting 100  $\mu$ s, at the rate of 20 per second, 90 dB (about 5 min); 4) silence (10 min); 5) continuous white noise (10 min): Leq 80 dB SPL; 6) four series of clicks as in period 3); 7) silence (10 min).

Tape 2. as tape 1), but period 5) consisted of white noise bursts lasting 200 ms at 87 dB SPL, at the rate of 1 per second with Leq 80 dB SPL.

Tape 3. as tape 1), except period 5) which contained 10 min silence.

Tape 4. as tape 1) but in period 5) the stimulus was made up of artificial impulsive noise with arise time of 1 ms, exponential decay of 100 ms, at the rate of 1 per second with peak level of 96 dB SPL (fast) and Leq 80 dB SPL.

Tape 5. as tape 4), except that period 5), was administered at an irregular rate.

Tapes 1, 2, 3, were prepared by the Acoustic Section of FIAT Research Laboratory (Turin, Italy), tapes 4, 5 by TNO Laboratory (The Netherlands).

The tests were performed at 9 a.m. in a silent faradic booth with the subject supine and his head extended. Needle electrodes were applied to the vertex, mastoid process, and forehead (earth). The pass band was set between 200 and 2000 Hz, with a 10 $\mu$ V gain and a 10 ms analysis time.

The click stimulus and the fatiguing stimulus were administered unilaterally through standard TDH 39 headphones from a Revox A 77 twin-track recorder. Intensity levels were measured in the headset with a Brüel & Kjær type 2209 phonometer.

##### b) Study of blood parameters and heart activity

The subjects were 10 males aged 18-24 yr. The audiometric test and the tapes were as described in a) above.

The following parameters were investigated:

blood sugar, azotemia, uric acid, cholesterol, triglycerides, diastolic and systolic blood pressure, heart rate, NEFA, and catecholamines.

The subjects were examined while lying down in an anechoic room. The stimuli were administered unilaterally through TDH 39 headphones.

Blood samples were withdrawn via a cannula inserted in the right basilic vein.

Laboratory analyses were performed at Fiat-Auto's Health Service Laboratory, with the exception of catecholamines, which were titred at the University of Pisa Clinical Physiology and Medical Pathology Department.

Homeral blood pressure values were taken with a Diasist T.

Heart rate was calculated from ECG tracings obtained with an 8-channel Mingograf (Enema-Schönander).

For technical reasons, all parameters were determined in accordance with the following pattern:

- I) 1 minute before commencement of noise
- II) 1 minute after commencement of noise
- III) 1 minute before end of noise
- IV) 1 minute after end of noise
- V) 15 minute after end of noise

In addition, the heart rate of 4 volunteers was measured under the same conditions as those employed in the study of brain stem potentials.

The tapes were recorded in FM with a Brüel & Kjaer recorder at 6.5 ips on 1/4" tape. For technical reasons connected with processing of the data, the recordings were subsequently transferred to 7.5 ips tape. They were then sent to the University of Erlangen Physiology and Biocybernetics Department for processing.

## Results

### a) Brain stem potentials

Latency, amplitude and some components of the characteristic wave train were evaluated. Waves I, II, III and the IV-V complex were evaluated in accordance with the mean morphology of the responses obtained.

The mean response of the 10 subjects was calculated, and the standard deviation (SD) was determined for each mean value (latency and amplitude). Arithmetical and graphic comparison of the values obtained before and after each fatiguing stimulus is illustrated in figs 1, 2 for W.N. continuous and for W.N. impulse random.

Next, each subject was analysed by comparing the mean ( $\pm$  SD) before and after the presentation of each stimulus.

This analysis was made separately for latency and amplitude.

Lastly, the latency between wave I and the IV-V complex was examined. Once again, the mean and SD were calculated for the values observed before and after the stimuli (Fig. 3).

Next, the results were processed to ensure an objective comparison between the responses before and after the fatiguing stimuli. Two typical curves (templates) were obtained from 20 responses before and after exposure to each stimulus respectively. These were compared and some cross-correlation coefficients were calculated (figs. 4A, B, C, D).

Direct morphological comparison and the numerical values made it clear that the brain stem potentials were in no way altered by the fatiguing stimuli.

#### **b) Blood parameters and heart activity**

Blood parameters patterns were evaluated from the percentage variation of the mean for the 10 subjects examined, and the absolute variation for each subjects.

With the exception of the catecholamines and NEFA, the percentage mean variation and SD were always within  $\pm 5\%$  and  $\pm 9\%$  respectively, including the 2% margin of error present in the analysis technique. The data for blood sugar (fig. 5A), for cholesterolemia (fig. 5B) and for NEFA (fig. 6) are presented by way of exemple. Statistically significant alterations were also absent in the case of the absolute values.

Catecholamines increased more markedly after continuous noise than any other stimulus, and this increase was still present 15' after the stimulus ceased.

The considerable spread of the data may be due to the particularly difficult and complicated analysis technique, and also to the wide fluctuations catecholamines may display even in the absence of specific stimuli, owing to emotional and psychological factors.

NEFA values could not be directly correlated to any particular stimulus, but tended to increase during and after all of them.

The heart activity results will be presented by the Erlangen University Physiological and Biocybernetics Department, along with the data concerning their own subjects.

#### **Conclusions**

None of the stimuli employed resulted in significant modifications in the electrical responses derived from the central acoustic pathways, between Corti's organ and the medial geniculate body. In particular, there was no significant difference between the effects of continuous and impulsive noise.

The responses to continuous, interrupted, and impulsive noise did not display distinct patterns when compared with those obtained in the absence of stimuli.

Similar conclusions can be drawn with regard to the blood parameters. In the case of NEFA and the catecholamines, particularly owing to the technical difficulties associated with the determination of the latter, and their frequent fluctuations owing to casual individual psychological factors, no clear conclusions can be drawn as to their patterns in relation to the time characteristics of the stimuli employed. In general terms, it can be said that their values tend to increase, and that this increase persists for as long as 15' after removal of the stimulus.

In our opinion, the intensity of the fatiguing stimulus in particular was not great enough to bring about significant changes in the parameters considered.



Contractor : Centrale Organisatie TNO  
Contract n° ENV-352-80 N  
Project leader : Drs. R.G. de Jong  
Title of project : Research on the effects of impulsive sounds on human beings. Summary report of a pilot study

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## 1. OBJECTIVE OF THE RESEARCH

*The aim of the project and the method used are described briefly. It appears that reactions to impulsive sounds vary widely. Daily occurring impulsive sounds seem to cause no more annoyance than daily occurring non-impulsive sounds do. The temporary nature of pile driving seems to make people adopt a rather lenient attitude to the admitted noise. Some remarks are made about the noise measurement techniques, the concept of impulsivity, the samples and the questionnaire.*

1.1 Enquiry into impulsive sounds: The objective of this research is a pilot study in the Netherlands to determine the methodology, the questionnaire, the techniques and instruments for a large scale enquiry on the importance of impulsive sounds in the environment.

## 2. MATERIALS AND METHODS

2.1 Choice of the noise: At first we planned to execute our survey in the vicinity of industrial plants. However, a combination of flats in a populous district and industrial impulsive sound of any perceptible level was very scarce in the Netherlands. This forced us to direct the enquiry to noise from pile driving.

2.2 Locations: Two areas were selected, not far apart, in The Hague. The distance between the houses and the noise source at the time of the survey and the measurements varied from sixty to eighty metres. Pile driving at one area stopped one day before the survey started, at the other one it had been going on for several weeks before the survey started.

2.3 Fieldwork and questionnaire: The fieldwork was carried out by face to face questioning from the second half of September to the first half of October 1980. On both locations fifty respondents (twenty-five on each) were interviewed. The questionnaire consisted of 104 questions. Interviewing time was about 45 to 50 minutes. From six preceding pilot

interviews it appeared that the questionnaire was not boring for the respondents. The questionnaires in the four participating countries were as identical as possible. In the Dutch questionnaire were the following variations. The health section, though shortened, was maintained. Registration of the time needed for each section of questions was dropped. Some other relevant questions were inserted.

2.4 Noise measurements: Impulse noise measurements were carried out on different spots in the two locations. Because of high frequency components and the short duration of this type of noise, attention had to be paid to the instruments and the measuring method. Making a registration of impulsive sounds and other environmental sounds without losing information can only be done by recording on tape, so noise records were made at the locations and analysed in the laboratory.

The EC directive 79/113 defines noise as "impulsive" when the difference between the sound levels measured on "slow" and "impulse" response settings exceeds 4 dB or more. Nothing was said about the duration of an impulse or the moment at which the 4 dB difference between the sound levels had to be reached. So in order to meet the EC directive some analysing methods were tried out:

- to find the "EC impulse" by means of a sound level meter and a level recorder using the DC output of the measuring device on "slow" and "impulse" response settings. In this case the decay time constant of the impulse response is longer than at slow response. So the difference in sound level grows after the maximum value has been reached. The sound levels could be registered simultaneously by two level recorders. Also the difference in sound level could be registered on one level recorder by means of a differential amplifier
- The response setting can be done with the level recorder instead of the measuring device (sound level meter or measuring amplifier) by means of the setting of the "writing speed": 31.5 mm/s is in accordance with impulse response. In this case the rise time and decay time of a meter response are exactly the same.

### 3. RESULTS

3.1 Analysing: We did not analyse in depth the results of the pilot study, only the most salient outcomes are presented. No statistical significance tests were carried out; the approach is a qualitative one.

3.2 Characteristics of the sample: The majority of the respondents belong to the higher and middle social classes. There was no difference between the samples of the two locations in the variables of age and sex of the respondents. The samples differed, however, in the variables of having

children (younger than eighteen only presented at one area), marital status, the average time spent at home, working in evening and night shifts and living on a higher floor. These differences must be kept in mind if one would like to compare the reactions in both areas.

3.3 Pile driving and other sounds: The 40 specific (groups of) sounds of our data were divided into: 22 sounds from outside the blocks of apartments; 12 from neighbouring apartments; 6 from inside their own home. From this total of 40 sounds the most frequently mentioned are listed in table 1.

A striking result is that pile driving is only mentioned by 23 of the 50 people who had been exposed to this noise for at least several weeks! This can partly be explained by the fact that some people in the sample are working elsewhere in the daytime (19 of the 50). However, also some people who usually are at home in the daytime do not mention the noise. Another striking result is that bells and churchbells and, very surprisingly, also police, fire or ambulance sirens, appear to have a very deviant psychological meaning to the respondents. Mopeds and motorbikes are the most annoying noise sources in the two locations.

TABLE 1

	I	II	III	IV	V
1 Police, fire or ambulance sirens	41	00	05	04	12
2 Mopeds	36	86	81	17	1
3 Tyres and brakes screeching	29	69	66	12	4
4 Lorries passing by	24	46	50	13	7
5 Motorbikes	23	78	74	22	2
6 Pile driving	23	57	30	03	9
7 Cars or vans passing by	19	42	47	12	8
8 Churchbells	19	00	00	02	13
9 Car, van or lorry horns	18	56	61	16	5
10 A neighbours' plumbing system including WC cistern	18	33	33	13	10
11 Car, van or lorry doors being slammed	17	76	71	11	3
12 Neighbours carrying out (other) do-it-yourself work	16	63	38	13	6
13 Vacuum cleaner (own home)	15	00	00	00	14/15
14 Pneumatic hammers or drills	14	57	14	06	11
15 Bells	12	00	00	00	14/15

I = number of respondents mentioning the sound; II = proportion of respondents mentioning the sound and rating it on the positions 8 to 11 on a rating scale from 1 = like very much to hear the sound at all, III = proportion of the respondents mentioning the sound and rating it as very much or moderately annoying, IV = an index of 6 activity interferences (called the Mean Relative Interference Index), V ranking from 1 = most annoying to 15 = least annoying, derived from a summation of the columns II, III and IV

3.4 Impulsive sounds versus non-impulsive sounds: The 22 sounds from outside were divided into sounds with a probably impulsive character and sounds with a probably non-impulsive character. This is not done with the other sounds because these are often too ambiguous to decide whether they are prevalently impulsive or not. By mutual comparison of the data from table 2 (though with the necessary caution because of the limited sample, which moreover is not representative of the whole population) the follow-

TABLE 2

	I less than one day or one day or several days or most days or all day		II mean score on 11- point scale		III very much or moderately annoying only a little annoyed		IV not at all annoyed		V MRAII	
Impulsive	22	24	54	6.6	38	20	38	4	08	
Non-impulsive	11	24	65	7.5	44	19	35	2	13	
Churchbells bells	39	51	10	2.5	0	0	97	3	01	
Building activities	39	20	41	7.4	25	34	34	7	04	
Other impulsive	6	16	78	7.7	61	21	15	3	12	
Police fire or ambulance sirens	12	32	56	6.1	5	27	66	2	04	
Other non-impulsive	10	22	68	7.9	58	16	24	2	16	
Pile driving	30	22	48	7.5	30	30	35	5	03	
Other sounds from building area	50	21	29	7.0	17	33	38	12	03	
Daytime sounds	37	26	36	6.2	19	24	50	7	04	
24-hours sounds	7	21	71	6.9	55	17	26	1	11	

I = their frequency of occurrence (in percentages), II = their mean score on an eleven-point scale (1 = like very much to hear, 11 = don't like to hear at all), III = degree of annoyance (in percentages), IV the Mean Relative Activity Interference Index.

ing conclusions may be drawn:

- a) the daily occurring impulsive sounds appear to cause no more annoyance than the daily occurring non-impulsive sounds do. This might be used as a working hypothesis for a next stage;
- b) pile driving is the most frequently heard sound from the building areas, and also the most annoying sound;
- c) sounds occurring only during the daytime cause less annoyance than sounds which occur every now and then during 24 hours.

3.5 Noise measurements: For pile driving impulses the difference between the maximum sound levels measured on impulse and slow response settings reaches 5 to 9 dB(A) at an outside microphone position at about 80 metres from the pile frame. The maximum sound levels reaches 77 dB(A) on one slow and 85 dB(A) on impulse response setting.

A-weighted equivalent sound level L(Aeq) measurements were carried out by means of a noise level analyzer and the equivalent sound level was also calculated by sampling the impulse (with 50 kHz sampling rate) with a transient recorder and a computer.

The L(Aeq) measurements with the noise level analyzer on slow and fast response settings and the L(Aeq) calculation show a good agreement.

The L(Aeq) value reaches 72 dB(A) on slow and 81 dB(A) on impulse setting. Because of the longer decay time the L(Aeq) measurement with the noise level analyzer is higher on impulse response than on slow response setting, the difference is about 7 to 9 dB(A).

#### 4. CONCLUSIONS AND ADDITIONAL COMMENTS

4.1 Some thoughts about impulsivity: In view of the objective, mentioned under 1, the following remarks can be made:

- a) Though the number of different sounds in our checklist is rather extensive, it cannot possibly be complete because of practical reasons. This makes the outcome of the comparison rather arbitrary as it depends on the sounds which happened to be included in the questionnaire. So there is only a relative importance. Second, the sounds from a given source can in some situations be "typical impulsive", in other situations this will certainly not be so. Distance from the source, for instance, is such a variable. A given noise source can only be decided to immit "a typical impulsive sound" in a given area, when the source is either a stationary one or always follows the same route, so

the immission levels are rather stable (apart from any meteorological conditions).

- b) The effects on health can only be related to a noise source under very specific conditions. For a study of this type it appears to be not useful to include a health section.

As far as the bothering effects are concerned, the techniques used in the questionnaire appear to be adequate.

- c) A difference of 4 dB in noise levels measured by two sound level meters on "slow" and "impulse" response as specified is achieved very easily for many kinds of noise sources. However, the duration of impulsive sounds is not specified. So when a continuous noise is started it may also cause a difference of 4 dB in the "slow" and "impulse" settings. The moment at which the difference of 4 dB must occur is not specified either. Because the "impulse" response has a higher decay time constant than the "slow" response it is possible that the difference in the noise levels measured by these meter response grows just after the impulse. It is not clear whether the difference in noise levels measured simultaneously by "slow" and "impulse" response is a good measure for the annoyance caused by impulsive noise. For measurements during long periods, the frequency of the impulse noise and the difference in the level distribution, for instance  $L_1-L_{95}$ , might be a better measurement. In this way the background noise is also accounted for.

This survey shows a good agreement between  $L_{eq}$  measurements of regular impulse noise using a noise level analyzer at "slow" and "fast" settings and the  $L_{eq}$  measured and calculated with a transient recorder.

#### 4.2 Important points about kinds of sounds:

- a) Time of the day: Sounds should be differentiated by those occurring only in the daytime, those occurring only at night and those occurring both in the daytime and at night;
- b) The meaning of a sound: Enquiries on specific sounds should be made more significant by adding questions about what a respondent him/herself considers to be the most distinctive feature of the sound at issue;
- c) Sound description: The description of a sound should not be applicable to varying sounds, but should allow only one single explanation (e.g. "drilling" instead of "do-it-yourself-work"). Moreover the sound question should apply to only one sound source and not to two or more sources together (e.g. not "doorbell or doorknocker" but

"doorbellen" and "doorknocker" separately).

4.3 Pile driving as a noise source: It seems plausible that the temporary nature of pile driving makes people adopt a relatively lenient attitude to the imited noise. On this ground the noise of pile driving cannot be regarded as a representative of "the impulse noises".

4.4 Selection of locations and sample: The results show that locations and samples are very important issues in this particular study: the area in which the study is to be carried out determines the outcomes.

4.5 Integration of the data: In carrying out this pilot study every participant was free to choose any situation in which impulsive sounds occur. For the main study it may be good to match the acoustical environments as well as possible. Only then it is feasible to integrate the data.

## 5. PUBLICATIONS OF THIS RESEARCH

- |   |  |
|---|--|
| [1] Berg, R. van den.<br>Impulsive Noise Measurements<br>in the Environment,<br>Memo 80-78, GLB-IMG-TNO,<br>Delft, March 1980.    | Research on the Effects of<br>Impulsive Sounds on Human<br>Beings (six-monthly report),<br>IMG-TNO rapport D 52,<br>Delft, November 1980.  |
| [2] Berg, R. van den.<br>Impulse Noise Measurements<br>in the Environment,<br>Memo 80-168, GLB-IMG-TNO,<br>Delft, September 1980. | [4] Jong, R.G. de, and R. van<br>den Berg, and J.W. Stolk.<br>Research on the Effects of<br>Impulsive Sounds on Human<br>Beings,<br>IMG-TNO rapport D 56,<br>Delft, February 1981. |
| [3] Jong, R.G. de, and R. van<br>den Berg.  |  |

## 6. ORAL COMMUNICATIONS

On behalf of this survey we participated in meetings at:

Southampton (January 29/30, 1980)	Paris (June 13, 1980)
Brussels (February 5, 1980)	Dublin (October 27/29, 1980)
Delft (April 1, 1980)	Southampton (March 3/4, 1981).

**Contractor:** Rijksverdedigingsorganisatie TNO, The Hague, The Netherlands

**Contract no.:** ENV 351-80-N

**Project leader:** G.F. Smoorenburg, IZF-TNO

**Title of project:** Effects of Impulse Noise on Human Beings  
Group 3: Annoyance in the Laboratory

#### OBJECTIVE OF THE RESEARCH

A joint pilot study of annoyance ratings in laboratory conditions for impulse sounds compared with traffic noise was initiated and partially funded by the Commission of the European Communities, Directorate-General for Research, Science and Education, under the Environment and Raw Materials Research Programme. Four laboratories took part (ISVR, Southampton University, England; The Acoustics Laboratory, Technical University of Denmark; Institute for Perception TNO, Soesterberg, Netherlands and Institut für Lärmschutz, Düsseldorf, Germany). The study was carried out in accordance with a protocol devised by team leaders. This report describes the main findings of that part of the study carried out at the Institute for Perception TNO.

#### MATERIALS and METHODS

Four master tape recordings were prepared and identical copies distributed to each of the participating laboratories. The tapes were, with their original source: Gunfire - Soesterberg, Pile driving - Lyngby, Synthetic impulses - Soesterberg and for comparison purposes Traffic - Southampton. Each of these noises was presented for five minute periods at four different levels ( $L_{eq} = 49, 56, 63$  and  $70$  dBA) to sixteen subjects according to a pre-determined experimental design sequence. Normally hearing ( $< 15$  dB re ISO R389 from 250 Hz - 8000 Hz) male subjects between 20 and 30 years of age listened to the sounds one at a time, whilst seated in a room with concealed loudspeakers. The complete test procedure, including a hearing test at the beginning and a coffee break halfway through, took approximately  $2\frac{1}{2}$  hours for each subject who was paid Dfl 40,-- on completion of the session. Full details of this pilot study are described in an IZF Report. In the present report the principal results and conclusions only are given.

## RESULTS and CONCLUSIONS

1. In the main experiment to follow this pilot study more attention should be paid to the quality of the sound recordings. In the pilot study tape hiss was clearly audible, in particular playing the pile-driving sound but also for the synthetic impulse noise. Also, there should be more consistency in the spectra of the sounds. Only the spectrum of gun-fire noise was tailored to meet indoor conditions. The tape hiss can be reduced by recording outside and inserting into the reproduction system some high-frequency attenuation which should correspond to the outdoor-indoor transfer function. In addition, the traffic-noise recording should be improved. The traffic noise sounded artificial because of compression of the level fluctuations. The fluctuations in level should fit in with the absolute sound level: large and rapid fluctuations at high sound levels (close traffic) and small and slow fluctuations at low levels (remote traffic). Unnatural sounds, like the synthetic impulse noise included in the pilot study, should not be included into the main experiment.

2. True A-weighted equivalent levels were obtained by numerical integration of the squared sound pressure and compared with other (approximate) measures of the equivalent level. For the four sounds equivalent levels calculated from samples taken every 200 msec from a Rohde and Schwarz analyzer in the position "fast" were 0.6 to 1.4 dB lower than the numerically determined true equivalent levels. Equivalent levels calculated from samples taken every 500 msec from the analyzer in position "slow" were 0.8 to 2.4 dB lower. The median level of the samples taken in the position "slow" differs by no more than 1 dB from the true equivalent level; the median level for the position "fast" is 0.8 dB lower for traffic noise but 34 dB lower for gun-fire noise. Equivalent levels measured with the Brüel & Kjaer sound level meter type 2218 were  $0.29 \pm 0.07$  dB lower than the numerically determined equivalent levels.

3. In the pilot study each condition was measured only once for each subject. This implied for the analysis of variance that we had to take the highest order interaction term as an estimate of the error in the ratings which impeded the interpretation of the results. In the main experiment this should be avoided by presenting each condition twice to each subject. The order of presentation of the conditions, replicas included, should be perfectly balanced.



4. Analysis of variance of the ratings given by the IZF-TNO subjects to the questions "How annoying did you find the noise you have just heard, if you heard it all the time?" and "How annoying would you find this noise if you heard it all the time in your own living room in the evening?" shows for both questions that there are no significant differences in the ratings given to the four types of sounds. In this analysis "subjects" was considered a random factor; listed volunteers, not employed by our institute, were asked to serve as a subject. A significant effect was found for the ratings given at different levels. In addition, differences amongst subjects appeared to be a significant source of variation. The variance in the ratings due to intersubject differences is about three times the variance due to the difference in sound levels. The intersubject differences affect the ratings in the same way as differences in noise level of about 16 dB.

5. Analysis of variance of the ratings collected by the four institutes together shows a significant interaction between "sounds" and "levels". This interaction implies that the rating as a function of noise level depends on the type of sound. The IZF-TNO data do not show this interaction. Since there is no significant interaction among "institutes", "sounds" and "levels" the significant interaction found for the combined data should be taken as the general result. This significant interaction is due to a difference in increase of the rating with noise level which is faster for traffic noise than for the impulse noises. The mean ratings for traffic noise, gun-fire noise and pile-driving noise are about the same at  $L_{eq} = 63$  dBA. Thus, at lower equivalent levels the ratings for the two impulse sounds are higher than those for traffic noise. Extrapolating the results to traffic noise at  $L_{eq} = 40$  dBA indoors shows that the equivalent levels of the two impulse sounds should be 11 dB lower in order to get the same rating as traffic noise. In addition to this result synthetic impulse noise appeared to get somewhat higher ratings than the other two impulse sounds. Similarly to the IZF-TNO results, the combined data show that intersubject differences are an important source of variation. They affect the ratings in the same way as differences in noise level of about 11 dB.

6. For each condition, the lowest noise level included, more than 50% of the subjects would be very much annoyed if the noises were heard all the

time in the living room in the evening. This shows that the range of equivalent sound levels (49 - 70 dBA) was chosen too high.

7. The question: "What point on the rating scale (from 0 up to 9) as you used it would correspond to a little, moderately and very much annoying?" asked at the end of the experiment appeared to be not very useful. The answers were inconsistent with answers given during the experiment.

#### REFERENCES

Smoorenburg, G.F. and de Vries, W.H. (1981). Effects of impulse noise on human beings: a pilot study on annoyance ratings in the laboratory, Report of the Institute for Perception TNO.

Smoorenburg, G.F. (1981). Evaluation of shooting noise with regard to annoyance, Invited paper to be presented at Inter-Noise 81, Amsterdam, October 1981.

**Contractor** : TNO, on behalf of the TNO Research Institute for  
Environmental Hygiene at Delft, the Netherlands.  
**Contract no** : 353.  
**Project co-ordinator** : W. Passchier-Vermeer.  
**Project leader** : A.J.M. Rövekamp.  
**Title of project** : Effects of impulsive noises on human beings.  
Group 2 : Physiological effects.

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## 1. OBJECTIVE OF THE RESEARCH.

A pilot study has been carried out in 1980 in the laboratory of the TNO Research Institute for Environmental Hygiene at Delft, in cooperation with laboratories in Germany and Italy. The aim of this pilot study was to determine those physiological parameters which are most sensitive to noise exposure. Some of the physiological parameters were measured by two or all three participating laboratories and others by only one laboratory. This study was also carried out to determine whether physiological effects result from exposure to artificial impulsive sounds and if so, to determine the magnitude of these effects relative to effects from constant noise. For this purpose, the effects on parameters of blood circulation and respiration have been studied by the TNO Research Institute for Environmental Hygiene at Delft.

## 2. MATERIALS AND METHODS.

### 2.1 Experimental method.

In the pilot study, 50 experiments were carried out with ten young persons (5 males and 5 females). They were exposed to artificial impulsive noises, constant white noise and quiet. The exposure lasted ten minutes and the noise had an equivalent sound level of about 80 dB(A). During the experiments, a number of parameters of blood circulation and respiration were recorded. From these recordings, the following physiological parameters were determined :- heart beat frequency  
- sinus- arithmia  
- the maximum value of the relative impedance plethysmogram  
- systolic and diastolic blood pressure  
- respiratory frequency.

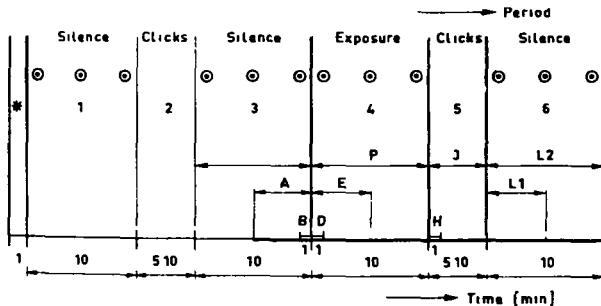
Five experiments were executed with each subject on five different days. The sequence of four experiments was given at random. Only the fifth exposure was always given on the last day. All subjects were in the age of 15 to 30 years and had a good health and a hearing loss less than 15 dB between 250 Hz and 8000 Hz, measured by means of a pure tone audiometer. During an experiment, a test subject was alone in an air-conditioned room, in a sitting position on a chair. The subject had to sit there during the whole experiment. The background noise level in the room was about 35 dB(A). On the five different days, the subject was exposed to noise recorded on tape and presented through TDH39 headphones :

- TAPE 1 - Constant white noise (sound level of 80 dB(A))
- TAPE 2 - White noise bursts (lasting 200 ms at 87 dB(SPL))
- TAPE 3 - Quiet (about 35 dB(A))
- TAPE 4 - Artificial regular impulsive sound (peak level at 96 dB(SPL-fast), duration about 100 ms per impulse).
- TAPE 5 - Artificial irregular impulsive sound (with the same characteristics as TAPE 4)

Each experiment is divided into six periods (see Figure 1):

- PERIOD 1 - First 10 minutes of the experiment : quiet.
- PERIOD 2 - For 5 minutes the subjects were exposed to click trains for measurement of neuro-physiological parameters.
- PERIOD 3 - Again 10 minutes of quiet.
- PERIOD 4 - Exposure to one of the noises recorded on tape.
- PERIOD 5 - Equal to period 2.
- PERIOD 6 - Again 10 minutes of quiet.

For analysing the test results, a further division of each experiment is also given in Figure 1. During all periods the subject was wearing the headphones. At the end of each experiment the subject was asked for his or her subjective experience of the noise exposure concerned.



- ⊙ Measurement of blood pressure
- \* Calibration period

Figure 1 Division of the experiments into several periods

## 2.2 Processing of the physiological signals.

During all periods the following physiological signals were recorded on

tape:

- 1 - Respiration
- 2 - Blood pressure
- 3 - Relative and absolute impedance plethysmogram
- 4 - ECG

From the signals 1 and 4, the number of respirations and heart beats per minute were determined. The arrhythmia quotient was calculated for every 32 heart beats. The arrhythmia quotient was based on the variability in the time between two heart beats. From the relative part of the impedance plethysmogram, the maximum value of this signal, occurring as a result of a contraction of the heart, was determined for each heart beat and the mean of these values was calculated for every 30 seconds.

## 2.3 Processing of data.

For each period as mentioned in figure 1, the mean value and standard deviation were calculated of the following quantities: one-minute values of the respiration rate, the one-minute values of the heart rate, the thirty-second values of the relative impedance plethysmogram, the arrhythmia quotient of 32 heart beats and the systolic and diastolic blood pressure. Thereafter, the differences between the mean values for the several periods were determined. To make these differences in the mean values of the several periods more comparable, the calculated differences were taken relative to the mean value for period A; see Figure 1.

The results derived in this way were called "normalized differences between several periods". To show statistical significant effects of noise exposure, the sign test, which is suitable for a small amount of data, was used. In most cases it was very instructive to know the differences in each parameter expressed in the specific dimension. For instance, the heart rate in beats per minute and so on for the other parameters. Therefore, per exposure the mean values of the normalized differences between several periods have been multiplied with the mean value of period A of that exposure. Tables of the results are given in TNO-IMC report B 466 [1,2].

## 3. RESULTS.

In this chapter the results for each physiological parameter are summarized. A difference in the effects caused by impulsive noise and constant noise or quiet is given.

3.1 Respiration rate.

The respiration rate is increased slightly (about 1%) during exposure to impulsive noises, whereas this rate decreases during constant noise and quiet. These effects are not statistically significant. The effects of the clicks used for brainstem responses after the exposure to white noise pulses and quiet are comparable to the effects of the impulsive noises.

3.2 Heart rate.

During the exposure period, there is a slight decrease in the heart rate. The impulsive noises cause a larger decrease (about 1.5%) than constant noise and quiet. The decrease is statistically significant for the experiments 1,3,4 and 5.

3.3 Arithmia quotient.

The arithmia quotient increases (about 15%) more during exposure to constant noise and quiet than during impulsive noise. Only quiet and irregular impulsive noise show statistically significant differences.

3.4 Relative impedance plethysmogram.

During exposure to constant noise and quiet, this parameter decreases statistically significant (about 10%). During exposure to impulsive noise, it also decreases, but not statistically significant and with smaller values (about 5%).

3.5 Blood pressure.

In all cases, there is a decreasing tendency in the systolic blood pressure during the exposure period. The diastolic blood pressure slightly increases (about 1%) during exposure to impulsive noises and decreases (about 1.5%) during quiet and constant noise exposure.

4. CONCLUSIONS AND ADDITIONAL COMMENTS.

The changes of the measured physiological parameters of persons exposed to constant white noise for a period of ten minutes are in most cases not statistically significantly different from those due to impulsive noises. Impulsive noises cause an increase in the respiration rate, whereas during constant noise and quiet this rate decreases. It also causes, compared with constant noise and quiet, a larger decrease in the heart rate, less increase of the arithmia quotient and less decrease in the relative impedance plethysmogram. Therefore exposure to ten minute impulsive noises form a

larger load on the physiological parameters measured than ten-minute exposure to constant noise or quiet. From former experiments [ 3 ] with a two-hour exposition to impulsive noise, it was concluded that impulsive noise cause larger effects than other environmental noises and quiet.

Most probably the total exposition period of ten minutes in this pilot study is too short to show clear differences in effects of impulsive noise and constant noise.

5. LITERATURE.

- [ 1 ] Rövekamp, A.J.M. Physiological effects of impulsive noises on human beings. Results of a ten-minute exposure.  
Report B 466, Volume 1, Delft, IMG-TNO, March 1980.
- [ 2 ] Rövekamp, A.J.M. Physiological effects of impulsive noises on human beings. Tables of the results of a ten-minute exposure. Report B 466, Volume 2, Delft, IMG-TNO,  
March 1980.
- [ 3 ] Rövekamp, A.J.M. Invloed van woonomgevingsgeluid op de mens. (Effects of environmental noise on human beings-Dutch Report-). Experimenteel onderzoek naar de invloed van woonomgevingsgeluid op de bloedcirculatie en ademhaling van de mens. Report B 432, Delft, IMG-TNO, 1980.

Contractor: ISVR, The University, Southampton, England?  
Contract No: ENV-364-UK(N)  
Project leader: C.G. RICE  
Title of project: Effects of Impulse Noise on Human Beings  
Group 3: Annoyance in the Laboratory

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#### OBJECTIVE OF THE RESEARCH

A joint laboratory study of reported annoyance to impulsive sound compared with traffic noise was initiated and partially funded by the Commission of the European Communities, Directorate-General for Research, Science and Education, under the Environment and Raw Materials Research Programme. Four laboratories took part (ISVR, Southampton University, England; The Acoustics Laboratory, Technical University of Denmark; Institute for Perception TNO, Soesterberg, Netherlands and Institute für Lamschutz, Dusseldorf, Germany). The study was carried out in accordance with a protocol devised by team leaders at a series of meetings chaired by Dr.P. Guillot of the CEC, Brussels. This report describes the main findings of that part of the study carried out at ISVR.

#### MATERIALS and METHODS

Four master tape recordings were prepared and identical copies distributed to each of the participating laboratories. The tapes were, with their designation and original source: Gunfire (G) - Soesterberg, Pile driving (P) - Lyngby, Synthetic impulses (S) - Soesterberg and for comparison purposes Traffic (T) - Southampton. Each of these noises was presented for five minute periods at four different  $L_{Aeq}$  levels to sixteen subjects according to a pre-determined experimental design sequence. Normally hearing (<15 dB re ISO R389 from 250 Hz - 8000 Hz) male subjects between 20 and 30 years of age listened to the sounds one at a time, whilst seated in a simulated domestic living room with concealed loudspeakers. The completed test procedure, including a hearing test at the beginning and a coffee break halfway through, took approximately 2 hours for each subject who was paid £5 on completion of the session. Full details of the study are described in ISVR Contract Report 81/12 which was prepared in accordance with contractual requirements. In this report the principal results only are described.

#### RESULTS

##### Question 1: Reported annoyance in laboratory

An analysis of variance for the data on question one is shown in Table 1. The main effects of both SOUNDS and LEVELS are significant; but their inter-action is not. The means for SOUNDS and LEVELS are shown in Table 2, the physical data in Table 3. With a standard error of the difference between means of 0.2157, a difference between means of about  $2.6 \times 0.2157 \approx 0.56$  is significant at the 5% level. Hence, there is a difference between sounds S and G and between sounds S and T. No other differences are significant.

The analysis of variance table shows that the difference between these responses can be explained almost entirely by a linear component, that is by a straight line regression of means on levels. For each 7 dB increase in LEVELS there is an average increase of 0.9625 in subjective response.



The trends for each sound are given in Figure 1, although the differences between slopes are not significant.

#### Question 2: Annoyance projected to home environment

An analysis of variance for the data on question two are shown in Table 4. The significant interaction between SOUNDS and LEVELS is due primarily to the interaction of SOUNDS with the linear component of LEVELS. This is significant at the 1% level. Both main effects are also significant. The two way table of means is shown in Table 2, the slopes being G 0.081, S 0.090, P 0.099 and T 0.162. It is clear that the slope for sound T is different from the other sounds; the standard error of the difference between two slopes is 0.025. For sounds G, S and P there are no differences between slopes but the mean response on sound S is significantly higher than those for G and P; the standard error of the difference between means is 0.199.

The sounds, therefore, fall into three groups, namely S, T and the other two sounds G and P together. There are interesting differences between the groups both in overall means and in the way subjective response changes over levels. The results can be plotted as shown in Figure 2.

#### Other Questions

Questions relating to the percentage of subjects annoyed 'a little', 'moderately' or 'very much' were also asked, but the results they produced were not as definite as those for questions 1 and 2, and these techniques require further development. The ability of subjects to recognise impulsive sounds was not high, for example of the sixteen subjects who took part only one recognised the synthetic sound, five the gunfire and eight the pile driving, compared with fifteen who recognised the traffic noise. Most of the impulsive sounds were referred to in terms of 'non-specific banging'.

#### CONCLUSIONS

The techniques used in this study have shown that laboratory experiments can be designed to distinguish significant differences between the relative reported annoyance of impulsive and other sounds.

In general, the impulsive sounds were more annoying than the traffic noise at the same  $L_{Aeq}$  level, although their rate of growth of annoyance was less. This means that the impulsive sounds were relatively more annoying at lower levels.

The ability of subjects to recognise the type of impulsive noise source is not as good as their recognition of traffic noise.

#### RECOMMENDATIONS

In planning further studies consideration should be given to: allowing more time for discussing experimental design and data analysis; making minor modifications to the response questionnaires; re-assessing the role of session length, choice of reference and synthetic sounds; using a wider selection of community impulse sounds, and recording all sounds at their true outdoor levels with their subsequent attenuation to meet indoor requirements.

#### REFERENCE

- I.H. Flindell & C.G. Rice (1981) Effects of Impulse Noise on Human Beings Group 3: Annoyance in Laboratory.  
ISVR Final EEC Contract Report 81/12.

QUESTION 1: REPORTED ANNOYANCE IN LABORATORY

SSV

Source of Variation	d.f.	s.s.	m.s.	F
Between subjects	15	536.48	35.77	24.03 ***
Between order	15	17.98	1.20	0.80
Between sounds (S)	3	24.70	8.23	5.53 **
Between levels (L)	3	296.95	98.98	66.51 ***
Linear L	1	296.45	296.45	199.19 ***
Quadratic L	1	0.25	0.25	0.17
Remainder	1	0.25	0.25	0.17
S x L interaction	9	19.20	2.13	1.43
S x Linear L	3	9.66	3.22	2.16
S x Quadratic L	3	1.47	0.49	0.33
Remainder	3	8.08	2.69	1.81
Residual	210	312.53	1.488	
TOTAL	255	1207.86		

\* Significant at 5%, \*\* 1%, \*\*\* 0.1% levels

Table 1. Analysis of Variance

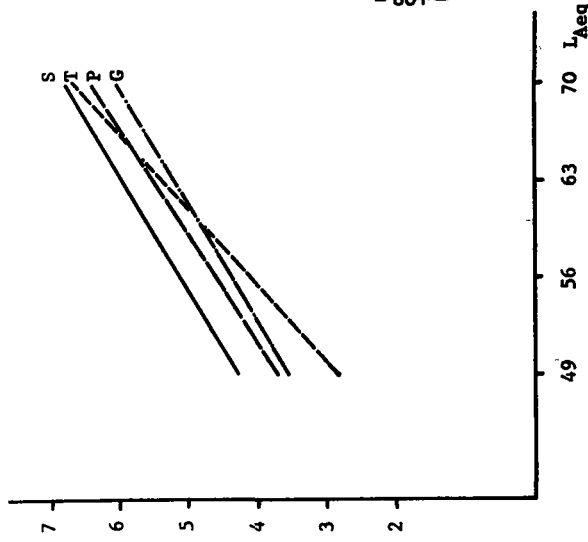


Figure 1. Comparison of traffic and impulsive sounds.

Q.1 How annoying did you find the noise you have just heard, if you heard it all the time?

Grand Mean		5.023			
Sound		Gunfire	Synthetic	Piling	Traffic
		4.781	5.516	5.063	4.734
Level		49	56	63	70
		3.563	4.531	5.578	6.422
Level		49	56	63	70
Sound	Gunfire	3.625	4.125	5.438	5.938
	Synthetic	4.250	5.188	5.750	6.875
	Piling	3.438	5.000	5.438	6.375
	Traffic	2.938	3.813	5.688	6.500

Q.2 How annoying would you find this noise if you heard it all the time in your own living room in the evening?

Grand Mean		7.137			
Sound		Gunfire	Synthetic	Piling	Traffic
		7.000	7.641	7.047	6.859
Level		49	56	63	70
		6.031	6.734	7.484	8.297
Level		49	56	63	70
Sound	Gunfire	6.438	6.375	7.125	8.063
	Synthetic	6.813	7.313	7.625	8.813
	Piling	5.813	6.938	7.500	7.938
	Traffic	5.063	6.313	7.688	8.375

Table 2 Tables of results

Meter time constants

Meter Time Constant	Tape	L <sub>Aeq</sub> 2218	L <sub>Aeq</sub> 4426	L <sub>01</sub>	L <sub>05</sub>	L <sub>10</sub>	L <sub>50</sub>	L <sub>90</sub>	L <sub>95</sub>	L <sub>99</sub>
Fast	Gunfire	70.3	70.4	79.0	78.5	77.0	54.8	36.3	36.3	36.3
	Synthetic	70.3	70.4	76.8	76.5	76.0	64.8	57.8	50.0	48.8
	Piling	69.9	70.0	78.0	76.8	76.0	61.5	47.8	47.0	46.3
	Traffic	70.0	70.2	76.0	74.3	73.3	69.5	64.8	63.5	59.8
Slow	Gunfire	70.3	70.4	73.8	73.3	73.0	70.3	66.3	64.8	61.5
	Synthetic	70.3	70.4	72.0	72.0	71.8	70.5	69.0	68.8	68.5
	Piling	70.2	70.3	72.8	72.5	72.0	70.3	68.5	68.0	67.8
	Traffic	70.1	70.2	75.3	73.8	73.0	70.0	65.8	64.5	61.5
Impulse	Gunfire	70.3	78.9	81.5	81.3	81.0	79.0	76.5	75.5	73.5
	Synthetic	70.3	78.1	79.5	79.3	79.3	78.3	77.3	77.0	77.0
	Piling	70.2	79.1	81.3	81.0	80.5	79.3	77.8	77.5	76.8
	Traffic	70.1	71.4	77.5	75.3	74.3	70.8	66.5	65.3	61.3

Notes

1. All levels dB(A) weighted at subject's head position
2. The 2218 is a true L<sub>Aeq</sub>
3. All levels at nominal 70 L<sub>Aeq</sub>

Maximum levels on 2218 meter 70 L<sub>Aeq</sub>

Tape	Peak hold	Impulse	Fast	Slow
Gunfire	93.0	81.5	79.0	73.0
Synthetic	92.0	80.0	77.5	72.0
Piling	95.5	81.5	78.5	72.0
Traffic	87.0	76.0	75.0	73.0

Impulsive Character

Gunfire	8.5 dB
Synthetic	8.0 dB
Piling	9.5 dB
Traffic	3.0 dB

Table 3 Physical Data

QUESTION 2: ANNOYANCE PROJECTED TO HOME ENVIRONMENT

Source of Variation	d.f.	s.s.	M.S.	F
Between subjects	15	393.03	26.20	20.77 ***
Between order	15	18.40	1.23	0.97
Between sounds (S)	3	22.89	7.63	6.05 ***
Between levels (L)	3	182.45	60.82	48.22 ***
Linear L	1	182.26	182.26	144.49 ***
Quadratic L	1	0.19	0.19	0.15
Remainder	1	0.00	0.00	0.00
S x L interaction	9	26.57	2.95	2.34 *
S x Linear L	3	15.82	5.27	4.18 **
S x Quadratic L	3	8.86	2.95	2.34
Remainder	3	1.90	0.63	0.50
Residual	210	264.88	1.261	
TOTAL	255	908.22		

\* Significant at 5%, \*\* 1%, \*\*\* 0.1% levels.

Table 4. Analysis of Variance

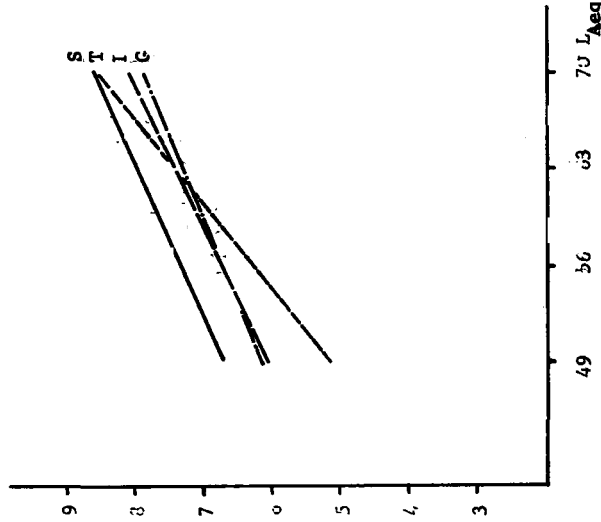


Figure 2. Comparison of traffic and impulsive sounds.

Contractor: Institute of Sound & Vibration Research,  
University of Southampton, Southampton, SO9 5NH, UK.  
Contract No: ENV.362 UK  
Project Leader: Professor J.B. Large  
Title of Project: EFFECT OF IMPULSE NOISE ON HUMAN BEINGS: REPORT OF U.K  
FIELD STUDY

---

## 1. Introduction and background

1.1 As part of a wider preliminary investigation of the effects of impulse sounds on exposed individuals, the Institute of Sound and Vibration Research (ISVR) at the University of Southampton, UK, has been collaborating for the past year with researchers from TNO (Holland), SEDES (France) and ESRI (Ireland), the research being sponsored by Environment and Raw Materials research programme of the Commission of the European Economic Community. This report concerns the contribution from ISVR towards the work of the field study subgroup. This work involved the development and piloting of an appropriate questionnaire for a proposed large scale social survey to measure the magnitude and nature of the problems generated by impulsive sounds within the populations of the participating countries. In parallel with the questionnaire development, ISVR has been considering suitable ways to measure the levels of impulse noise.

One progress report (Ref.1) and a draft final report (Ref.2) have been submitted to the Commission during the study.

## 2. Development of objectives and research design

2.1 The aim of the current research is to work towards an understanding of the extent and nature of the effects of impulsive sound in the community. This aim could be achieved by two complementary approaches. One is to secure estimates of the numbers and proportions of the populations of each participating country that (a) hear, with some measure of how often, and (b) incur some kind of discomfort as a result of being exposed to impulsive sounds. This approach would yield estimates of the overall prevalence, in the populations of interest, of these characteristics

(the prevalence approach). The other approach is to study individuals residing in areas known to be exposed to varying levels of sound from particular impulsive sources and to attempt to derive dose/response relationships including (a) an examination of the relationship between subjective response and various parameters of the sound (e.g. number of events, level in excess of background, various measures of absolute level, etc.) and (b) a comparison between dose/response to impulsive sounds and dose/response relations obtained for steady state noise e.g. road traffic, for individuals exposed to both types of sound (the dose/response approach). These two complementary approaches require, for reasons of efficient use of resources different research designs.

- 2.2 The prevalence approach could be achieved through some kind of probability sample of individuals, the aim being to make descriptive statements. The dose/response approach would necessitate the inclusion of sufficient individuals from areas exposed to a wide range of levels of the impulsive sounds of concern and road traffic in order that the dose/response relationship may be satisfactorily estimated. In the former case we would be interested in making descriptive statements about the population as a whole in each participating country whilst in the latter case we would be interested in studying dose/response relationships for individuals living in areas known to be exposed to some level of the sounds of interest.
  
- 2.3 During discussions, representatives of the EEC Commission expressed their preference for the prevalence approach. They considered that the proposed large scale social survey should attempt to yield descriptive estimates of proportions of the population who (a) hear and (b) experience various forms of intrusion and annoyance etc., in each participating country. They also advocated the compilation of an extensive and comprehensive list of impulsive sounds, which, on the basis of earlier work, had been identified as giving rise to annoyance. These impulsive sounds were to be combined in the questionnaire with a similar number of 'steady-state' sounds to provide a comparative perspective.

- 2.4 The adoption of the prevalence approach has, however, led to some conceptual and practical problems in the use of the questionnaire in the pilot study. The nature of the impulsive sounds at present included in the questionnaire is such that a substantial number have characteristics that present basic problems for the measurement of subjective response and the noise exposure; in these circumstances it can be meaningless to ask a respondent to give an 'average' response and to measure the typical noise exposure. These issues are of fundamental concern in the context of the present questionnaire.
- 2.5 The prevalence approach has other problems. Many of the impulsive sounds are likely to be heard by a relatively small proportion of the populations. Hence, the social survey and noise measurement programmes will be relatively expensive.
- 2.6 Even if the study were limited to regularly occurring impulsive sounds then many problems of research design would remain. However, the inclusion of sounds that occur irregularly, unpredictably or transiently means that the problems of the physical measurement of sound exposure and subjective response are substantially increased.

### 3. Questionnaire development

- 3.1 The initial draft of the questionnaire was prepared and modified prior to pre-testing. This pre-test identified outstanding problems before the final version for the pilot survey was produced. Both the pre-test version and the final version of the pilot questionnaire were included in the draft final report dated January 1981 (Ref.2). The final version did not include questions on perceived health.

### 4. Pilot study for the social survey

- 4.1 Ten sampling areas in the vicinity of Southampton were selected from information obtained from Environmental Health Officers in the areas concerned. A variety of sound sources were experienced in the areas. Eight addresses were selected in each area by systematic selection from a random start on a list of all dwellings located on maps of the areas



surrounding the noise sources. A further 6 addresses were selected in one area to compensate for ineligible addresses. Five professional trained interviewers were used to pilot the questionnaire.

## 5. Noise measurements

- 5.1 The piloting of the questionnaire was complemented by a preliminary study of techniques for the measurement of impulse noise in the environment.
- 5.2 Measurements and analyses were made on two groups of noises: (a) three specific sources including impulsive and non-impulsive noise; (b) neighbourhood noises containing impulsive components. The EEC directive 79/113 defines noise as impulsive if the peak level on 'impulse' response exceeds the 'slow' response peak level by 4 dB or more. This approach was investigated for both groups of noise. In addition the equivalent continuous noise level (Leq) was measured for the Group (b) noises with the Leq responses set to 'slow', 'impulse' and fast.
- 5.3 The results obtained were predictable from a consideration of the characteristics of the measuring instruments and the sound sources. The definition of impulsive as used in the EEC directive would apply to some clearly non-impulsive sounds.

## 6. Summary and conclusions

- The current study has adopted a prevalence approach. A questionnaire to measure the incidence of and attitudes to impulsive noise has been developed. There has been no consideration of a sample design for a full scale prevalence study.
- 6.2 Impulsive sounds can be usefully categorised into (i) those which are irregular, unpredictable and transient in occurrence and (ii) those which have a pattern of occurrence in time and place and will persist for an extended time. It is suggested that this division would help in the overall design of a full scale study and in both the measurement of respondents' attitudes and the physical measurement of the sounds.

- 6.3 Questions on the noise/perceived health link dropped from the pilot questionnaire could be re-introduced to a full study if the experience of using these questions in other studies is first examined.
- 6.4 The use of large scale Ordnance Survey maps to draw a sample of addresses is satisfactory. Some caution must be exercised in the use of older maps. The use of the Kish grid gave rise to some non-response. There is no alternative if maps are to be used to sample addresses.
- 6.5 The pilot study indicates a low rate of refusal (about 2%), and that an overall response rate of about 85% might be expected in a full scale study. The average length of interview was 58 minutes with a range of about 30-90 minutes. Respondents found the topic and its presentation interesting. Interviewers found the questionnaire tedious and repetitive. Great care is needed in briefing interviewers, especially in connection with the use of grids and rating scales. All questions were adequately understood by respondents. There was no evidence of particular questions giving rise to repeated 'not ascertained' or 'don't know' responses.
- 6.6 The questions following all checklists of sounds should be re-ordered to incorporate section (f) into section (e). SHOW CARD C was unsatisfactory in use. The question to identify the most annoying sound in each checklist should be reworded. Amendments are suggested to questions 2(b), 7, 9(b), 10(b), 15, 19, and 38. The transient nature of some sound sources caused problems in section (a) of each checklist. This problem needs further consideration. Some items of the activity interference scale are not appropriate for particular sounds. Simple blanking out of these boxes in the grids is suggested.
- 6.7 The trial checklist of sounds from domestic appliances gave rise to some difficulties. Attitudes to such noises are influenced by who is using the appliance. Amendments are suggested to take account of this problem.
- 6.8 The frequencies of all pre-coded questions have been reported, together with the median response of some rating scales (Ref.2). These data may help in subsequent sample design, when used in conjunction with the reported sound environment of each sampling area. The reported data

giving the number of positive responses to each checklist of sounds can be similarly employed.

6.9 Objective measurements and analysis of impulse noise have been carried out. The results suggest that the classification of a noise as 'impulsive' made in the EEC directive 79/113 needs some qualification and may need restricting to certain types of noise.

6.10 Recommendations for the development of objective measurement techniques, should a consideration of human reaction to impulse noise show it to be necessary, include proposals that a more precise objective classification of 'impulsive' noise be made, and for the development of instrumentation for noise measurements and validation of measurement techniques in field studies.

## 7. References

1. Impulse noise study: field study. Report by ISVR, University of Southampton, UK. Progress report: to 30.10.80.
2. M.M. Hawkins, P.J. Cooper, J.G. Walker (1980). Impulse noise study. Field study: draft final report. Prepared for the Environment and Raw Materials Research Programme, Commission of the European Communities, Brussels.

Contractor: Institute for Industrial Research and Standard  
Ballymun Road,  
Dublin, 9, Ireland

Contract No.: ENV 410-80 EIR

Project Leaders: Bernard Hayden (IIRS) and Brendan Whelan (ESRI)

Title of Project: Joint Research Project on Impulsive Noise - Pilot  
Field Study

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1. Objectives

- 1.1. The Pilot Field Study on Impulsive Noise was one of three Pilot Studies organised by the European Commission on Impulsive Noise. The other Studies were laboratory based studies of Subjective Response and Physiological Reaction. Four laboratories participated in the Pilot Field Study. These were ISVR, Southampton, SEDES, Paris, TNO, Delft and IIRS, Dublin.
- 1.2. The Institute for Industrial Research and Standards was assisted by the Economic and Social Research Institute, Dublin (ESRI) in the questionnaire development, interviews and questionnaire results analysis.
- 1.3. The basic objective of the Pilot Social Survey was to test the feasibility of carrying out a full scale enquiry to evaluate 'the importance attributed to noises which would be classified as impulsive ..... The questionnaire should contain a number of questions common for all teams plus specific ones dealing with the environment in general. Noises from different categories should be quoted (transport, domestic, construction, industrial, leisure) among which some impulsive noises would be included and general questions on subjective feeling on noises

would be asked'. (Quotation from Annex I to Contract).  
The main emphasis in the pilot enquiry was, therefore, on highlighting the problems which are likely to arise in the full scale study and to suggest ways in which they may be overcome. It was not possible with a sample of the size used in the pilot enquiry to attempt to describe the population under study with any degree of accuracy.

1.4. The basic objectives of the Pilot Noise Survey were:

- i) To examine measurement methods which could be used for a large scale survey on impulsive noise.
- ii) To examine the method for impulsive noise measurement laid down in EEC Directive 79/113.
- iii) To carry out these measurements in the environment of some of the people participating in the Social Survey interviews.
- iv) As far as possible to use commonly known measurement parameters and commonly available equipment.

2. Materials and Methods

- 2.1. The questionnaire design and various measurement and analysis possibilities were discussed and developed at meetings between representatives of the participating laboratories. The major part of the questionnaire development and synthesis was undertaken by M. Hawkins and P. Cooper of ISVR, Southampton.
- 2.2. In Ireland, trial tests of the questionnaire were carried out by Brendan Whelan of ESRI. As a result of these and similar trials by other participants a major change to the questionnaire was to reduce the time needed for interviews by shortening the section on Health.

- 2.3. Tests on various types of noise analysis, in particular measurements according to EEC Directive 79/113, were carried out by Bernard Hayden of IIRS. Measurement results were also reported by other participants and general guidelines for noise measurements and analysis were agreed.
- 2.4. A Pilot Social Survey and a Pilot Noise Survey were then carried out in two city centre areas, one suburban area and one rural area. A total of 52 people, selected at random from the Electoral Register in each area, was interviewed for the Social Survey and noise measurements were made at eight locations.
- 2.5. The objective in sampling was to represent in the sample as wide a range of variation as possible in the variables of interest. In addition, special emphasis was placed on ensuring that those sub-groups of particular interest to the EEC (i.e. 'persons living in high-density, low-income areas') should be well represented. Some individuals from a rural area were also selected because rural residents constitute a higher proportion of the population in Ireland than in most Community Countries and it was felt that the Irish contribution to the project should include some of these residents.
- 2.6. Noise measurements were made at the homes of questionnaire respondents in each sample area. Noise levels were recorded on magnetic tape. A seven minute sample was recorded every hour for 24 hours at each location.
- 2.7. An automatic measurement system was developed to give noise analysis results for each seven minute sample. Each sample was analysed to give L(1), L(10), L(95) and L(eq) for both Slow and Impulse response. A system was also developed for automatic analysis of each sample according to EEC Directive 79/113.

### 3. Results

- 3.1. The data from the Social Survey was coded and punched, and subsequently analysed by means of the SPSS package.
- 3.2. It must be emphasised that the sample was selected to test the feasibility of the survey and that the results of such a sample cannot be assumed to be representative of the population from which it was selected. However, some tentative indications did emerge from the study.
  - i) Relatively few people give noise or its converse, quietness, a substantial weight in evaluating their house or environment.
  - ii) When noise is specifically mentioned to respondents, the numbers who report being bothered by it are considerably greater.
  - iii) The sounds which most clearly impulsive in nature are among the least frequently heard (e.g. shooting, bird scarers, pneumatic drills, pile driving, blasting, metal working etc.).
  - iv) Quite high proportions of respondents said they liked hearing many of the sounds, even such unlikely ones as factory hooters, police/fire/ambulance sirens, dogs barking etc. However, those sounds which are clearly impulsive in nature (though heard quite rarely) elicited almost universal dislike from respondents.
- 3.3. The results of the noise analysis were presented in tabular and graphical form.
- 3.4. The measurement system adopted gave good samples of the noise environment in several different types of location. The analysis of these samples gave sufficient data to enable assessment of various measurement methods.

#### 4. Conclusions

- 4.1. Both the Pilot Social Survey and the Pilot Noise Survey suggest that data collection of the type envisaged is technically feasible. However, if full scale surveys are attempted, some major problems of research design will have to be overcome.
- 4.2. The results of the Pilot Social Survey indicate that a very low proportion of respondents hear sounds which are clearly impulsive. This problem can be exacerbated by the fact that sources of impulsive sounds are often temporary in nature. Hence, a very large sample would be required to draw any conclusion about reactions to these sounds which could be generalised to the population as a whole.
- 4.3. The results of the Pilot Noise Survey suggest that measurements to EEC Directive 79/113 give useful insights into the impulsive character of the noise environment. The selection of a 4 dB difference criterion does, however, appear to be arbitrary. Evaluation of the Slow/Impulse difference criterion is needed.
- 4.4. No correlation could be found between the number of Impulses per sample and any of the Slow and Impulse differences or any of the statistical parameters within either response.

- References
1. Hayden, B and Whelan, B. J., 1981, EEC Joint Research Project on Impulsive Noise, Pilot Field Study Draft Report, EEC Contract 410-80.
  2. Whelan, B. J., 1981, Economic and Social Review, Vol. 10, No. 2, 169-174. RANSAM: A National Random Sample Design for Ireland.



Contractor: The Acoustics Laboratory  
Technical University of Denmark

Contract N<sup>o</sup>: ENV-364-DK

Project leaders: O. Juhl Pedersen, Torben Poulsen

Title of project: Annoyance of Impulsive Noise

---

### 1. INTRODUCTION

This report provides information on the procedures and results of the authors' participation in a joint project to study annoyance due to impulsive noise in the laboratory. The research comprises objective as well as subjective measurements. In this report emphasis is laid on the objective measurements.

Subjective measurements under this contract which expires June 30, 1981 are in progress and will be reported separately.

### 2. OBJECTIVE OF THE RESEARCH

The objective of the research is to find relations between the results of objective measurements on impulsive noise and the annoyance of such noise as assessed by normal subjects under laboratory conditions.

It is hoped that such research will result in means for predicting the subjective annoyance from objective measurements on noise signals. In particular, it will be investigated whether the addition of a correction to the A-weighted equivalent continuous sound pressure level of the noise will be useful for this purpose.

The research carried out under this contract is regarded as a part of a pilot study for a more comprehensive study. This pilot study is established as a joint project with participants from Denmark, England, Germany and The Netherlands coordinated and partly sponsored by EEC, see [A] for further details.

### 3. MATERIALS AND METHODS

The materials (including test room and electroacoustic equipment) and methods utilized are briefly mentioned in this report, for further details see [A].

### 3.1 NOISE SIGNALS

Four noise signals are used in the research, they are recorded on magnetic tape by the participants. The noise signals are:

- G Computer generated noise from a shooting range
- S White noise pulses
- P Noise from a pile driver
- T Traffic noise

The tapes contain suitable calibration signals.

Noise P is identical with noise No. 12 from the ISO Round Robin Test on evaluation of loudness level of impulsive noise, [1].

### 3.2 TEST ROOM

The test room is a laboratory room adapted as a living room, [2]. The floor is  $4.5 \times 3.5 \text{ m}^2$ , the height 2.8 m.

The reverberation time of the furnished room without test subjects is 0.38 s from 63 Hz to 10 kHz.

### 3.3 PLAYBACK SYSTEM

The playback system consists of a studio tape recorder (Telefunken M10a), an attenuator with 1 dB steps (hp 350D), a power amplifier and an electrostatic loudspeaker (QUAD) placed near a corner of the room. The distance from the surface of the loudspeaker to the position of the centre of the test subject's head is 2.15 m.

The frequency response of the playback system is within  $\pm 5$  dB in the range 70 Hz to 10 kHz.

### 3.4 MEASURING SYSTEM

The sound pressure levels at the position to be occupied by the centre of the test subject's head are measured by a  $\frac{1}{2}$  inch free-field microphone (B & K 4133) with preamplifier placed in the room, two cascade-coupled measuring amplifiers (B & K 2606 and 2607), and integrating sound level meter (B & K 2218) and a storage oscillograph (Tektronix 531). This system is connected via a coax-cable to the computer room where a measuring amplifier

(B & K 2607) is placed to drive an 11 kHz low-pass filter connected to the A/D converter of a PDP-8/e Computer with peripherals as described in [3].

#### 4. RESULTS

For each noise signal, objective quantities are determined at a level corresponding to an A-weighted equivalent continuous sound pressure level of 70 dB at the test subject's position in the test room. All quantities are determined with A-weighting.

The following quantities are measured with the computer (for definitions, see [3]):

$$L_{eq} , L_{Mean} , L_{sigma} , NPL , L'_{eq} , \bar{L} , L_{New} , L_{0,1} - L_{99} .$$

All computer measurements are taken over a time interval of 5 minutes and with an integration time of 250 ms corresponding to time-weighting F. This value of the integration time is an approximation to the integration time of the hearing mechanism for evaluation of loudness, [4].

The A-weighted equivalent continuous sound pressure levels are also measured with an integrating sound level meter (B & K 2218). The same instrument is used for measurements of A-weighted sound pressure levels with time-weighting F, S and I (cf. IEC Publication 651). The figures given are average values from the readings by two skilled observers.

A-weighted maximum peak levels are taken from a measuring amplifier (B & K 2607).

For all noises, probability density functions and cumulative distribution functions of A-weighted levels are computed and plotted. An example of a cumulative distribution function is showed on page 5.

##### 4.1 LEVELS IN DB(A)

Noise signal	$L_{eq}$	$L_{Mean}$	$L_{sigma}$	NPL	$L'_{eq}$	$\bar{L}$	$L_{New}$
G	70.5	54.3	14.0	106.3	99.4	111.7	98.3
S	70.2	56.9	14.1	106.2	100.8	113.7	103.6
P	69.9	55.3	13.3	103.9	100.0	113.1	103.4
T	70.4	69.2	3.1	78.3	88.1	80.1	78.3

Noise signal	$L_{eq}$	$L_{0.1}$	$L_{0.5}$	$L_{1.0}$	$L_{10}$	$L_{50}$	$L_{90}$	$L_{99}$
G	70.5	79.9	79.8	79.6	77.5	48.0	41.2	41.0
S	70.2	76.9	76.9	76.8	75.9	54.8	41.3	41.0
P	69.9	78.8	78.4	77.9	76.1	47.3	44.1	41.3
T	70.4	76.7	75.9	75.5	73.4	69.5	65.1	62.0

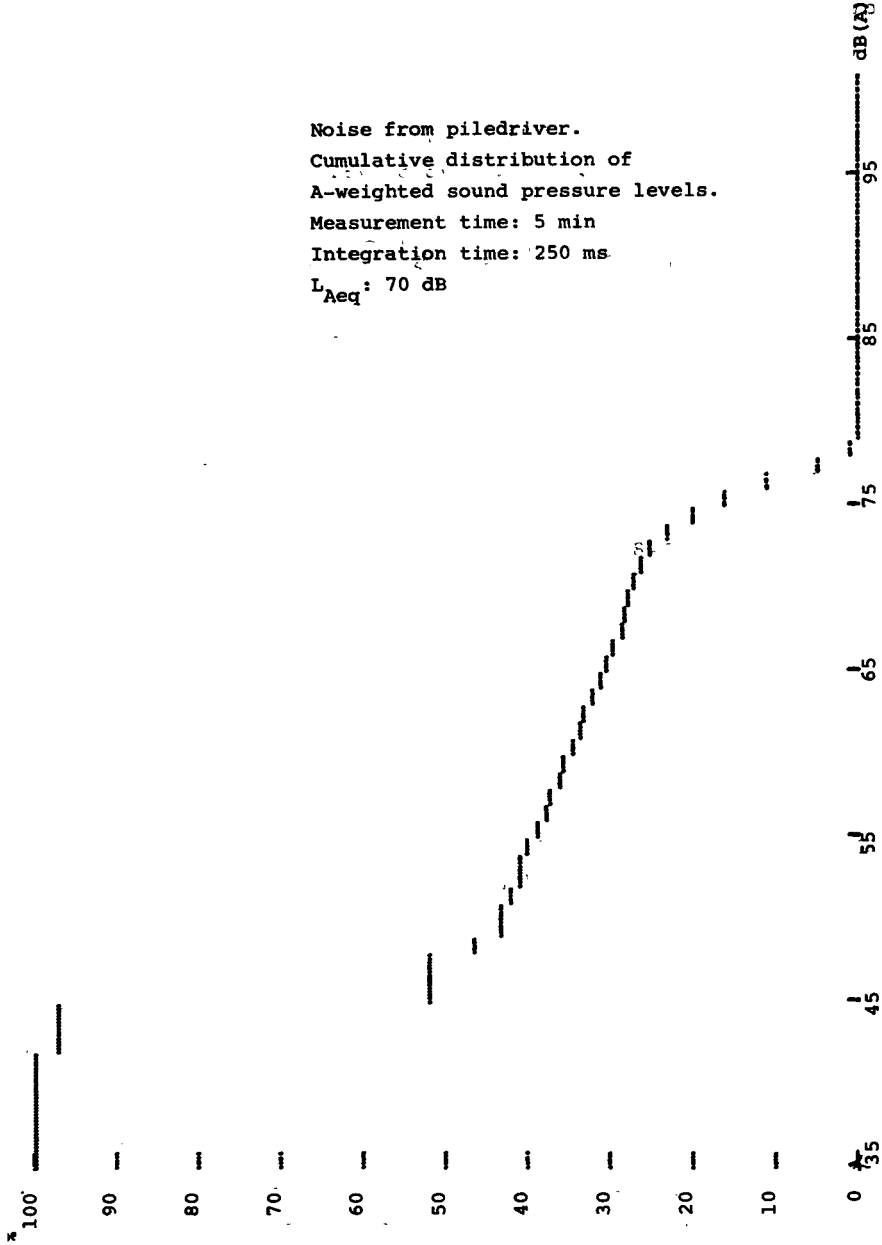
Noise signal	$L_{eq}$	$L_F^{*})$	$L_{Fmax}$	$L_S^{*})$	$L_{Smax}$	$L_I$	$L_{maxpeak}$
G	70.5	-	78	-	73	81	88
S	70.2	-	75	-	72	79	86
P	69.9	76	77	-	72	81	91
T	70.4	70	76	74	76	72	84

\*) average of "max" deflections.

Noise signal	PDP-8	2218	Impulsiveness
	$L_{eq}$	$L_{eq}$	$L_I - L_S$
G	70.5	69.9	8
S	70.2	69.7	7
P	69.2	69.4	9
T	70.4	69.7	2

[Time weightings F, S, I (IEC 651) correspond to responses "Fast", "Slow", "Impulse" (IEC 179 and 179A).]

Noise from piledriver.  
Cumulative distribution of  
A-weighted sound pressure levels.  
Measurement time: 5 min  
Integration time: 250 ms  
 $L_{Aeq}$ : 70 dB



Previous publications on contract research

- A. O. Juhl Pedersen, T. Poulsen: Preliminary objective measurements of annoyance of impulsive noise under laboratory conditions.  
Report to EEC. Internal report No. 12, February 1981.  
The Acoustics Laboratory, Technical University of Denmark.

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- 1 O. Juhl Pedersen, P.E. Lyregaard, T. Poulsen: The Round Robin Test on evaluation of loudness level of impulsive noise.  
Report No. 22, 1977, The Acoustics Laboratory, Technical University of Denmark.
- 2 Torben Jacobsen: Measurement and Assessment of Annoyance of Fluctuating Noise.  
Report No. 24, 1978, The Acoustics Laboratory, Technical University of Denmark.
- 3 P.K. Møller: Digital evaluation of fluctuating noise.  
Report No. 23, 1978, The Acoustics Laboratory, Technical University of Denmark.
- 4 T. Poulsen: Temporal Loudness Summation of Tone-Pulses.  
Report No. 8, 1975, The Acoustics Laboratory, Technical University of Denmark.

Contractor: Consiglio Nazionale delle Ricerche - Italy

Contract n. ENV/392 I

Project Leader: Piero Borruso

Title of project: Influence of noise on human behaviour.  
Study on the disturbance indexes by means  
of numerical techniques

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#### Project program

According to the aforesaid contract, our Laboratory has undertaken objective analysis on a set of noises by means of the following measurements:

- a) FFT analysis in the frequency domain .
- b) Statistical analysis of noise levels. Time constants of the RC - inte -  
gration process and the duration of the true integration process range from  
10 ms to 1 s .
- c) Measurement of the signals in the time domain (rise-time, decay-time,  
repetition rate etc.).
- d) Evaluation of some objective noise indexes ( $L_{eq}$ ,  $L_{NF}$ ,  $L_{DI}$ ).

#### Noise features

From October 1980 to January 1981, according to previous agreements ,  
this Laboratory received the following noise samples :

- a) Road traffic noise, slowly varying (70 dB(A)  $\pm$  5) from Mr. I. Flindell  
(Southampton University).
- b) Synthetic pulses and gun shots from Dr. F. Smoorenburg (Institute for  
Perception , TNO, Netherlands).

c) Pile driver noise from Dr. O.J. Pedersen (Lyngby University, Denmark)

The Technical University in Lyngby provided a second copy of all noises to this Laboratory. The beginning of each tape consists of a calibrating noise (pink or white noise) which was used in order to set absolute levels of all subsequent measurements.

Analysis programs

In the period within September 1980–April 1981 several routines were elaborated and tested on a minicomputer Digital PDP11/04; the main tasks performed by this software are:

a) Analog to Digital conversion of noise samples; acoustical intensity calculation and subsequent integration in a time window in the range 1 ms to 10 s. The total acquisition time can vary from 1 ms to several minutes.

The integrated samples are stored on a hard-disk.

b) Program for direct visualization of data from disk in time domain. The ordinate values can be switched from the linear scale to the logarithmic one; the corresponding dynamic range was 51 dB. The number of displayed samples was automatically ranged for fitting with the total noise duration. Moreover this program allows to evaluate rise-time and decay-time of pulses and their repetition rate.

c) Statistical analysis and indexes evaluation: this program takes samples from disk and performs their statistical distribution in 512 bins and the corresponding cumulative one. These distributions are displayed and then reproduced on a hard copy device. The main purpose of this program is



however the calculation of indexes  $L_1$ ,  $L_{10}$ ,  $L_{50}$ ,  $L_{90}$ ,  $L_{peak}$ , the standard deviation and possible underflows and overflows. Moreover indexes  $L_{NP}$ ,  $L_{eq}$  are calculated.

d) Frequency analysis: the signal samples are taken from disk, weighted with rectangular or Hamming's window and FFT-processed in order to obtain the power density spectrum.

#### Present Status

After testing the software and the analysis chain, the tapes containing the aforesaid noises have been processed. At first, integration times of 1ms and 10 ms were used on all tapes; the obtained results outlined the measurement quality, values and errors of impulsive noise physical parameters.

The drawback of this procedure is a low dynamic range and, generally, a slow processing speed. This is due to the lack of the Extended Instruction Set on the PDP 11/04 system, that forced to use a table of squared values of the instantaneous samples, stored in the main memory. Moreover, the real time processing of these squares compels a simple precision storage with loss of the two less significant bits of the 12 bits A/D converter.

Because of the impossibility of improving these performances the scheduled program has then been stopped and resumed only after computer upgrading with PDP 11/34 CPU and Floating Point Processor.

After a time spent for software fitting to the new hardware, measurements are again carrying out. At present, with this improvement, the dynamic range is extended from 51 to 72 dB and the sampling and processing speed are substantially increased.

Contractor: THE GREATER LONDON COUNCIL

Contract No: ENV - 363 - UK

Project Leaders: J. Simson, G. Vulkan, J. Hyde

Title of project: Acoustic Comfort and Remedial Acoustic Treatments -  
Stage 1

---

1. Objective of the research

The objectives of the project being undertaken as part of the European Community Environmental Research Programme are to establish the extent of increased satisfaction with the acoustic environment provided by an improvement in sound insulation for a dwelling, and to evaluate this in terms of 'Acoustic Comfort Class' (Ref. 1). The research is to be conducted in two stages, each lasting one year. In Stage 1, covered by the present contract, a social survey questionnaire has been designed, interview techniques formulated, a pilot survey to test these is being undertaken and approximately 40% of a main survey will be completed. In Stage 2, to be completed, subject to approval, in 1982 during the 3rd CEC research programme, the remaining 60% of survey work will be undertaken.

This report covers work undertaken in the first six months up until 31 December 1980 corresponding to the end of the second CEC research programme and concerns itself mainly with the pilot social survey.

Double windows with acoustically treated mechanical ventilation have for long been widely used to insulate against noise from road traffic. Whereas the acoustic performance of a wide variety of such treatments is well known very little is presently known about the benefits of insulation in subjective terms. The initial task has been to develop and test a social survey questionnaire and acoustic monitoring programme which can then be applied to a number of sites, as they become available in London, where double windows have been installed. As far as possible the social survey relies on experience from previous survey work to relate external traffic noise to subjective reaction in situations where no acoustic insulation is present. The survey developed in so far as this contract is concerned is intended to provide data on the way in which subjective reaction under everyday living conditions is modified by the acoustic performance of noise insulation packages installed against various levels of traffic noise. For this survey it is the actual noise environment and the real improvements achieved by double windows which is particularly relevant rather than the optimum design performance of sound insulation under ideal conditions of use which is generally well known. For this reason acoustic performance has been monitored by direct measurement at a representative sample of dwellings used in the survey and questions have been included to help determine the actual day to day use patterns.

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(1) Commins DE & Meier A.v. Classes of Acoustical Comfort in Houses. Report No. 7 September 1977 for the environment and consumer protection service, ECC.

At the request of representatives of the EEC Commission the pilot survey included one set of questions on people's reaction to 'impulsive noise'. This was drawn from the draft survey questionnaire being developed by the Institute of Sound and Vibration Research at Southampton University (EEC Contract ENV 362)UK(N). It proved possible to include only one question from the ISVR work because of limitation on the practical length of the draft questionnaire for this project.

## 2 Materials and Methods - The Field Studies

### 2.1 Background information about the study area - Holland Estate

Pilot surveys using both the pilot questionnaire and the trial acoustic monitoring techniques were carried out at Holland Estate which is situated in the London Borough of Tower Hamlets. This estate consists of three blocks of dwellings all overlooking Commercial Street, a very heavily trafficked road carrying a high percentage of heavy vehicles.

Holland estate was built by the GLC in January 1972 and is owned by the Council. There have been a series of complaints from tenants about noise from traffic ever since the buildings were first occupied and this led to the Council deciding in 1978 to install double window insulation at its own cost. Because of practical difficulties and because of views previously expressed by the tenants, the installations, with one exception, were not provided with any means of mechanical ventilation. Installation was carried out in 1979 and prior to that 12 tenants were interviewed 'in depth' by members of the Council's Behavioural Sciences Unit on their attitudes to double glazing before it was put in.

### 2.2 The Social Survey

A pilot questionnaire was designed to test the various hypotheses previously discussed. It is intended that this pilot work should lead to a questionnaire which can be used at a large range of sites where sound insulation has been provided by the Council under its Major Technical Problems programme and under the provision of the Land Compensation Act 1973. Consequently the questionnaire has been designed to cover a range of conditions not all of which were encountered at Holland Estate. Future work will cover multi-occupancy and single family dwellings, owner occupied and tenanted dwellings and also installations that have been paid for entirely out of public funds or installed partly at the cost of the house holder. There will be a wide variety of noise exposures at future sites and it is expected that there will also be a wide range of acoustic performance from the insulation.

The pilot questionnaire was fully structured for the most part and it had been intended that the main survey would consist entirely of structured questions. However, as a result of the Holland Estate test it has been decided to include more open ended questions in the later survey. Fieldwork was carried out by experienced interviewers before any acoustic tests were started.

### 2.3 The Acoustic Survey

The acoustic survey was designed to retain maximum flexibility for subsequent analysis of noise data and at this stage does not attempt to measure all parameters required to determine acoustic comfort but

concentrates on the performance of window insulation and external traffic noise. It was therefore decided to measure as many parameters as possible with the resources available. The statistical parameters  $L_1$ ,  $L_{10}$ ,  $L_{50}$ ,  $L_{90}$  and  $L_{95}$  were measured in terms of A-weighted decibels on an hourly basis over a 24 hr period. The hourly  $L_{eq}$  could also easily be determined. Facade insulation measurements were also taken by tape recording unweighted external and internal noise levels simultaneously. Spectral information was available from further analysis allowing for correction for reverberation time in the receiving room where necessary. Fifteen minute samples of the noise inside the room and the external facade level were used to determine facade insulation values. Each sample used three positions of the internal microphone in order to give an approximate space averaged result.

### 3 Results

#### 3.1 Findings of the Social Survey

Before considering any of the detailed results from the survey one important finding must be recognised. The dominant feature of the flats, so far as the majority of the people living in them was concerned, was the general unpleasantness of the area, particularly at night, with noise as a relatively minor annoyance, and many of those interviewed would rather have talked about these other matters. This must be borne in mind when considering the results.

It seems that double glazing is certainly effective in reducing noise annoyance. A list of 14 possible sources of environmental dissatisfaction was presented, and the results, with the percentages objecting to them, are shown in the Table below.

	<u>Percent dissatisfied</u>
1 Absence of open spaces nearby	68
2 Rents too high	58
3 Surroundings unpleasant	51
4 Local shopping facilities	50
5 Noise other than traffic	17
6 Friends/relatives too far away	17
7 Local public transport (other than work)	16
8 Traffic noise	13
9 Number of amenities in home	10
10 Travel costs to work	9
11 Type of dwelling	9
12 People around	9
13 Age of flat	2
14 Difficulty of getting to work	0

In spite of the fact that the site was definitely a noisy one, as shown in section 3.2 traffic noise was only 8th on the list. Without double glazing this would presumably have been much more annoying and there is other evidence to bear out this view.

It also seems that people tend to become more favourable to the idea of double glazing once they have it. Questions were formulated to get people to think right back to the time when they first heard that double glazing was going to be installed. The comparative figures for initial reaction to double windows compared with those after installation were as follows:-

Reactions to Double Glazing	When first heard about the idea	Now
	percentage	percentage
Very favourable	37	50
Favourable	22	25
No feelings either way	13	7
Unfavourable	19	10
Very unfavourable	6	5
Can't remember	3	3

It can be seen that positive responses rise from 59% to 75%, and that negative responses go down from 25% to 15%. This must mean that, for most of the people involved, double glazing was a good thing. It meant that - as the respondents made clear in their answers to a later question - closing their windows was an adequate answer to traffic noise, whenever it did get bad.

In another question, it was actually possible to quantify the difference between the way the noise was perceived without double glazing and the way it is now, with double glazing. On a scale running from 0 (not bad at all) to 10 (bad as it could be) a mean rating of 6.2 was obtained before double glazing, and 1.5 after. This is a striking result, indicating a very big perceived change in the situation, from distinctly negative to very positive.

No less than 61% of these people agreed that 'Noise is one of the biggest nuisances of modern times' and 44% agreed that 'Certain noises sometimes get on my nerves'. One question which was put in to try it out turned out to be very useful: 49% of them agreed with the statement - 'I am a very light sleeper'. This question, which has not, as far as is known, been used before in this kind of survey, on the main stage of the work will therefore be retained. (Another question which seems worth retaining is 'I think I am slightly hard of hearing', which 19% agreed with; obviously someone with impaired hearing is less likely to be bothered by noise.) And 35% thought that 'too little fuss' was made about noise nowadays.

On the question of how the double glazing is actually used, there were some surprises. Of the 74 people who had double glazing, the results were as follows:-

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	<u>Living room</u>	<u>Bedroom</u>
	percentages	percentages
Double glazing is closed all the time	12	12
open very seldom	19	15
open about half the time	32	27
usually open	14	19
open all the time	19	23
Other answers	4	4

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It can be seen from this that in fact the double glazing was more often open than shut. Since it only works as an antidote to noise when shut, this means that most of the time, these people are without protection from traffic noise. How can we reconcile this with the enthusiasm and sense of real benefit which we found earlier?

One hypothesis would be that it is the sense of control which is important - the feeling that we can shut out the noise when we want to or need to. There is a good deal of psychological evidence to show that this feeling of being in control of one's environment can be very important to people's sense of stability.

Another hypothesis would be that people want a balance between noise control and ventilation: 52% of the whole sample thought that there were problems about ventilation with the windows closed. It is a great pity here that no one had the fans installed, because it would then have been possible to test the importance of this hypothesis. The main survey should however give information on this point.

It will be desirable in the main survey to check whether it is this feeling of control which is the important thing, and whether it takes the particular form of wanting a balance between noise protection and adequate ventilation of the room.

The remainder of the questions were mainly required to check if they would be suitable for cross-tabulation in the main survey.

### 3.2 Results of the acoustic survey

The  $L_{10}$  and  $Leq$  dB(A) results were determined on an hourly basis together with the 18 hr, daytime and nighttime averages.

The results shown below are typical of external noise levels measured on the front facade and insulation provided by the double windows.

	L10	L50	L90	Leq dB(A)
External level	76.8	71.8	67.8	73.5
Internal level	<u>40.3</u>	<u>35.3</u>	<u>32.3</u>	<u>37.7</u>
Insulation	36.5	36.5	35.5	35.8

The estate is very badly affected by road traffic noise with an 18 hr L10 of 77 dB(A) at first floor level. Even at night time (10.00 pm to midnight) the level is 75 dB(A), a reduction of only 2.5 dB(A) on the daytime level. The noise levels drop 2 to 6 dB(A) along the side facade of Denning Point. The measurements on the back facade are about 15 dB(A) lower than those on the front facade.

#### 4 Conclusion

##### Progress of Study

The general approach to both social and acoustic surveys proved adequate for use in the planned investigation into the performance of double windows and with additional acoustic tests acoustic comfort can be adequately classified. There were however, some problems on the social survey questionnaire. The general flow of the pilot questionnaire was not as good as had been anticipated and was thought to be somewhat repetitive. The section on impulsive noise has proved to be very time consuming and will therefore need to be substantially reduced. Greater use of open ended questions is proposed to understand better how the insulation and ventilation systems are actually used. The pilot has demonstrated that the use of 'before and after' sampling is not required. Further questions on the neighbourhood are necessary.

A new questionnaire which should overcome the difficulties of the pilot has now been developed and tested. Full details of the test will be published during the next reporting cycle.

Contractor: Institute of Psychiatry, University of London

Contract no. ENV - 451 - UK

Project Leaders: Prof. M. Shepherd, Dr. A. Tarnopolsky

Title of Project: Annoyance by aircraft noise and mental health

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Objective of the research

The Aircraft Noise and Psychiatric Morbidity Research Project was a large interdisciplinary study undertaken in the United Kingdom with the sponsorship of the Department of Trade and the Medical Research Council to establish if there were any reliable associations between aircraft noise and mental health indicators. It has shown that noise in isolation cannot be considered an important cause of psychiatric disorders, except for the case of some respondents of high educational and occupational standing who are prone to suffer symptomatically in noisy environments. The frequency of individual acute and chronic symptoms was also investigated. Many acute symptoms showed an increase with noise, and this was particularly evident for waking at night, irritability, depression, difficulty in getting to sleep, swollen ankles, burns/cuts/minor accidents and skin troubles. Two chronic symptoms, tinnitus and ear problems, also increased with noise. It was clear that the health effects of noise are better assessed examining "annoyance" reactions and with the postulate of an intermediate factor related to personal vulnerability or "sensitivity" to noise. Results from the surveys demonstrated a clear association between annoyance and psychiatric status. Moreover, subjects exhibiting high annoyance - even if not psychiatrically ill - used psychotropic drugs and other medicines more frequently than those less annoyed; they also attended out-patient departments more frequently and generally complained more often of bad health. (Further details in: Tarnopolsky and Morton Williams, 1980; Tarnopolsky et al, 1980; Watkins et al, 1981)

Annoyance, however, is a complex concept comprising at least three different responses: a) a global emotional expression of discomfort, i.e. feelings of being bothered; b) special reactions which are the consequence of noise interfering with the subject's activities, and c) symptoms of psychosomatic distress such as irritability, sleep difficulties, headaches, fatigue, etc. We call this dimension "symptomatic annoyance". These symptoms are not easy to classify in existing psychiatric categories,



belonging as they do to an area of epidemiological and public health concern which has attracted increasing research only in the past few years. The E.E.C. is currently supporting further research in this area, with the following aims:

1. To study the interrelations of the three components of annoyance - symptoms, feelings of being bothered and interference with activities.
2. To investigate the existence of individual differences in the expression of annoyance according to the predominance of particular components.
3. To study the association of each component with health variables (use of psycho-tropic drugs, use of medical services, declared psychiatric illness).
4. To study the relationship between health variables, components of annoyance and specific characteristics of the source of noise (intensity, frequency, meaning).
5. To lay out the basis of a follow-up study that would investigate the long term relationship between annoyance by noise and frank psychiatric morbidity.
6. To determine the importance of some particular noises.

#### Materials and Methods

The present investigation uses material collected in the course of a community survey reported in detail in the papers quoted above. A domiciliary survey (sample size circa 6000) was conducted in 1977 in areas of different aircraft noise exposure affected by London (Heathrow) Airport. Respondents were urban dwellers aged 16 +. Aircraft Noise Number Index (N.N.I.) measures were provided by the Civil Aviation Authority (C.A.A.). Road traffic noise was controlled for. Each subject answered a questionnaire in three parts. One section covered health, including reports of symptoms, the use of health care services and an assessment of mental status; another section explored attitudes to the environment, satisfaction, annoyance scales and sensitivity to noise; and the third collected social and demographic data. A sample of respondents were reinterviewed in 1980 and will be incorporated in the current analysis.

#### Preliminary Results

The work is in progress and will be reported in full towards the end of 1981. Preliminary findings refer to 1) annoyance and 2) noise exposure.

- 1) The three components of annoyance are related in a hierarchical manner.

The most common manifestation is a global feeling of being bothered by noise which is reported by 4000 + subjects. Of these, 3000 + report in addition, activity interference; and of those, 1900 + report they also suffer from symptoms. Thus, symptomatic annoyance is an extreme expression of annoyance with notably few exceptions: only 40 subjects report symptoms without the accompanying lower levels of distress. The relationships between noise levels in N.N.I. and the three components of annoyance was weaker for symptomatic annoyance than for activity interferences or feelings of being bothered. We considered the effect of thirteen other variables in these relationships examining demographic, attitudinal, socioeconomic and noise exposure factors. All the relationships were weak, and even weaker for the symptoms than for the other two components of annoyance.

Although all three components of annoyance were associated with psychiatric scores, symptoms exhibited a noticeably stronger linear trend than the other two components.

2) The noise measures available for this study (N.N.I., 1977 day-values provided by the C.A.A.) were compared with two other sets of data including night exposure estimates obtained by other investigators for their research on noise annoyance. It became clear that although they were all valuable, they could not be utilized outside the study for which they were originally produced, because one or more of the following applied: 1) the area in each case was very different, and smaller than ours; 2) individual monitoring points in other studies could only be used to attribute noise exposure to a very small number of our respondents; 3) there was a large gap between the date of the noise assessment and the date of our interviews; 4) night-time data without daytime assessment would underestimate the total exposure of our sample. It was therefore concluded that C.A.A. - N.N.I. 1977 data was, and still is, the most representative noise assessment available for the analysis of our sample.

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**RESEARCH AREA 2 : ENVIRONMENTAL INFORMATION MANAGEMENT**



**TOPIC 21 : ECDIN PROJECT**

Contractor: Bundesforschungsanstalt für Landwirtschaft  
Institut für Bodenbiologie, Braunschweig

Contract No. 186-77-1 ENV D

Project leader: Prof. Dr. K.H. Domsch

Title of Project: Effects of environmental chemicals on microorganisms;  
Degradation of environmental chemicals by soil micro-  
organisms

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### Objective of the research

A substantial amount has been published on pesticide side-effects and degradation and its potential hazards by long-term applications. However, most data in the literature are derived from experiments concerned with pesticide effects on isolated cases. Any direct projection of these findings into an ecosystem as a whole is questionable. Particularly the terrestrial soil ecosystem is such a heterogenous environment with manifold and complex interactions of soil components, eg. variability of microorganisms and their functions, which concertedly are the make-up of a dynamic equilibrium. Therefore, potential effects in the field cannot per se be predicted from toxicity tests or tests based only on one parameter.

Since chemicals seem to be an integral part of our life it became imperative to look for criteria which will allow immediate recognition of potential harmful substances and to evaluate the type of hazard found or expected to its relative importance, in order to take necessary precautions.

Two ECDIN projects were taken up in the years 1977/78 and were continued up to 1979/80. The work on "Effects of environmental chemicals on microorganisms" and "Degradation of environmental chemicals by soil microorganisms" included a) assessment of published material b) establishment of parameters for data analysis and c) establishment of ecotoxicological profiles for single compounds based on data extracted from the literature followed by resynthesis.

The latter is a tentative model; its suitability must still be proven after a series of compounds have been tested. The advantage of such an ecotoxicological profile is seen in the fact that it will provide an overall guide of the general effects of a substance not only for individual chemicals but also for groups of chemicals.

### Material and Methods

It is generally assumed that hazardous effects on populations of microorganisms or their functions in soil are not common or transient when the compound is applied at rates recommended for practical use. This assumption is based on the fact that soil, when viewed as an ecosystem, possesses a considerable buffer capacity against negative influences of any kind. Production and consumption of each component are in a balanced condition and the concentration of all components remains constant. This so-called dynamic equilibrium reflects steady state conditions when the soil is observed for a long period and over a large enough scale. However, oscillations and temporary changes occur on a small scale between interacting populations and their functions. It is at this point where prospective monitoring must assess potentially negative changes under the influence of chemicals, particularly pesticides. The soil microflora is directly responsible for soil productivity with the recycling of nutrients from organic materials as the most prominent function. A critical evaluation of the influence of a chemical involves more than a determination of its toxic potential. It must include the evaluation of the amount and duration of exposure, the specific behaviour of the substance and the physical condition of the environment under which exposure occurs, in order to avoid an overestimation of the effect. Therefore, adequate assessment of effects should consist of a resynthesis of information such as shown, for example, by an ecotoxicological profile as discussed below.

For that reason the published material on Side-effects of environmental chemicals on microorganisms and on Degradation of environmental chemicals by soil microorganisms was collected, analyzed and evaluated according to a set of parameters already reported on.\*) The principles will not be repeated here, except for those which were included or revised during the last term:

#### Pesticide side-effects

In addition to the earlier practice, soil type and, when applicable, soil amendments were included in the data sheets. Also on the side of the soil organisms effects on mycorrhiza and lichen were registered. With regard to effects on microbial populations or functions the "deficit" definition as one of the concluding remarks on "ecotoxicological significance" has been

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\*) Comm. Eur. Communities (Rep.) Eur., No. (Eur. 6388), Environ. Res. Programme 1980, p. 656-662.



revised. Deficits up to 30% are regarded as negligible if they have not been monitored for longer than 60 days; deficits up to 99% are regarded as tolerable if it has not been shown that they last longer than 30 days.

#### Pesticide degradation

For the data survey on degradation no changes were made. However, data from degradation studies which were based on bioassays were not longer included.

In general, it was not attempted to resolve any questions which occasionally remain when data from published sources are evaluated. Data out of a dubious context, i.e. missing controls, unclear rate of application, unclear time of exposure etc. have been neglected. Rates of commercially formulated pesticides were only converted to rates of active ingredient, when the percentage of active ingredient has been given by the author. Rates of application have been converted to kg or litres ha<sup>-1</sup> or as kg (a.i.) or litres (a.i.) ha<sup>-1</sup>. Except for "no-effect" data only experiments done with pesticide concentrations at or 10 times above the recommended amount for agricultural use were considered for the side-effect survey, while no such restrictions were applied for the pesticide degradation study.

#### Results

##### Range of chemicals

Of the c. 3000 publications so far registered, approximately 400 single pesticides were extracted and listed. Of those the following compounds have been analyzed and transferred onto data sheets for ECDIN during the term 1979/80:

Aethyl-Hg-acetat, Aldrin, Arochlor, Atrazin, BFMC, Camphechlor, Carbofuran, Chlordane, Chlordecone, 4-Chloroanilin, 2,4-D, DDA, DDT, Dihydroheptachlor, Dodecylbenzosulfonat, Endosulfan, Endrin, Ethiophencarb, Fentin-acetat, HCH,  $\gamma$ HCH, Heptachlorepoxyde, Hexachlorbenzol, Hexachlorcyclopentadien, HgCl<sub>2</sub>, Isobenzan, Isolan, Isoproc carb, Metal-kamate, Metasol, Methomyl, Methylarsinsulfid, Methyl-Hg-chlorid, Mexamcarbate, Mirex, Oxamyl, Panogen, PCMB, PCMC, Phenyl-Hg-acetat, Propoxur, TEIO, TDE, Thiophanox

Literature on the compounds Carbaron, DDE, DCEE, Dioxacarb, Ethyl-Hg-phosphate, Isodrin, Phenyl-Hg-chlorid, Phenyl-Hg-triethanolamine, Semesan, Toly-Hg-acetate, has been surveyed and examined. It did not contain suitable data for this purpose.

### Ecotoxicological profile

The severity of a negative influence ranges from no-effect to negligible, tolerable and critical. In each group a residual deficit has a higher weight than a delay period. If the data from over 300 individual experiments are allocated to those categories it can be shown that some of the investigated microbiological parameters are consistently more sensitive to randomly chosen groups of pesticide chemicals than others. If some of the insufficiently documented parameters (<5 sources) are excluded for the time being the following groups emerge:

- High sensitivity:      Population Nitrifiers  
                         Population Rhizobium  
                         Population Actinomycetes  
                         Function Organic matter degradation  
                         Function Nitrification
- Medium sensitivity:    Population Algae (total)  
                         Population Bacteria (total)  
                         Function CO<sub>2</sub> Production  
                         Function O<sub>2</sub> Uptake  
                         Population Fungi (total)  
                         Function Denitrification  
                         Function Ammonification
- Low sensitivity:        Function N<sub>2</sub> fixing capacity (aerobic)  
                         Population Azotobacter  
                         Population Ammonifiers  
                         Population Microorganisms (general)  
                         Population Proteolytics

Schemes for ecotoxicological profiles can progressively be improved with every additional piece of information and finally reach a reliable degree of stability. From the information available at the present time a practical example of an ecotoxicological profile is given below:

Allyl alcohol

Tested parameters	Dosage l/ha	Categories of negative influences						
		NO-effect	Negligible Delay	Deficit	Tolerable Delay	Deficit	Critical Delay	Deficit
Popul. Actinomycetes	300							80% 105 d
Funct. Org. matter deg.	555							77% 112 d
Function Nitrific.	555					50% 21 d		
Popul. Bact. (total)	300					73% 35 d		
Function CO <sub>2</sub> prod.	555					65% 2 d		
Popul. Fungi (total)	150		4 d					

**Explanation:**

Based on the principles developed for data evaluation the entities should read as follows: The recovery of the population of fungi after allyl alcohol application ( $150 \text{ l ha}^{-1}$ ) is completed within 4 days; or 21 days after the application of  $555 \text{ l [allyl alcohol] ha}^{-1}$  the process of nitrification is still 50% below that of a comparable untreated soil sample.

Most of the recorded effects are found in the "Tolerable" and "Critical" categories. The synoptical profile indicates a toxic potential.

Conclusions

Predictions on the ecological effects following pesticide application are difficult to make. Biological indicators are missing which could universally be used as an evidence of damage. Since pesticides will affect every level of organization - from the cellular over to the individual, up to the level of populations and ecosystem - the evaluation of effects must consist of a resynthesis of data obtained from parameters which would sufficiently cover this spectrum. An ecotoxicological profile could provide this type of scheme.

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Contractor : Technische Universität München  
Contract n°: ENV/471 D  
Project leader : Prof. Dr. Ivar K. Ugi  
Institut für Organische Chemie  
Title of project : Correlations and dynamic retrieval in chemical  
structure files and in hierarchically organized  
chemical substructure files

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The problem of substructure access in data bases of chemical compounds and of substructure-activity selection has been investigated, employing an algebraic model of constitutional chemistry which was developed by UGI et al. A program system for determining and correlating effectors (i.e. substructures marked as "active" substructures) has been developed and tested against data bases of various sizes. The program is able to produce lists of effectors with probability values assigned to each effector. A unique feature of the program is its ability to evaluate synergism and antagonism. Such data are important in screening the environmental impact of "unknown" or "new" substances, i.e. substances whose chemical structure is known, but whose environmental properties are by and large unknown.

An earlier version of the fragmentation and retrieval system has been implemented in FORTRAN on a PDP 11/45. It had shown that a file of about 1000 molecules occurring in the environment can be processed interactively.

Based on experience gained with that system, an improved algorithm for substructure generation has now been developed. The new algorithm avoids duplicated generation and storage overflow. It can handle molecules with up to 50 atoms. Improvements were also achieved on the structure/property locating procedures, which are of the same time-complexity as the substructure generation, but have much smaller step entities. This new version has been implemented in PL/1 on an IBM 360/91 and AMDAHL 470/V6.

Conventional substructure matching algorithms are based upon classifying of atoms and their valencies, followed by refinement of this classification. The exponential time requirement of these algorithms is somewhat alleviated by the use of fragment codes or screens: Matching is speeded up, but nevertheless, each molecule must be inspected. Complications arise when molecules in the data base do not contain the given substructure, but give a hit in screen-matching. The reason is that it is not possible to define any graph with a delimited number of (actual) subgraphs invariants. A final time-consuming atom-by-atom comparison is therefore necessary. The

same situation occurs with fragment codes, but in addition, there may be misses if the query fragment is "hidden" under a combination of other fragments.

Our approach overcomes the described deficiencies by creating all possible substructures beginning from the largest molecule, level by level in a breadth-first manner. If the largest molecules have  $n$  bonds, the system first generates all substructures with  $n-1$  bonds and puts them on level  $n-1$ . Then, starting from level  $n-1$ , including all data base molecules with  $n-1$  bonds, the next level  $n-2$  will be generated, and so on, until at last only single-bond fragments are obtained in level 1.

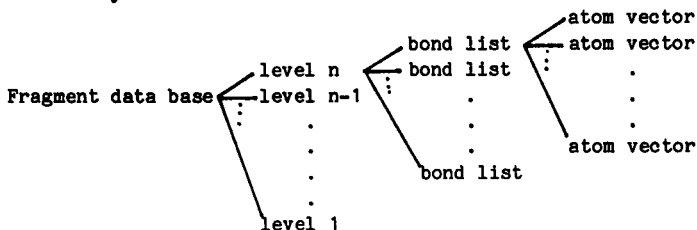
If used naively, this method may give rise to two substantial problems: first, more and more duplicates are generated with increasing molecules size and, second, the number of different substructures may become larger than the storage capacity available.

The first problem is solved by a level-by-level generation process. No generation step spans more than two levels. A two-step graph analysis of each (sub-) structure prohibits duplicate generation. The first step determines the constitutional symmetry of the (sub-) structure. The algorithm is an extension of the canonisation algorithm that was developed in the previous project and can now mark constitutionally different atoms. The second step employs an algorithm, which is based on a linear depth-first search. It processes the molecular graph by looking for "marginal nodes", i.e. a node that can be removed from a graph leaving the remaining graph connected. The intersection of the constitutionally different atoms from the first step and the marginal nodes from the second step gives a special set of atoms with the property that by taking away these atoms one after the other from the considered (sub-) structures while replacing the preceding atoms, no duplicates are generated, and that these new fragments are all the possible fragments of size  $n-1$ . Furthermore, because the substructure relation is transitive, it is superfluous to generate substructures across more than one level. Thereby each level contains all possible substructures which can be generated from the next higher level, whereas the redundant generation of duplicates is restricted to a minimum.

The second problem, storage overflow, is attacked by heuristics. A heuristic function was implemented which tells the generation system whether a generated substructure is chemically meaningful, i.e. relevant information. Another function computes the frequency of occurrence of a given substructure in the entire data base. Higher-frequency-fragments can be eliminated when storage occupancy approaches the limit of capacity. These two functions have been optimized in the process of fine-tuning the system. They not only are an efficient tool in avoiding storage overflow, but also speed up the generation process, because dropped substructures do not have to be

stored or searched for.

The generation process builds a data base of fragments, which internally is further subdivided as follows:



This data structure allows access to structures that are defined by their connectivity list only.

The user may further define atom names. These are used to build a mask. The fragment network is entered at the defined bond list. By cancelling undefined atoms, the access algorithm proceeds downward level by level until all substructures with fully defined atom vectors are obtained. By this process of backtracking, all substructures are retrieved that have the query substructure mask in common. From these substructures the search procedure goes upwards in the same way as with a query for a fully defined substructure.

If the input molecules are tagged with property or activity information, a fragment data set is generated as described before. Investigating a certain property then involves working out the fragment network from top to bottom, starting with those molecules in the data base that exhibit the desired property or activity.

A merit number is assigned to each substructure as follows:

- (1) Determine the number of tagged molecules with the desired property  $p$  containing the substructure.
- (2) Determine the number of entire molecules containing the substructure.
- (3) Compute the confidence value from the values obtained in step 1 and 2, and from the number of all molecules having the desired  $p$  and of all not having it.

Selection of the most confidential substructures then follows in two steps:

- (1) Determine the rank of all substructures according to the confidence values.
- (2) Beginning with the highest value, select the substructures with the highest degree of confidence by eliminating from the rank all involving and contained substructures of lower value.

An effector with a 0.97 confidence value being contained in a better p effector is not selected.

Substructures which are responsible for one or several properties are called effectors. They may yield an effect, an activity, or a property in an independent manner. A special kind of interdependent effectors are named synergistic effectors, if they yield a property only or preferably through joint occurrence.

For any given effector e, the best synergistic substructure can be selected:

- (1) Determine all molecules with property p and containing e.
- (2) Weigh and select the substructures of the molecules obtained in step 1.
- (3) Eliminate e and all father fragments of e from the set of substructures obtained in 2.
- (4) Improve the confidence value of the remaining e according to the maximal confidence value of 2 and 3.

Antagonistic effectors occur where a substructure suppresses a property of an effector. An algorithm for locating these effectors is similar to the one above. It deviates only in the determination of the starting molecules:

- (1) Determine all molecules without p, but containing e.
- (2) - (4) analogous to step (2) to (4) above

The retrieval and correlation system is now fully operational and is extensively documented in a users manual.

#### Publications:

I. Ugi\*, J. Bauer, J. Brandt, J. Friedrich, J. Gasteiger,  
C. Jochum, W. Schubert  
"Neue Anwendungsgebiete für Computer in der Chemie",  
Angew. Chem., 91, 99-111 (1979)

I. Ugi\*, J. Bauer, J. Brandt, J. Friedrich, J. Gasteiger,  
C. Jochum, W. Schubert  
"New Applications of Computers in Chemistry",  
Angew. Chem. Int. Ed., 18, 123-147 (1979)

I. Ugi\*, J. Bauer, J. Brandt, J. Friedrich, J. Gasteiger,  
C. Jochum, W. Schubert  
"Ein mathematisches Modell der konstitutionellen Chemie  
und darauf beruhende Computer-Programme"  
Informal Commun. Math. Chem., 6, 159-176 (1979).



- J. Friedrich, I. Ugi  
"Substructure Searching and Structure Property Locating  
by Means of Subgraph Generation",  
Informal Commun. Math. Chem., 6, 201-211 (1979).
- J. Friedrich  
"Retrieval von Substrukturen und Struktur-Wirkungs-  
Beziehungen mittels Teilgraph-Generierung"  
Thesis, Techn. Univ. München (1979)
- J. Friedrich, I. Ugi  
"Substructure Retrieval and the Analysis of Structure-  
Activity-Relations on the Basis of a Complete and  
Ordered Set of Fragments",  
J. Chem. Research (S), 7Q (1980)  
J. Chem. Research (M), 1301-1360, 1401-1497, 1501-1550 (1980).

Computer Simulation of Radical Reactions

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The simulation of reactions by reaction operators (R-matrices) that span one entire step going from one stable molecule to another stable molecule has been elaborated in some detail by our group in the past. For reactions with a high degree of branching between initialisation and stable intermediate, this approach is less favorable. Radical reactions are a typical case.

In the present project, just started, we are implementing a program whereby a network of instable but mechanistically plausible structures is built up. The chemical boundary conditions which prevent a "combinatorial explosion" are still to be detailed. Parameters under investigation are:

- number of breakable bonds in dependence of reaction conditions to be simulated
- number of instable intermediates allowed between (in case of radicals:) initiation, propagation and termination
- choice of bonds marked as non-reactive
- degree and optimum method of user interaction.

The latter point, interaction with a "knowledgable chemist", is highly attractive as a means of restricting the width and depth of a simulation and is extensively used in popular synthesis planning programs of the retrieval type. Its use, however, has to be carefully restricted in order to limit the degree of prejudice in simulation programs of the deductive type like the one we are developing here.

Contractor : A R D I C

Contract N° : 286-77-1 ENV F

Project Leader : Mr. Jean-Claude BONNET

Project Title : PISCINE ("Projet Pilote d'Information Structurale et de Comportement Intégré et Evolutif").

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#### I. OBJECTIVE OF THE RESEARCH

Continuing research and development on applications of the DARC System in coordination with the EDCIN project ; study on integration of DARC software specific to structural data base (SDB) interrogation into a data base management system (DBMS) capable of managing all types of data which can be associated with a chemical structure.

By recording structural data in topological code form (CAS code or DARC code), it is possible to implement interrogation systems (searching by chemical structure or sub-structure)) whereby questions are directly formulated in the natural language of the chemist, i.e. the structural diagram of a chemical compound.

Prior studies have made possible the creation and organization under the DARC system of an SDB of ca. 3 500 chemical structures which were initially available in CAS RIII/SDF code. This SDB can currently be interrogated on a trial basis at the JRC at ISPRA through the DARC software in graphic and alphanumerical versions on the IBM 370/165 computer at the JRC. Currently limited, the bank should evolve to 25 000 compounds in a first stage, and reach over 50 000 compounds by several successive updatings.

This SDB is one component of the EDCIN bank. The other data gathered regarding each compound of the SDB are managed and processed in an independent component, the Information Data Base (IDB) implemented under the DBMS used by the JRC at ISPRA. We have studied the possibilities of a coordination operation, i.e. integration of the DARC software specific to SDB interrogation into a DBMS capable of managing all types of data (scientific, legal, ... numerical, textual,...) which can be associated with a chemical compound.

## II. MATERIALS AND METHODS

The unredundant introduction of new substances into the SDB can, if necessary, be carried out in two ways :

- for substances for which the CAS Registry Numbers (CAS-RN) are available to the ARDIC : by extracting the DARC Reference Structural Data Base (DARC-RSDB) from the CAS Registry Structure File (CAS-RSF) used by the ARDIC for the "Centre National de l'Information Chimique" and "Télésystèmes-Questel".
- for structures without a CAS-RN (1-2 %) : by direct acquisition of the structural diagram of a molecule through the "TOPOCODEUR". In this latter case, the ARDIC receives the structural diagram of each structure on an entry sheet which complies with the specifications provided by the ARDIC. The CAS-RN can be assigned to a structure if the latter is present in the DARC RSDB held by the ARDIC at the time of processing.

At the same time as these SDB updating tasks are being carried out, the possibility of integrating, by successive stages, the DARC application into a DEMS is being studied.

This integration is the ideal solution for coherent interrogation and management of the bank under a single system. It required :

- a prior study of the possibilities offered by DEMS's such as the one used by the JRC at ISPRA in order to implement the SDB structure and access functions (essentially files of structural access keys - SAK -) currently accomplished by the specific DARC software.
- an assessment of the consequences that the possible solutions could have on system performances.

It is only on the basis of such a study on the feasibility and on the basis of similar studies conducted for other projects that it can be decided whether programming the DARC application under the DEMS should genuinely be carried out.

### III. RESULTS:

#### III.1 - Applying the DARC software to the ECDIN Data Bank

The CAS-RF currently contains 5 million chemical substances recorded at a given moment with their unique RN. The DARC transcoding software has retained 4 250 000 defined chemical structures integrated into the DARC RSDB. Among the substances temporarily included in a reject file, about 45 % are coordination complexes and 17 % have no connection table (only a molecular formula and/or a trivial name).

Extracting a sub-file from a finite file of RN's involves obtaining the DARC code for each RN and the structural access keys (SAK) for the compound corresponding to that RN, and, in certain cases, the explicit connection table which is an intermediary between the CAS code and the DARC code.

This management function of the RSDB is geared not only to the RSDB organized for conversational interrogation (available via TELESYSTEMES-QUESTEL as of 1981), but also to basic files which have made this organization possible and which are simply structured so that they will be readily accessible for batch processing through use of a list of RN.

There are two main problems :

- a) Handling synonymous RN's (different RN's assigned to the same substance)
- b) Searching for an item of information in a large volume of data.

Both of these problems have been solved by creating, for the main file, a file of ties between the RN and the series number, sorted by RN. The intersection between the sorted list of RN's being sought and this file yields the sequence numbers of the main file corresponding to the RN of the list. This new partial file is sorted by series number. If there are RN's which have been replaced by other RN's, the same sequence number is given for the substituted RN and its replacement ; the list of synonymous RN's can therefore be deduced.

Searches of DARC connection tables (intermediate work code between the CAS and DARC codes) corresponding to the RN's is made easier by the very organization of this file (index + block format tables). Access to the index blocks corresponding to RN's, as well as access to tables via the index minimize the number and time of accesses. Once the tables are extracted, the SDB corresponding to the initial list of RN's can be generated.

### III.2 - Assigning a Registry Number to a Chemical Substance

Several non-exclusive procedures have been elaborated for this assignment :

- comparison of the biunivocal DARC code for a substance with the codes obtained by DARC transcoding of the CAS-RSF,
- assignment of an RN through the name of a substance, which entails availability of the complete CAS Registry Nomenclature File with the "synonyms" option,
- assignment of an RN by interrogation of the RSDB, preferably in conversational mode, given the small volume of substances with an unknown RN.

### III.3 - Integrating the DARC Software into a General DBMS

Except for the SDB, the overall set of data is currently managed by the ADABAS software. The ARDIC has for its part been able to conduct an experiment using this same software for a pharmacological data bank with 12 000 chemical structures.

The structural data on the whole are so specific and so complex that programming its management, interrogation and input under ADABAS would entail major intervention on both the DARC software and the ADABAS software.

Despite the restrictions imposed on the handling of numerical values by ADABAS, the management of these values remains achievable. It is with this in mind that the problem of interfacing the DARC software and the ADABAS software (which respectively continue to manage the structural data and other information associated with chemical compounds) has been viewed.

To each logical recording, ADABAS assigns an Internal Sequence Number (ISN) which remains transparent to the user. Whatever the treatment to be applied (updating, destruction, reading of data), ADABAS accedes to the data via the ISN. In point of fact, the ISN can only be delivered as a result of a search over a given field. This is the only means of communication that ADABAS has with the outside. Thus, any relationship between "ADABAS data" and an external program of use will be rendered concrete by a search interface which will transform the internal ADABAS numbers into external DARC numbers and vice-versa.

Actually, it is not possible a priori to conserve a list of ISN's from one question to the next, therefore each intervention from an input program, for example, would trigger a complete search of the entire bank ; this would have unacceptable consequences on the performance of the system and would seriously encumber the possibilities of the software at the interrogation level. Possible solutions to this type of problem are being studied.

#### IV. CONCLUSIONS

The research under way will make it possible to progressively ensure the more and more complete integration of both sub-sets constituting the bank (the SDB and IDB) and of the corresponding software sub-systems for their maintenance (updating and management) and interrogation.

The establishment of a bidimensional automatic connection between both interrogation sub-systems will make possible the interactive processing of true mixed questions (structural criteria selected from the SDB, plus criteria on associated information selected from the IDB) so that the user will benefit simultaneously from all the possibilities offered by the DARC structural interrogation software and the general interrogation software elaborated by the researchers from the JRC.

Contractor: University of Pisa, Italy

Contract n. 266-77-1 ENV I

Project leader: A. Abbondandolo

Title of project: The induction of DNA damage by environmental chemicals. Tests for detecting DNA repair synthesis in human cell lines and strains

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#### Objective of the research

The detection of DNA repair synthesis has been proposed as one of the short-term assays for the screening of chemicals suspected of being mutagenic or carcinogenic. Such an application of DNA repair studies has stressed the opportunity of setting up experimental methods more rapid than the classical autoradiographic procedure used to detect unscheduled DNA synthesis (UDS) and at the same time simpler than isopycnic centrifugation or chromatography on extracted DNA. Scintillation counting of radioactive thymidine incorporated into DNA during repair synthesis appears as the ideal technique, on account of its simplicity and rapidity.

The aim of this research was to compare a simple test based on suppression of semiconservative DNA synthesis by hydroxyurea and scintillation counting of unscheduled  $^3\text{H}$ -TdR incorporation, with autoradiographic detection of the same unscheduled incorporation.

#### Materials and Methods

Most of the experiments were carried out on EUE human heteroploid cells. HeLa cells and human diploid fibroblasts (AH, stock from Rijswijk) were also used.

With the scintillometric method, cells were seeded on coverslips 1 day before treatment, at a density of  $2 \times 10^5$  cells/coverslip (12x12 mm). Cultures were exposed to mutagens in

Hanks' balanced salt solution + HEPES buffer, pH 7.4 (HBSSH) for 1 hour at 37°C. 10 mM hydroxyurea (HU) was added to half of samples and, 15 min. later, <sup>3</sup>H-TdR (10 µCi/ml, 20 Ci/Mole) was added to all samples. After 4 hours incubation at 37°C, coverslips were washed serially in cold HBSSH, ethanol/ether 1:1 and ethyl ether. They were incubated overnight in scintillation vial with 1 ml Soluene (Packard) and counted in a standard scintillation cocktail.

Autoradiographies were performed on parallel samples treated as explained before up to the washing in ethyl ether. Coverslips were fixed on slides, dipped in photographic emulsion, incubated for 3-5 days at 4°C, developed and stained with toluidine blue. The number of silver grain per nucleus was then determined by microscopic observation. The number of grains present in equivalent cytoplasmic areas was subtracted. Significance of differences between control and treated cells was assessed on the basis of a t test.

The 9,000xg supernatant (S9) of liver homogenates from PB-induced mice (Swiss albino, males) was used for the in vitro metabolic activation of chemicals. 5mM NADP, 20mM glucose-6-phosphate and 20mM MgCl<sub>2</sub> were present as co-factors. S9 for med 33% of the total incubation mixture.

All chemical tested were of reagent grade.

## Results

In table 1, a summary of the results obtained with a number of physical and chemical agents is presented. Agents included in class A are mutagens acting through different mechanisms of action. In particular, acridine orange and hycanthone have been reported as frame-shift mutagens, mitomycin C is known to cross-link DNA strands and griseofulvin produces aneuploidization in mammalian cells. The remaining chemicals are alkylating agents whose mutagenicity has been extensively studied. Class B included compounds which have consistently shown lack of mutagenicity in one or more tests. Class C included mutagens requiring metabolic activation. Compounds in class D are DNA synthesis inhibitors.



Radioactivity counting gives a measure of  $^3\text{H}$ -TdR incorporation in each of the following experimental series: controls (C), controls plus hydroxyurea ( $C_{\text{HU}}$ ), cultures treated with the agents under test (T) and treated cultures plus hydroxyurea ( $T_{\text{HU}}$ ).

Stimulation of unscheduled  $^3\text{H}$ -TdR incorporation was indicated by an increase of  $T_{\text{HU}}$  over  $C_{\text{HU}}$ . The mean percent residual synthesis due to inhibition by HU in controls in 50 different experiments was  $3.69 \pm 1.73$  and, accepting the 3x or 2x S.D. as confidence limits, 8.89 and 7.16 are obtained as the limits over which the increase of DNA radioactivity in  $T_{\text{HU}}$  is significant ( $p \leq 0.01$  and  $p \leq 0.05$ , respectively). Given the marked variation between experiments in  $C_{\text{HU}}$  residual DNA synthesis, the above limits were normalized by dividing them by the ratio between the percent  $C_{\text{HU}}$  residual synthesis of a given experiment and the mean value 3.69. This procedure allows to state  $T_{\text{HU}}$  radioactivity as significantly increased when it is at least 2.4 ( $p < 0.01$ ) or 1.94 ( $p < 0.05$ ) times higher than  $C_{\text{HU}}$  radioactivity. The ratio  $T_{\text{HU}}/C_{\text{HU}}$  when significantly increased is referred to as absolute increase of DNA radioactivity in the presence of HU, and taken as an indication of stimulation of DNA repair synthesis.

Among compounds listed in class A (Table 1), radiations and alkylating agents produced a significant increase of  $T_{\text{HU}}/C_{\text{HU}}$ . With the remaining compounds, a negative result was found. It will be noted that the latter compounds, when tested, also failed to induce UDS in the autoradiographic assay. Non-mutagenic substances (class B) were all negative with both assays. Among the compounds requiring metabolic activation, only cyclophosphamide gave a positive result.

On the assumption that the inhibition of replicative synthesis by HU is the same in control and treated cultures, also the increase of the ratio  $T_{\text{HU}}/T$  in comparison with  $C_{\text{HU}}/C$  has been taken as an index of the induction of DNA repair synthesis. It is reported in Table 1 as relative increase of DNA radioactivity in the presence of HU.

As shown in the table, a relative increase was found with most mutagenic substances but with none of the non-mutagenic compounds. One of the agents which showed only a relative increase was (activated) DMN; this has been described by other Authors as a repair-inducing agent. For this reason, DMN, as well as mitomycin C, a known mutagen which failed to give an absolute increase in our test, were investigated more extensively.

Several experiments on DMN and MMC were performed during a stay of the project leader at the Medical Biological Laboratory of TNO, Rijswijk, the Netherlands. EUE, HeLa and AH, a strain of human diploid fibroblasts, were used with both scintillometric and autoradiographic assays. Metabolic activation for DMN was provided by S9 fractions from arochlor-induced rats, S9 fractions from PB-induced mice and pure microsomes from PB-induced mice. Two different batches of DMN were tested. All experiments gave negative results. MMS was inactive also when tested by the isopycnic centrifugation analysis, although 4-nitroquinoline-1-oxide, used as a positive control, gave the expected positive result.

We are tentatively concluding that agents failing to induce both an absolute and a relative increase in the scintillometric assay, can safely be classified as negatives. Compounds producing only a relative increase should be considered with some caution, since (1) most of them (acridine orange, hydroxylamine, mitomycin c, activated 2-naphtylamine and DMN) are mutagenic in many test systems and (2) some of them (DMN) have been found active even in DNA repair tests by others. Finally, compounds producing an absolute increase should be regarded as repair synthesis inducers.

### Conclusions

Scintillometric detection of  $^3\text{H}$ -TdR incorporation in hydroxyurea-inhibited human cells is a rapid and simple test for detecting DNA repair synthesis. On the basis of our results, there is no reason to assume the information given by this test to be qualitatively different from that provided by the classi-

cal autoradiographic assay. With both methods, a number of known mutagens escape classification as DNA damaging agents. Therefore, these methods can be safely used only in association with other complementary tests.

#### Publications

Bianchi, V. et al., Scintillometric detection of DNA repair synthesis: A critical appraisal (in preparation).

Abbondandolo, A. et al., Molecular dosimetry of alkylating agents. Problems encountered in relating DNA damage (single strand breaks) to cell survival (in preparation).

TABLE 1. SUMMARY OF DATA OBTAINED WITH 26 PHYSICAL AND CHEMICAL AGENTS

AGENT (1)	DOSE RANGE	% RESIDUAL DNA SYNTHESIS (T/C)	MAX. ABSOLUTE INCREASE OF DNA RADIOACTIVITY ( $T_{HU}/C_{HU}$ )	MAX. RELATIVE INCREASE OF DNA RADIOACTIVITY ( $T_{HU}/T$ $(C_{HU}/C)$ )	UDS
<b>Class A</b>					
X-rays	0.2-50 KR	12.7	4.3*	35.6	+
UV light	2.5-20 J.m <sup>-2</sup>	29.6	4.2*	14.6	+
EMS	20-100 mM	11.8	3.5*	29.2	+
MMS	0.5-2 mM	59.7	4.1*	6.8	+
MNNG	1-100 uM	13.5	6.6*	48.7	+
Styrene oxide	4.4 mM	21.3	2.0**	9.4	ND
Acridine orange	0.1-10 uM	14.4	1	4.8	-
Hycanthone	0.01-1 mM	25.0	1	3.2	-
Hydroxylamine	0.1-10 mM	85.1	1	1	NT
MMC	0.5-10 uM	42.6	1	2.4	-
Griseofulvin	0.07-0.28 mM	100	1	1.8	ND
<b>Class B</b>					
Praziquantel	0.5-5 mM	85.0	1	1	-
Praziquantel+M	0.5-5 mM	100	1	1	ND
PABA	0.1-10 mM	100	1	1	NT
DBT	0.1-10 mM	100	1	1	-

MSDS	0.1-10 mM	100	1		4.8	NT
Phthocol+M	1-4 mM	24.6	1			
<u>Class C</u>						
CFA	0.01-0.1 mM	100	1		1	NT
CFA+M	0.01-0.1 mM	81.5	2.2**		2.8	NT
2-naphthylamine	5-20 mM	57.5	1		1	-
2-naphthylamine+M	5-20 mM	71.3	1		3.3	-
DMN	2-400 mM	26.2	1		1	-
DMN+M	100-400 mM	19.7	1		4.3	-
Styrene+M	14-28 mM	59.6	1		1	INT
<u>Class D</u>						
5-BUdR	0.1-1 mM	31.5	1		1	-
5-FUdR	1-5 mM	9.1	1		1	NT

(1) Abbreviations: EMS, ethyl methanesulfonate; MMS, methyl methanesulfonate; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; MMC, mitomycin c; PABA, para-aminobenzoic acid; DBT, dibromotyrosine; MSDS, menadiol-sodium disulfate; CFA, cyclophosphamide; DMN, dimethyl-nitrosoamine; 5-BUdR, 5-bromo deoxyuridine; 5-FUdR, 5-Fluoro deoxyuridine; M, micro-somes.

Contractor : ICI BRIXHAM LABORATORY

Contract No : 180-77-1 ENV UK

Project Leader : C R PEARSON

Title of project : Contribution to the ECDIN Pilot Study. Elaboration  
and testing of a draft format for data on ecological  
fate and effects

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### 1. Objective

The objective of the work was to provide support for the ECDIN group at JRC, by

- (a) devising formats for certain types of data on ecological fate and effects in the freshwater aquatic environment
- (b) testing these formats by trial inputs of data on a selected range of chemicals.

### 2. Materials and Methods

Draft formats and the trial data sets using these formats, were submitted to JRC as typescript tables.

An essential part of the preparation of draft formats was a series of meetings between the contractors who were working together on a related group of contracts.

### 3. Results

#### 3.1 Effects on aquatic organisms

The work was shared between Brixham, who handled freshwater, and the Marine Biological Association, Plymouth, who covered saline conditions. A trial format was agreed and Brixham submitted tabulated data on the first 40 compounds in alphabetical order on the ECDIN list of 100 (Refs 1 and 2); at this stage PCB, DDT and metals were excluded.

The basis of the format was to display a quantitative dose response relationship with details of test conditions as appropriate. Sufficient data were found to be available to show that the format was basically acceptable. A revised and updated version, with minor modifications, was prepared.

In order to test the use of the format for compounds of current interest, about which it was known the literature was large, tables

were prepared for Aldrin, Dieldrin, Methoxychlor, Chlordane, Parathion (Ref 4).

### 3.2 System requirements

Early work had shown that programming arrangements would be needed to cover listing of references, glossaries of terms, nomenclature of isomers, and hierarchies of biological nomenclature, if any form of regular updating was needed. Provisional agreements on actions on all these were arrived at.

### 3.3 Bioaccumulation, metabolism, excretion

Early discussions revealed it would not be possible to prepare a single tabular format for all these elements ; 2 trial formats were agreed for Bioaccumulation, and for Metabolism/Elimination.

Tables prepared for the 40 compounds covered in Section 2 above showed that there was so little data available that no test of the formats was possible ; it was therefore decided to proceed to the 5 compounds known to have data (Aldrin, etc) and a tabulation of data was presented (Ref 4).

### 3.4 Abiotic transformations

There is very little information in the literature at present, but the number of relevant papers is growing. Some approaches to format are discussed in the body of the final report.

### 3.5 Sewage treatment and biodegradation

The nature of the literature is such that most work on biodegradation of industrial chemicals has been carried out in relation to behaviour in biological sewage treatment ; the normal criterion of toxicity of a compound is suppression of biodegradation. Some studies of single species cultures have been reported in which the effect criterion is suppression of growth ; there is also common ground with mutagenesis testing, which uses the same, or closely related, species, as does work on aspects of sewage microbiology.

A trial of a single tabular format, which included all the factors described above, was carried out on the first 18 compounds in the ECDIN list ; enough data were available to draw reasonable conclusions. (Ref 3)

All references to direct effects on individual micro-organism species were transferred to the Effects on Aquatic Organisms tables ;

and references to mutagenicity testing have been removed, without loss of relevant data.

A modified format, with a glossary of terms, is presented and discussed in the final report. This format is compatible with proposals made for the soil environment by Institut für Bodenbiologie.

4. List of Reports Submitted

1. Brixham Laboratory Report No BL/B/1919
2. Brixham Laboratory Report No BL/B/1926
3. Draft Report - March 1980
4. Brixham Laboratory Report - Effects of and Accumulation/Metabolism/  
Elimination of 5 chemicals. To be issued.
5. Brixham Laboratory Report - Effects of, and Accumulation/  
Metabolism/Elimination of, 40 chemicals. To be issued.
6. Final report and Discussion. To be issued.



Contractor : Natural Environment Research Council - Marine  
Biological Association of the United Kingdom,  
Citadel Hill, Plymouth PL1 2PB, Devon

Contract n° : 181-77-1 ENV UK

Project Leader : Allen Varley

Title of project : Contribution to the ECDIN pilot study - Elaboration  
and testing of a draft format for ecological data,  
and collection of data on fate and effects of  
chemicals in the marine and estuarine environment.

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#### Objectives of the research

The work carried out was a continuation and extension of that undertaken and initiated during the previous contract period (January 1977 to December 1978). It was concerned with the collection and presentation of information and data on the fate and effects of chemicals in seas and estuaries, and it consisted mainly of developing and testing formats, with the emphasis on dispersion and transformation, and collecting and tabulating information and data from the literature, which was structured and submitted to the Commission for computer processing.

#### Materials and methods

The library of the Marine Biological Association of the United Kingdom (MBA) and its Marine Pollution Information Centre were used to access the scientific and technical literature. The MBA holds one of the most comprehensive collections in the world of literature on marine and estuarine pollution, and staff have built up special indexes and developed expertise and methodologies aimed at facilitating the work of reviewing and analysing the literature pertinent to ECDIN's requirements. A new indexing system, to retrieve information on specific compounds and to assist in updating, was put into operation, and continuing efforts were made to achieve comprehensive coverage of the literature, with the Library and Marine Pollution Information Centre aiming to collect and index all relevant scientific and technical publications on marine and estuarine pollution.

### Results

The tables for data on effects on aquatic organisms (ECDIN Category 9) were agreed to be satisfactory for the present, with Effects on Ecosystems remaining in free text, but probably requiring further consideration. Data which had been supplied in the earlier pilot versions of the tables was reformatted.

Formats for the presentation of data on dispersion and transformation (ECDIN Category 8) continued to cause difficulties, but by the end of the contract period the problems had been resolved and the following tables were accepted as satisfactory:

- Dispersion Pathways (free text)
- Abiotic Degradation
- Biodegradation
- Transformation of Elements
- Biological Accumulation
- Metabolism and Elimination

During a major study on Cadmium which was undertaken as a separate contract, the large amount of data highlighted problems, particularly in connection with biological accumulation, which apply to other metals and persistent chemicals. A succession of formats were tested and it was not until late 1980 that an acceptable Biological Accumulation table was agreed. Testing of the Abiotic Degradation and Biodegradation tables continued, and the additional table which had been devised and introduced for Transformation of Elements was further tested and used successfully. Minor alterations were made to the Metabolism and Elimination table in order to accommodate information on translocation within organisms. Although new and additional information may make it desirable to introduce new tables, strong justification and careful testing is necessary, bearing in mind the need to stabilise formats so that computerisation may proceed unhindered.

Data collected by exhaustive searches of the literature was provided on the following :

- Chlordane
- Dodecylbenzene Sulphonic Acid, Sodium Salt
- Endosulfan
- Endrin
- Heptachlor
- Heptachlor Epoxide
- Hexachlorobutadiene

Pentachlorophenol  
Sodium Pentachlorophenatè  
Trichlorophenol  
Chlorinated Paraffins  
Searched as a group, with data provided on 15.  
Chlorinated Benzenes  
Searched as a group, with data provided on 11.  
Organophosphates  
Methyl Parathion  
Parathion  
193 compounds searched as a group, with data  
provided on 72.

The "group" approach introduced in 1980 was successful, and proved to be an economic and logical way to assemble and handle data. During the study on organophosphates which was undertaken as a separate project, the number and variety of alternative names (over 1400) caused some difficulties.

A process of continuous updating was developed, with new data on all compounds which had been dealt with earlier being formatted and despatched regularly to the Commission and to the Joint Research Centre, Ispra. As the number of compounds covered increased, there was an inevitable and corresponding increase in the amount of time devoted to updating. The ECDIN database was accessed periodically by Mr. D.S. Moulder, both through a direct computer link between Plymouth and Ispra, and via Euronet DIANE. Technical problems caused some frustration and limited the amount of time that could be spent on testing the database. Data extraction was carried out mainly by Dr. A. Louise Cowie, who also developed and tested the formats in cooperation with Miss Linda Noble, and undertook related work.

Occasional working meetings were held with ECDIN staff from the Joint Research Centre, Ispra, and these proved extremely beneficial. Contact was maintained with the other contractors and with UK government departments, and in 1980 there was substantial cooperation with staff of the Freshwater Biological Association, Windermere. The ready availability of MBA scientific research staff, particularly the chemists and biochemists, for discussion and advice, continued to be of great value.

#### Conclusions

A number of problems, both minor and major, require further examination.

These include: database requirements if the eventual possibilities of graphical display of data by the computer are to be considered; data obtained by measuring from graphs; the indication of statistical significance; treatment of field and laboratory data concerning dispersion and transformation within, and effects on, complex systems, e.g. microcosms, communities, ecosystems; the handling of data on some enzyme and metallothionein studies; the need to improve the presentation of information on Dispersion Pathways and to avoid undesirable repetition; mixtures of compounds; the standardisation of terminology; taxonomic hierarchies and relationships.

Many outstanding questions are related to data "quality" and data "selection", and these areas require detailed consideration and discussion.

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**CONTRACTOR:** UKAEA,  
Hazardous Materials Service,  
Harwell Laboratory,  
Harwell, Didcot,  
Oxfordshire,  
England

**CONTRACT NO:** 296-77-3 ENV UK

**PROJECT LEADER:** E.E. Finnecy

**TITLE OF PROJECT:** Contribution to the ECDIN pilot project - Elaboration and testing of a draft format for data on waste disposal.

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### Objective of Research

After some changes in the original specification, the final objective of the contract, as agreed with JRC Ispra, was to produce an input format that would allow for the entry of data relating to waste production, composition, treatment or disposal into the ECDIN system.

As a part of early work during the time that ECDIN was organised to use the SIMAS data management system, a tentative list of priority chemicals was produced.

In the original specification for this contract we were asked to identify those parameters of a waste that critically differentiate it from another, superficially similar waste. This was an important task so long as the SIMAS data management system was to be used. The change to ADABAS coupled with the proposed method of data input render this distinction unnecessary. We believe this to be a satisfactory outcome since, in our opinion, only the enquirer can ultimately decide when, for his specific purposes, one waste is different from another. The proposed input format gives him the ability to set his own boundaries.

### 2 Materials and Methods

The project was progressed by a series of meetings with staff from JRC Ispra and Scicon Consultants Ltd. The intellectual content of the work is original though note has been taken of our experiences - and those of others, in preparing information and data on wastes for computer processing. The nature of our everyday work has given us insights into what form this information typically takes and what sort of questions are asked of such data bases by potential users. In this we drew on our experience of doing similar - but crucially different - tasks for the UK

Department of the Environment and Cheshire County Council:

A particularly difficult aspect of this task has resulted from the delays in converting ECDIN from SIMAS to ADABAS and the resultant changes in the nature of the information on waste that needed to be processed. The whole original concept of this part of ECDIN had to be recast once the change to ADABAS had been made.

### 3 Results

As originally conceived the method of entry of information on waste, in which waste management was a sub-field in the Production and Use category, was seen to be inadequate, since major arisings of wastes containing chemicals of environmental significance would not be included where they arise from the production of materials that are not pure chemicals. This problem was eventually resolved after the change to the ADABAS data management system.

It was originally intended that ECDIN should include information on the waste produced at each stage of a production process. This was eventually rejected as leading to an unacceptable complexity in the input format structure - and information at this level would rarely be available. It was concluded that waste was a material that left the factory gate for treatment (including recycling) or disposal. Where information was available on the particular stage of a process that produced the waste it would be identified. The problem of waste treatment within a factory either prior to or as disposal was resolved thus:

- 1 where waste from only one process was treated in plant attached to the process, the waste treatment would be classed as a part of the process and only the products of treatment would be candidates for ECDIN entry.
- 2 where a treatment plant received wastes from several processes, it would be considered as a separate factory and its output wastes would be entered.

We conclude that, in the context of waste management, there is no rational definition of the term 'Environmental Chemical'. All materials, including wastes, are 'chemical' and all materials, in appropriate circumstances can have a deleterious environmental impact. In assessing the potential environmental impact of a waste material, chemical composition is important, but so are many other parameters. We therefore conclude that ECDIN must be capable of storing information on all wastes.

This has some implications for Category 1 of ECDIN. Many constituents of wastes will not be candidates for entry into Category 1 or for having ECDIN numbers assigned to them. To allow for retrieval of information on materials in the 'No ECDIN Number' Class we have devised two additional code lists for materials we have defined as Non-Specific Chemical Entities (NSCE) and Non-Chemical Components (NCC).

SCEs are specific, named chemical materials - those materials that will have Chemical Abstracts System (CAS) numbers and to which ECDIN Numbers are assigned, We recommend the expansion of this group, and the assignment of ECDIN Numbers, to include ions of defined SCEs eg sulphate, fluoroacetate etc.

NSCEs are 'chemical' descriptors but are not SCEs. This class includes:

- groups of SCEs eg hydrocarbons, 'heavy' metals etc
- a group consisting of minerals or mineral-like materials
- a group consisting of manufactured chemical products described in terms of their use, eg oils, pesticides etc
- a group consisting of extensive chemical properties that are not substance specific, eg BOD, suspended solids etc
- a group which contains data relating to elemental composition

NCCs are the rest of the materials that may be found in waste and consist of materials that are not described in chemical terms, eg vegetable matter, manufactured goods eg electroplated ware, batteries, paper, timber.

Waste is describable in several ways:

- by reference to the process producing it
- by reference to the name given to the waste by the waste producer
- by reference to its composition in terms of SCEs, NSCEs and NCCs.

Similarly the origin of the waste can be described in four ways:

- 1 - by reference to the product whose production gives rise to the waste
- 2 - by reference to the process whose use gives rise to the waste
- 3 - by identifying the geographical origin of the waste
- 4 - by reference to the class of activity carried on at the place producing the waste.

Descriptors 1,2 and 4 can be related to the NACE-NIPRO codes published by the Statistical Office of the EEC and by the International Standard

Industrial Classification (ISIC) of the United Nations, and the National Standard Industrial Classification used in the country where the waste arises. We note that, at the present time, NACE and NIPRO have disadvantages for the present purposes that render their use for the present purpose rather unsatisfactory. NACE, as yet, has a very variable level of detail and NIPRO only applies to manufacturing industry and does not cover primary raw material acquisition, construction or service industries. For this reason we have also recommended the use of the ISIC.

Sundry other items of information that are potentially valuable to an ECDIN user include:

- the date of the information
- the date of entry into ECDIN
- the source of the information
- explanatory comments.

In addition we believe it to be valuable to indicate the nature of the data entry. We have identified 5 different types of data:

- 1 data that refers to point source arisings
- 2 data relating to summarised data from more than one source
- 3 forecast data relating to point source arisings
- 4 forecast data relating to multiple source arisings
- 5 data relating to notional or 'typical' wastes that may or may not actually exist.

The final draft format arrived at contains fields for the following data:

- 1 A unique identifier for each entry
- 2 A code identifying the type of information contained in the entry
- 3 A code identifying the degree of confidentiality of the entry
- 4 A description of the waste by its origin
- 5 A description of the waste using the producers own terms
- 6 A description of the waste in chemical terms
- 7 A description of the waste in non-chemical terms
- 8 Information on the detailed composition of the waste
- 9 Information on up to 14 physical and chemical properties of the waste
- 10 The quality of waste expressed in time units and production units
- 11 The identity of the waste producer in terms of his name and address, geographical location and standard industrial classific-



ation

- 12 The origin of waste in terms of the specific process that produced it and the stage in the process at which it arose. This field provides a link to the ECDIN Processes file in some cases
- 13 The nature of any pre-treatment that has been given to the waste prior to its disposal
- 14 The means of transport used for the waste and its destination
- 15 The form of treatment given to the waste at its destination
- 16 The method of ultimate disposal used for the waste
- 17 Information relating to the dates of the data and its sources and a General Comments field.

4 Conclusions and Comments

1 It is important to consider what constitutes the minimum amount of data acceptable for ECDIN input. We have concluded that this is rather difficult to determine but have proposed the following criterion:

"No data is entered in ECDIN that, in effect, merely states that wastes arise because people live, or work, somewhere, or where the data would be self-evident to the intelligent, informed layman with an interest in environmental matters."

2 We have adopted the philosophy that the eventual ECDIN Management team will not be well placed to decide that certain available data about wastes is of no interest - and will never be of interest. Therefore we have constructed a format that, we believe, will be able to contain any information about waste that is likely to be available. If an enquirer requires less detail the hierarchical structure of the format is such that he can construct his search so that only less detailed data is produced. It is, however, impossible to recreate detailed data that has not been entered without recourse to the original data. Few ECDIN enquirers will, we suggest, have ready access to this material.

3 It may be said that the proposed format is over complex, Our experience suggests that this is not so, and that complex structures and code lists, properly arranged, make the work of data input faster, more reproducible and improve the value of retrieved data. We believe that close study of the worked examples that are included in the Final Report will show that the apparently complex system devised will enable trained people to prepare data for input comparatively easily. This is achieved by reducing to a minimum the number of occasions on which the

data processer is required to exercise judgement.

4 We conclude that maximum useful value will only be achieved from the Waste Management Category of ECDIN if it can have access to the statutory notifications of waste production required in several Member States of the European Community, along with the detailed results of surveys of waste production carried out by public authorities in the performance of their duties. If ECDIN must rely on data in the openly available, scientific, professional and technical literature then the data base will be very incomplete and extremely costly in the time necessary to prepare the data for input. It will also provide answers, to enquirers, that will often be difficult to interpret.

5 We recognise that the proposed format has not been extensively tested and we envisage that it will undergo dynamic modification over the next few years. We do believe that the structure is sound - but that the detail of the dictionaries included needs to be extensively tested.

6 We suggest that information on wastes is different in nature from that in the other categories of ECDIN and does not fit well into the ECDIN System as we understand it. We believe the proposed EWADAT System about to be investigated by the Commission of the European Communities represents, potentially, a more convenient place for this data.

#### 5 References

No particular source has proved outstandingly important though many have been of value.

#### 6 Reports and Meetings

Periodic Report No 1 December 1977

Periodic Report No 2 May 1978

Periodic Report No 3 November 1978

Meeting with Staff from JRC Ispra November 1977 (at Harwell)

Meeting with Staff from JRC Ispra May 1978 (at Ispra)

Meeting with Staff from JRC Ispra February 1979 (at Ispra)

Progress Report submitted to Ispra March 1979

Meeting with Staff from JRC Ispra + Scicon Consultants April 1979  
(at Harwell)

Meeting with Scicon Consultants September 1979 (at Harwell)

Progress Meeting with Staff from JRC Ispra May 1980 (at Ispra)

Meeting with Staff from JRC Ispra July 1980 (at Harwell).

Contractor: Natural Environment Research Council  
Freshwater Biological Association  
(Windermere Laboratory), U.K.

Contract no.: ENV/457 UK

Project Leader: John E.M. Horne

Title of Project: Contribution to the ECDIN pilot study - Elaboration  
and testing of a format for ecological data, and  
collection of data on fate and effects of chemicals  
in the freshwater environment

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#### Objectives of the research

The objectives of this research were two-fold.

1. Formats already in use for the presentation of ecological data on chemicals in the marine environment were to be modified and tested for the freshwater environment. New formats were to be designed where necessary.
2. The published literature was to be searched for information on the fate and effects of certain selected chemicals in the freshwater environment, and data were to be extracted and presented in forms suitable for computer processing by the Commission.

#### Materials and methods.

The work was done by the library of the Freshwater Biological Association at Windermere. This is the main freshwater library in Britain, and has a wide coverage of the literature of inland waters. Library staff also call on the expertise of research scientists in the fields of biology, physics and chemistry for assistance and advice. As well as the 700 serial titles received by the library, the wider field of scientific literature, the wider field of scientific literature is covered through current-awareness and abstracting journals and on-line information retrieval services, and copies of relevant papers are readily obtained from the British Library Lending Division.

The formats designed by the Marine Biological Association of the U.K. were taken as a starting point and modified in the light of experience. It was agreed that data should be collected on a list of six pesticides (aldrin, endrin, heptachlor, heptachlor epoxide, chlordane, parathion) and seven other compounds (aniline, anthracene, benzene, carbon tetrachloride, dichloromethyl ether, ethanol, trichlorophenol). Work on the project started in June 1980. A basic literature search on the listed compounds was carried out, and comprehensive searching and data extraction were commenced for aldrin, endrin, carbon tetrachloride and dichloromethyl ether. Advice and cooperation were received from the Marine Biological Association.

### Results

It was found that the formats used by the Marine Biological Association could in general be applied to freshwater data, with the substitution of a "hardness (CaCO<sub>3</sub>)" column for the "salinity" column. However, papers dealing with field trials of pesticides often yielded data which could not be fitted to existing formats. A new format dealing with the environmental partitioning of residues was devised, and this is currently being tested.

Over 600 references to aldrin and endrin were identified, and data extraction is continuing. About 70 references to carbon tetrachloride were identified and only 3 to dichloromethyl ether. Data extraction and compilation for these two chemicals were virtually complete by the end of 1980.

### Conclusions

The marine formats designed by MBA can, with minor modification, be used for freshwater data, but a number of new formats may be needed. The literature on chemicals in the freshwater environment is widely scattered and considerable searching effort is required. For example, out of about 70 references found to carbon tetrachloride, 24 proved relevant; these came from 17 different source journals, from 8 countries, and yielded 193 lines of data. For many pesticides the scatter is much greater.



**RESEARCH AREA 3 : REDUCTION AND PREVENTION OF POLLUTION AND NUISANCES**



**TOPIC 31 : WATER POLLUTION ABATEMENT**



Contractor : Centre des Sciences de l'Environnement  
Université de Metz - 1, rue des Récollets  
57000 METZ - FRANCE

Contract n° ENV/429 F

Project leader : BLOCK J.-C., Professeur in collaboration with  
M. FLORENTZ and M.A. DOLLARD

Title of project : (1) Phosphate removal by biological treatment of  
waste water.

(2) Limiting effect of suspended matter on disinfection.

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### I - Phosphate removal by biological treatment of waste water

Removal of phosphates from wastewater is important in order to protect the lakes and other surface waters from eutrophisation. Physical and Chemical treatment of wastewaters leads to a good removal of phosphorus but increases the volume of obtained sludge. Moreover one needs to add a module to a pre-existent system. Then a integrated and compact biological process keeping carbone, nitrogen and phosphorus would be appreciable. Different processes have been proposed so-called : "Phos-strip" (Levin et al, 1972), "Contact-stabilisation" (Jones, 1973), "Bardenpho" (Barnard, 1973) and "Phoredox" (Osborn and Nicholls, 1978). In much of those systems bacteria would stored phosphorus into the cells under the effect of a stress (as oxygen depletion) or after selection. It seems, within the treatment, that minimum phosphorus bacterial cell quota was increased dramatically and could be described by the term of luxury uptake (Brar and Tollfson, 1975 ; Mulbarger et al, 1974). In fact numerous parameters governing phosphate removal are unknown : what is the effect of carbon flux, temperature nitrogen concentration, anaerobic phase... or is exact, biochemically speaking, the term of the so-called luxury uptake. Then our research was conducted in three ways.

- 1) Isolation of different types of bacteria able or no to store phosphates
- 2) Study of the effect of successive phases of aeration and oxygen starvation (in pilot plant with pure culture or activated sludges)
- 3) Measure of the metabolic potential of selected bacteria or activated sludge against phosphorus uptake

This intermediary report gives preliminary results on the phases 1 and 2 of the contract.

Used bacteria were choosed after bibliographic study (for example Acinetobacter lwoffii) or selected from lagooned industrial wastewaters.

Growth study were conducted with a biophotometer of with chemostates. Two phosphate uptake mediums were used (a) diluted beef extract medium (initial concentration of phosphate # 14 mg/l) (b) Fuhs and Chen broth (Fuhs and Chen, 1975) with a high level of phosphate (# 400 mg/l).

Dissolved oxygen, pH, phosphate concentrations were regularly evaluated.

Six bacterial strains were isolated from the lagooned waste waters :

Pseudomonas mallei, Pseudomonas mendocina, Pseudomonas facilis, Kurthia sp, Arthrobacter sp and Bacillus sp.

Assays of growth in diluted beef-extract showed that after a 40 h-culture only Pseudomonas facilis and Acinetobacter lwoffii (used as a control) was able to store high quantities of phosphates (see Table 1). Surprisingly we did not observe, as Harold (1966), that phosphate accumulation in bacteria was promoted by growth limitation. In opposite (see Figure 1) significant phosphate uptake occurs with the exponential phase of growth.

Successive aerobic-anaerobic phases have been applied onto Acinetobacter lwoffii cultures in batch. Results presented on figure 2 shown that during anaerobic stage phosphate release occurs whereas in presence of oxygen phosphate removal is really effective. In comparison with a control experimentation one can demonstrated taht the anaerobic phase does not allow a better phosphate removal in the following aerobic phase but simply delays the bacterial storage. In consequence and as observed with Pseudomonas and activated sludges the anaerobic stage does not appear as a prerequisite stress for biological removal of phosphorus.

## II - Limiting effect of suspended matter on disinfection

Current water and wastewater treatment practices do not effect complete removal of microorganisms. The possibility that bacterial and viral pathogens associated with solids may be protected from disinfection during treatment and hence remain viable is a question of considerable importance. The extent of protection given to microorganisms by encasement, adsorption or competitive oxidation of turbidity causing materials, depends on a number of factors.

These include the relative innate disinfection resistance of the microorganisms, the type of particles causing turbidity, the size of the microorganism in question relative to particulate size... and so on. Most of these parameters have been evaluated for viral protection (Boardman and Sproul, 1977 ; Hejkal et al, 1979 ; Stagg et al, 1977). but more rarely for bacterial

protection (Carlson et al, 1975 Walsh et al, 1980) An overall view of this question has been discussed by Sproul (1979)

The principal objective of our investigation was to develop information on the relative resistance to chlorine of solid-associated bacteria and to know the relation between the size of these solid-associated bacteria and the inefficacy of the chlorination

The bacteria used for this study were indigenous total bacteria recovered from raw and secondary wastewater Total bacteria in untreated and chlorinated wastewater were isolated from on nutritive agar plate at 44°C The 1 l-wastewater samples were filtrated through different filters 364  $\mu\text{m}$ , 68  $\mu\text{m}$ , 35  $\mu\text{m}$ , 12  $\mu\text{m}$ , 0.45  $\mu\text{m}$  Total bacteria and suspended matter were evaluated in each filtrats and results expressed as the quantity of bacteria or suspended matter retained by the filters Results are given <sup>3</sup> in the figures 3 and 4

In raw wastewaters most of the suspended matter are in the size of 12  $\mu\text{m}$  to 35  $\mu\text{m}$  while in secondary wastewater the maxima of solids are found in the range of 68  $\mu\text{m}$  to 364  $\mu\text{m}$  In parallel solids associated-<sup>x</sup> bacteria are in higher number in the range of 12  $\mu\text{m}$  to 35  $\mu\text{m}$  whatever the origin of the wastewater (raw or secondary)

This intermediary paper does not report assays of chlorination on these different fractions They will be given in the final report

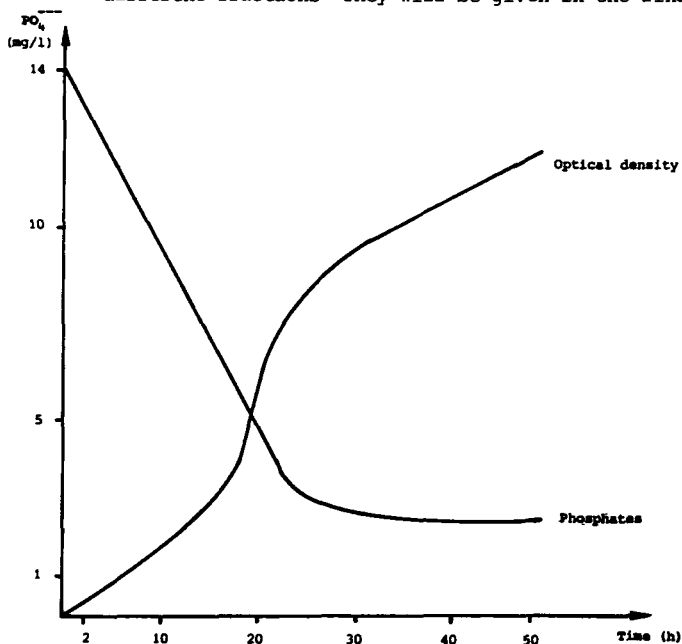


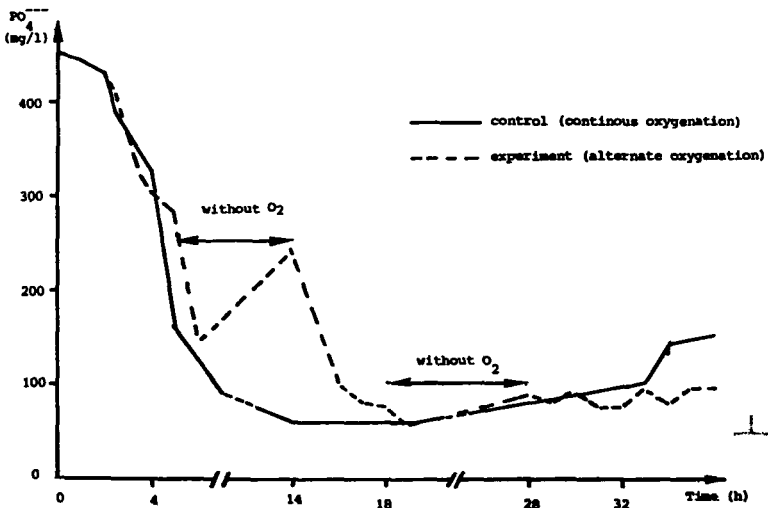
Figure 1 Relationship between the growth of Pseudomonas facilis and phosphates Removal (growth in diluted beef extract at 25°C)

**Table 1** Phosphate uptake by several strains of bacteria isolated from lagooned industrial wastewater  
(Growth for 40 h in diluted beef-extract ;  
 $PO_4^{---} = 14 \text{ mg/l}$  pH = 7.0 temperature = 25°C)

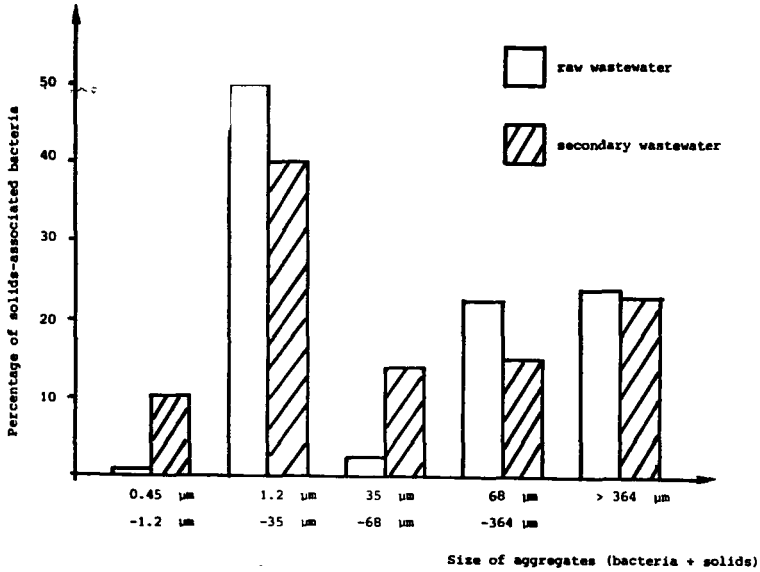
Isolated Bacteria	residual Phosphates (mg/l)	pH after growth
<u>Ps mallei</u>	11.6	6.4
<u>Ps mendocina</u>	7.6	7.45
<u>Ps facilis</u>	3	7.75
<u>Kurthia</u>	8.7	7.35
<u>Arthrobacter sp</u>	10.7	6.25
<u>Bacillus sp</u>	7.3	7.0

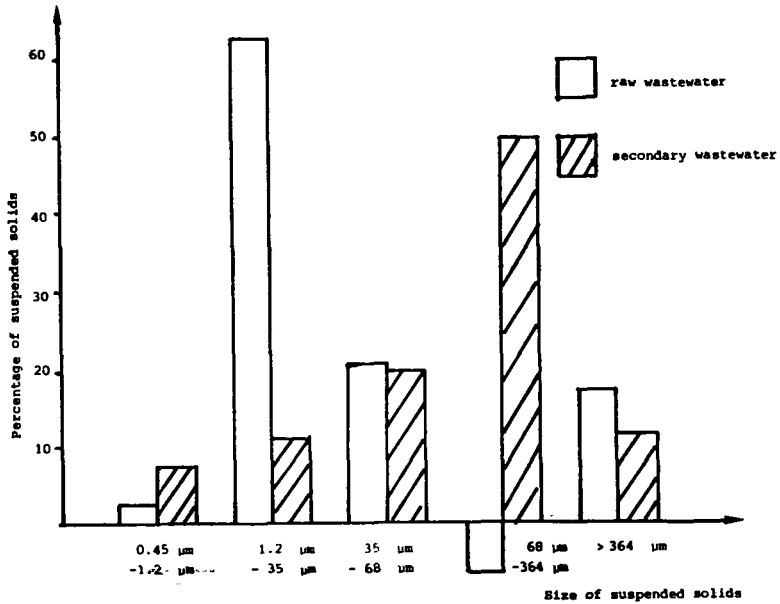
Control Bacteria	residual Phosphates (mg/l)	pH after growth
<u>E coli</u>	6.4	7.95
<u>A lwoffii</u>	2.5	7.95



**Figure 2** Phosphate removal during Acinetobacter lwoffii growth in Fuhs and Chen' medium at 37°C



**Figure 3 :** Percentage of suspended solids according to their size



**Figure 4 :** Percentage of solids-associated bacteria according to the size of the aggregates in raw and secondary wastewater.

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Contractant: INSTITUT BOUISSON BERTRAND

Contract N°: ENV-431 F

Project leaders: R. BAYLET, J. BONTOUX, R. BENAÏM

Title of the project: Conditions for the isolation or inactivation of bacteria and viruses in the flocculation process used for the purification of water

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#### Aim of the study

The aim of this work is to monitor simultaneously the development of physico-chemical and bacteriological features of water during a cleansing process based on flocculation.

Although several individual studies have examined the effect of flocculation on the physico-chemical or bacteriological features of water, no link has ever been established between these two types of data.

#### Equipment and method

##### 1. Preliminary studies

###### 1.1. Choice of water to be used

After preliminary studies of various types of water, it was decided to use an artificially-prepared effluent obtained by making a suspension of colloidal washed kaolin in tap water, and adding various strains of bacteria.

###### 1.2. Flocculation method

Flocculation took place in a jar-test type flocculator in which the velocity gradient was known as a function of the gradient for blade rotation.

##### 2. Experimental tests:

The tests focussed on two different aspects:

###### 2.1 Study of the distribution of germs depending on the quality of the floating substances and the floc after flocculation

The quality of the "floats" was monitored by measuring the following physical and chemical parameters: chemical oxygen demand (COD), AFNOR suspended solids, turbidity, pH and granulometric analysis. The floc is subjected to microscope examination and to a sludge cohesion test and given a Degrémont notation.

###### 2.2. Kinetic study of flocculation and its effects on the distribution of germs

Two strains of bacteria belonging to different families (Escherichia and Streptococcus) were added for this study.

The tests attempted to quantify the effect of the experimental conditions (pH, duration of rapid agitation, duration of slow agitation and applied velocity gradient) on the physico-chemical and bacteriological efficiency of flocculation.

For this purpose, the OD of the system being flocculated is continuously recorded by means of circulation tank; the bacteriological monitoring is achieved by taking isolated samples at typical stages of flocculation identified on the OD record.

To make things easier to understand, the various types of flocculation obtained have been classified according to the following three patterns:

Figure 1 represents the best flocculation efficiency obtained, Figure 2 represents an average flocculation efficiency and Figure 3 shows flocculation with a low efficiency. On these figures, zone (a) represents the duration of rapid agitation, zone (b) the duration of slow agitation and zone (c) the duration of the settling process.

### Results

1. In the case of flocculation with inadequate efficiency (reduction by about 80% in the various physico-chemical parameters tested), the results obtained for the study of the adsorption of microorganisms on the floc with specific physical and chemical features and formed under pre-determined conditions showed that the percentage of bacteria eliminated in the "floats" is also low at around 68%.

When flocculation has a higher physical and chemical purification efficiency, the percentage of the percentage of the bacteria eliminated in the "floats" is around 99%.

2. As regards the kinetics study, Figures 4,5 and 6 contain the findings of the tests in accordance with the three types of flocculation defined above.

The percentages for the elimination of the parameters controlled (behaviour of the strains of bacteria, optical density) are plotted on the y axis and the time on the x axis.

These three figures show that, in the first minutes of agitation, the number of germs detected drops and optical density increases.

Figures 4 and 5 representing flocculation with a high and medium efficiency show that the percentages eliminated during the settling process are very close for the strains of bacteria and optical density. But this phenomenon does not appear on Figure 6 which represents flocculation of inadequate efficiency.



### Conclusions

The findings obtained in the study of the kinetics of flocculation confirm those obtained during the first part of our work (3.1) when efficiency is high or medium; good efficiency at the physico-chemical level is accompanied by "floats" with a low germ content.

However, where the efficiency of flocculation is low, the kinetic study (conducted on a limited number of tests) does not confirm the previously obtained findings. More tests will have to be carried out if the conclusions are to be reliable.

### Development of work

The work is carried out along two main lines:

1. Study of actual effluent to check the findings obtained with simulated effluent
2. Study of the development of germs at floc level (isolation, destruction...).

Contractor : CENTRE TECHNIQUE du PAPIER  
B.P. 7110 38020 GRENOBLE Cedex (FRANCE)

Contrat : ENV/432 F

Project Leader : G. SAURET

Title of Project : Study of the biological pollution  
issued from the starch use in paper  
industry. How to eliminated it

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#### OBJECTIVE OF THE RESEARCH

Research of the ways, different of the biological treatments, to eliminate biological pollution resulting from the use of starch in paper industry.

The programme, composed of four stages, will examine

Biological pollution resulting from the different kinds of starch used in the paper industry.

- Direct biological pollution resulting from starch used as wet end additives.
- Indirect biological pollution issued from broke recycling (size press treatment) or waste paper recovery.
- Pollution reduction by a way different from the classical biological treatment.

At the present time, the first two stages and a part of the third one are achieved.

## MATERIALS and METHODS

### Materials

After taking an inventory of the starch products in current use in the paper industry, a representative sampling has been selected (starch and flour).

#### - Utilization as wet end additives

- . Cold water soluble products : anionic (ether/ester) cationic and non ionic.
- . Hot water soluble products : cationic.

#### - Surface treatment utilization (size press and coating)

- . Hot water solubles : oxidized hydrolysed (ester/phosphate) and enzyme converted.
- . Native cationic starch (after enzyme conversion or thermo-chemical treatment).

#### - Spraying utilization : Native starch

On the whole, fifteen samples industrially commercialized have been tested.

### Methods

#### Starch titration :

Starch used as an additive, is submitted to such chemical or biochemical degradations in the circuits of the paper machine, that it may be converted into sugars.

To take these phenomena into account, the following titration methods are developed :

Starch : TAPPI Standard method T 419 Sm70 (colorimetry)

Sugars : Phenol titration after hydrolysis with sulfuric acid

COD : AFNOR T 90 101 - Total oxidation of organic ma-

terials.

Simulation of the pollution in the laboratory

According to the methods used for hand sheet making, stock has to be about ten times more diluted than it is on industrial machines, and they lead to values of retention and drainage out of proportion with the practical reality.

For the purpose, the Centre Technique performed a hand sheet former with a lower dilution, which is able to simulate with a good concordance the industrial phenomena and to produce sheets suitable for physical tests.

Rising amounts of starch are introduced in a furnish for the printing/writing paper (fillers, size) and starch titrations are performed on the sheet and in the filtrates.

RESULTS

The industrial experiments show that some kinds of starch tend to decay and finally turn into sugars, and that makes starch titration and pollution treatment very difficult to achieve.

Research on size press starch degradation kinetics developed on industrial effluents allow to think that the origin of the degradation is biological but other tests must be performed in order to confirm this statement.

Taking into account a coefficient for correction, the C.T.P apparatus for hand sheet forming seems well adapted for the simulation of the phenomena which occur on industrial machines.

Specific pollution (BOD5 and COD) of starches used in paper industry is practically identical : no difference appears between wet end additives, size press and spraying products.

As far as retention is concerned, only non ionic starch is badly retained, the best retention being provided by phosphated starch.

Broke recycling containing starch used as wet end additives, cationic starch or phosphated starch does not generate pollution. The values of BOD5 and COD filtrates which correspond to a broke, incorporation of 40 %, are almost identical to those of the non recycled pulp.

The works referring to the fourth stage of the programme are in progress.

\*  
\* \*

ORAL COMMUNICATIONS :

- IRFIP - Papermakers East Syndicat - Nov. 1980
- Meeting in Centre Technique : Working Group with paper makers - Dec. 1980.

Contractor : Université de Clermont-Ferrand

Contract n°: ENV-433 F

Project Leader : Professeur D. BEYTOUT

Title of project : Recherches de moyens d'essai de l'activité virulicide de substances et procédés antiseptiques.

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By these experiments we aim at laying down rules for virus-denaturation-tests by antiseptics and disinfectants. In this task we must take 3 sorts of difficulties in account :

1°) THEORETICAL DIFFICULTIES

Viral suspensions are never quite dispersed so that they remains aggregates of viral particles which, yet, are counted as only single infectious units by titration procedures. Even if viral denaturation is a first order reaction, the log-titer-time curve of "surviving virus" will not be a straight line since the time of total destruction of a cluster of virions is size-de-pendant (1).

Multiplicity reactivation (M.R.) occurs, either when a receptive cell recieve 2 or more single particles (coincidence M.R.) or when it adsorbs an adhering group of virus particles (aggregates M.R.) (5).

Interference can be induced by inactivated as well as by viable viral particles : when inoculum contains many disinfectant-denatured particles this phenomenon perturbs titer evaluation ; this is related to the so called "Von Magnus phenomenon".

2°) OPERATIONAL DIFFICULTIES

It is not easy to stop suddenly the action of the antiseptic : the most suitable technic is a rapid dilution (with, if possible, addition of an antagonist material), and cooling.

The antiseptic, even diluted, is still present and can act on receptive cells ; we must check this residual activity on viability and ability of cell cultures to develop viral infection.

3°) STANDARDISATION DIFFICULTIES

Virus-cell couples will be chosen for antiseptic resistance of virus,

easiness of culture and accuracy of titration; assays must be reproducible : therefore stable strains of cells, easy to obtain, must be used. It would be of interest if assays could be the least expensive possible.

In assays with poliovirus type 2 (Sabín strain) and Vero cells previously published (3,4) :

- We have restricted our study to the early period of virus denaturation (50 % and 99 % denaturation) in order to reduce at the minimum the influence of C.M.R. and interference, so that log-titer -time curve could be considered as a straight line.

- We have codified rules for checking viability and virus receptivity of cells in the presence of residual antiseptic.

Now it seems to us of interest to use the couple coliphage-Escherichia coli in assays of disinfectants for several reasons :

- stability and availability of both virus and receptive cells.
- 3. - easiness and rapidity of titration methods.
- high efficacy of placking of the phages.
- absence of interference.
- 1. - cheapness of culture medium and material.
- morphological similarity of some strains of coliphage with animal viruses.

In addition, phage-contamination is a dangerous hazard in fermentation and biological industries so that disinfectant-assays on phages are important per se.

#### MATERIAL AND METHODS

Coliphages and E. coli used in our experimentation are

- T 7 - a double stranded DNA tailed phage — E. coli B
- MS<sub>2</sub> - a single stranded RNA cubic phage — E. coli Hfrh.
- $\Phi$  X 174 - a single stranded DNA cubic phage — E. coli ATCC 13706.

Phage preparation :

The ad hoc strain of E. coli, cultivated on TS broth, is inoculated with large amount of phages ; after three hours of incubation in agitated flask at 37°C, phage suspension is prepared by filtration through a 0,45  $\mu$  cellulose acetate filter. This raw preparation serves as the source of phages :

before storage at - 80°C it is titrated (for each strain of phage, we have obtained about  $10^9$  UFP/ml).

According to this high titer, we can dilute virus samples before assay to minimise the concentration of culture medium material.

Assay :

t = 0 : virus and definite concentration of antiseptic are mixed at room temperature.

t<sub>1</sub>, t<sub>2</sub>, t<sub>3</sub>... : a sample of mixture is 100 fold diluted in TS broth at 4°C, subsequent dilutions are performed and stored à 4°C before titration.

Titration :

E. coli is cultivated for 2 hours in TS broth in a 37°C incubator ; TS Agar Petri-dishes are appropriately dried in incubator during the same time.

We inoculate them by inundation with the 2 hours-cultures of E. coli.

After a few minutes of drying, we drop 0,025 ml of each dilution of samples t<sub>1</sub>, t<sub>2</sub>, t<sub>3</sub>... and check sample t<sub>0</sub>.

6 to 7 hours of 37°C incubation is a suitable time to observe plack formation and obtain a first approximation of titers : we can choose the dilution level of each sample whose precise titration will give maximal information.

Then, precisé titration is performed the next day by the same procedure, using only the chosen levels of dilution, with 8 replicas per level. Distribution of plack forming units is Poissonian : so we calculate titer either by exhaustive count of placks, or, in the case of large plack-forming viruses, by an appropriate method we have described elsewhere (2).

This procedure is very easy, sufficiently accurate and reproducible.

RESULTS

They are presented in table 1, where they are expressed by  $\log \frac{\text{titer at time } t}{\text{titer at time } 0} / t$  (in minutes).



1 - MS<sub>2</sub> and poliovirus 2 have similar high disinfectant resistance (the very low-pH resistance of the second is a characteristic property of enteroviruses) : their similar chemical composition and morphology may explain this analogy.

Φ X 174, more sensitive to chemical agents, is more thermo-resistant.

These two phage strains may be useful virus-tests to check activity of antiseptics and disinfectants. We note that neither these two phages nor poliovirus are inactivated by Tricresol, which is an usual disinfectant frequently recommended for stools or soil disinfection whereas it is not at all virucid.

T 7, very sensitive to every method of disinfection, is not a suitable virus for these assays.

2 - We have not expressed results by slope of log titer - time regression, or half or 99 % denaturation time, for the subsequent reasons :

With physical agents (temperature, pH), the log titer-time curve is linear, which may lead us to believe that virus denaturation is a single hit process which would permit us to use these expressions for an uniparametric law (fig. 1).

On the contrary with chemical disinfectants we have not observed linear but S shaped curves : denaturation velocity decreases during the time of assay. So we cannot characterize the denaturation law by a single parameter (fig. 2).

This difference is probably caused by presence of a high amount of organic compounds in the raw suspension of virus. Antiseptics and disinfectants reacting with these materials are partly and gradually neutralised and virus denaturation velocity slows down.

It seems necessary to make assays on purified virus suspension. So we have undertaken at present virus purification by gel filtration on sepharose and assays with purified viral suspension. Now it seems a bit premature to take these initial results into account.

ACTION OF VARIOUS AGENTS ON TITER OF RAW VIRUS SUSPENSION

(Log  $\frac{\text{titer at time } t}{\text{titer at time } 0}$  / time in minutes)

	VIRUS VIRUCID AGENT	POLIO II	OX 174	MS <sub>2</sub>	T <sub>7</sub>
PHYSICAL AGENTS	température 60°	- 4/5 m	- 1/10 m	- 2/5 m	<-4/5m
	70°		- 4/3 m		
	pH 3	0/60 m	- 1/60 m	-1,3/60m	<-4/5m
	3,5		0/20 m	0/20 m	
	11,5	0/60 m	0/60 m	0/60 m	<-4/5m
CHEMICAL AGENTS	Tricrésol 1 %	0/60 m	0/60 m	0/60 m	<-4/60m
	Glutaraldéhyde 0,07 %	-2/7m40	- 2/1 m	- 2/7 m	
	Bétadine 3 %	-2/25m	<-4/10m	- 2/8 m	
	Fréon 113 + (agitation)	0/60 m	0/60 m	0/60 m	0/60 m

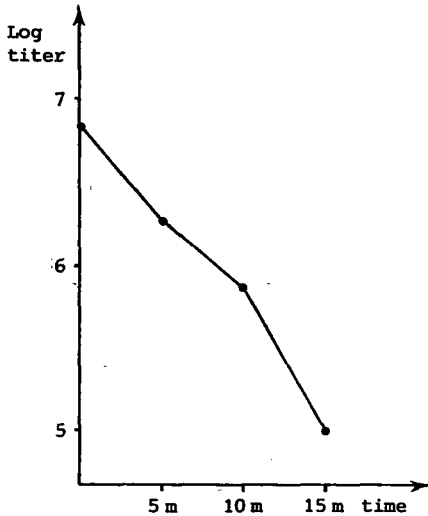


Figure 1

φ X 174 denaturation at 60°C

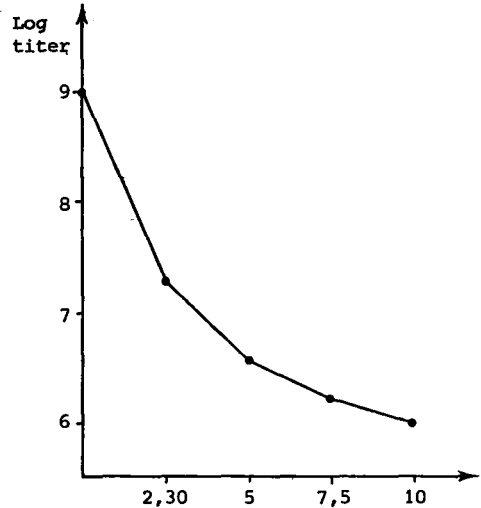


Figure 2

MS<sub>2</sub> denaturation by glutaraldéhyde Q07 %

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Contractor : Centro Sperimentale e di Ricerca per l'Industria Ceramica,  
Via Martelli, 26, I-40138 Bologna, Italy

Contract N° : ENV/437/I

Project leader : Professor Carlo Palmonari

Title of project : Problems of water pollution caused by ceramics industries.  
Disposal and possible reuse of the water used in processing  
and cleaning

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#### Objective of the research

Industrial ceramics processes for the manufacture of building products (particularly tiles, sanitary ware, etc.) involve the use of fairly large quantities of water, both for technological purposes (wetting the raw materials, wet-grinding, preparing suspensions of the raw material in water for the substrate and preparing enamels, motor cooling, etc.) and for washing all the various machinery.

Two different kinds of problem are involved: (i) water supply problems; quantities of water available are not unlimited, particularly in certain areas of Italy where there is an extremely high concentration of ceramics plants (e.g. the ceramics district of Sassuolo, where some 65% of the production of ceramic tiles in Italy and a little under 30% of world production is concentrated); (ii) environmental protection problems; waste water has to be properly treated in accordance with the legislation<sup>1</sup> before it is discharged, and the solid matter (sludge) remaining after processing and cleaning has to be disposed of.

These problems are of urgent concern, and to solve them requires considerable knowledge of the characteristics of the discharges and residues, and full and systematic experimentation with new treatment and disposal technologies.

This being the case, this research project, which concentrated on the production of ceramic wall and floor tiles, was assigned the following objectives:

- (i) to assess the water supply requirements according to the type of product and technology used;
- (ii) to determine the quantities and chemico-physical composition of waste water, and assess the specifications and efficiency of the purification plant currently in use;

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<sup>1</sup>In Italy this is Law N° 319 of 10 May 1976: "Regulations on protecting water against pollution".

- (iii) to determine the composition and quantity of sludge left after cleaning and processing;
- (iv) to investigate the possibilities of recovering or disposing of this sludge.

## 2. MATERIALS AND METHODS

In order to achieve these objectives, the following working methods were adopted:

- (1) A statistically significant number of ceramics producers representative of Italian ceramic wall and floor tile manufacturers were selected. These included:
  - (i) 5 makers of majolica (complete twice-firing cycle)
  - (ii) 7 makers of cottoforte (complete twice-firing cycle)
  - (iii) 5 makers of white body (complete twice-firing cycle)
  - (iv) 5 makers of once-fired red stone ware
  - (v) 5 makers of once-fired uncoloured stone ware
  - (vi) 7 enamel works (factories which carry out only enamelling and glazing).
- (2) Information was obtained from these firms in order to compile a water "balance sheet" using a special detailed questionnaire.
- (3) Statistically significant samples of effluent, cleaned water and sludge were taken from these firms.
- (4) The chemico-physical composition of the water was determined, i.e. the pH-value, the quantity of suspended matter, the content of Pb, Zn, Fe, Mn, Cu, Ni, Cr, F<sup>-</sup> and Cl<sup>-</sup> and the BOD and COD.
- (5) Analyses were made of the chemico-physical composition of the sludge: chemical analyses, diffractometric analysis, humidity, fusion point, dilatometric behaviour.
- (6) To determine the feasibility of disposing of or recovering sludge, tests were carried out on:
  - (i) the mixing of the raw material for the substrate
  - (ii) the chemical stabilization for controlled tipping;
  - (iii) reusing sludge in the preparation of enamels.

### 3. RESULTS

Given below are some of the most important results obtained.

#### (1) Consumption of water

Table 1 shows the specific water consumption levels, expressed as  $l/m^2$  (litres of water per  $m^2$  of tiles produced), given in the replies to the questionnaires. It can be seen that all in all between 13.2 and 20.5 litres are required for each  $m^2$  when preparing raw materials for the substrate dry, and between 31.2 and 38.5 litres per  $m^2$  if using the wet process. The waste water is basically that used for washing since the other water used evaporates in the drying and firing process.

The information on the amount of water used for washing is much less conclusive than the rest of the data so it was not possible to make any clear deductions about how water consumption is affected by types or methods of production. The reason is that washing is done manually and, even if all other factors remain unchanged, it varies according to the person concerned.

A large proportion of the firms questioned recycle at least some of the water after it has been cleaned and use it to wash the raw materials, and to wet-grind the raw materials for the substrate if the firm uses that process. At present, it is rather difficult to give an accurate estimate of amounts saved by recycling water after it has been cleaned.

The indications are however that only some 60 or 65% of the total requirements which can be worked out from Table 1 have to be drawn from water sources (wells or aqueducts).

	A	B
PREPARATION OF RAW MATERIALS FOR THE SUBSTRATE - wetting - grinding - washing mills	1	15 4
PRESSING (the water used to cool the presses is recycled)	0.5	
ENAMEL GRINDING	0.7-1	
ENAMELLING (washing lines and mills, wetting the substrate)	12-18	
Total	13.2-20.5	31.2-38.5

**Table 1** Specific water consumption (in  $l/m^2$ ) for the production of ceramic tiles

A = dry preparation of substrate raw materials

B = wet preparation of substrate raw materials

**(2) Chemical composition of discharged water**

Discharged water contains:

- (i) heavy metals in solution or suspension (mainly lead and zinc);
- (ii) solid matter in suspension (clay, sand, frit residue, generally insoluble silicates);
- (iii) anions in solution (fluorides, chlorides, boric oxides);
- (iv) traces of organic substances (serigraphy vehicles and adhesives used in enamelling).

The concentrations recorded vary considerably. (Fig. 1 gives an example of the various findings) and so do the volumes of water discharged. It was not possible therefore to establish any real links between the concentration of a pollutant and the type of product or the manufacturing technology used.

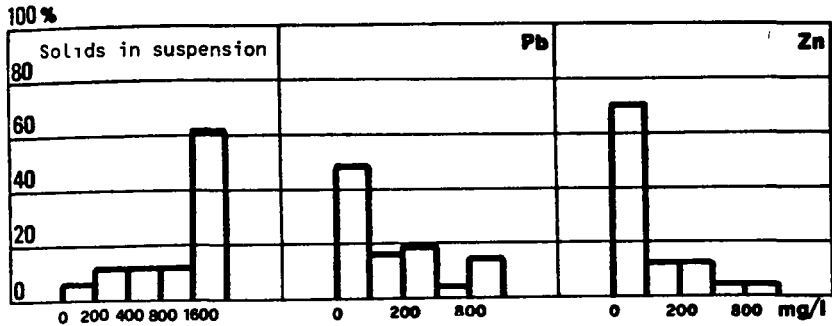


Fig. 1 Percentage distribution of concentrations (mg/Litres) of some pollutants in waste waters

The cleaning plant currently in use (which is almost entirely based on chemo-physical treatment of effluent using alkaline reagents, followed by flocculation and precipitation) is generally very efficient (see Fig. 2) even though sometimes it is not good enough to ensure that the legal limits are respected, because of the extremely high concentrations to start with.

There is no problem if this effluent is recirculated since for certain uses (e.g. wet-grinding the raw materials for the substrate) it is not strictly necessary to clean the metal beforehand.

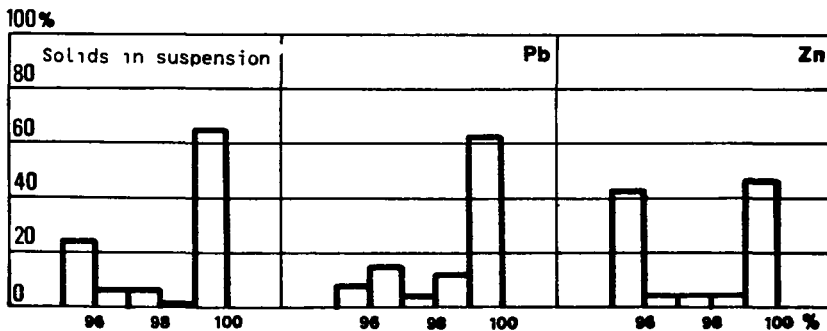


Fig. 2 Percentage efficiency in removing certain pollutants of the water cleaning plant investigated.



(3) Determining the chemico-physical composition of sludge

The same variations were encountered when assessing quantities of sludge as with waste water. On average, the amount of sludge fluctuates between 0.10 and 0.16 kg of dry matter per m<sup>2</sup> produced, with water content generally higher than 40% (in nearly half the examples it varied between 40 and 60%). Tables 2 shows the average compositions of the samples collected by type of production. The largest variations are in the lead content, which was fairly low in the sludge taken from firms producing tiles by the once-firing process. Diffractometric analysis then confirmed that this sludge contains a higher content of crystalline species than other sludge but relatively smaller quantities of vitreous and colloidal substances.

	A	B	C	D	E	F
P.f.	5.94	10.97	1.78	8.83	10.30	11.43
SiO <sub>2</sub>	49.05	39.81	52.94	41.48	44.02	34.15
Al <sub>2</sub> O <sub>3</sub>	9.25	12.12	9.92	11.76	13.12	10.77
TiO <sub>2</sub>	0.22	0.65	0.08	1.68	2.84	0.57
CaO	3.34	6.27	1.53	6.96	4.49	7.58
MgO	0.32	0.37	0.34	1.10	0.57	3.47
B <sub>2</sub> O <sub>3</sub>	8.02	6.40	8.63	5.37	3.91	6.42
Fe <sub>2</sub> O <sub>3</sub>	1.41	3.94	1.01	5.66	3.74	3.78
PbO	9.02	11.42	11.04	5.85	0.49	16.83
ZnO	5.40	1.98	1.95	4.02	4.44	1.75
K <sub>2</sub> O	2.42	0.88	0.95	1.67	0.92	0.71
Na <sub>2</sub> O	2.16	1.74	3.09	1.48	2.46	1.32

Table 2 Average percentage chemical composition of the samples of sludge from different types of production process

- A = Majolica (complete twice-firing cycle)
- B = Cottoforte (complete twice-firing cycle)
- C = White body (complete twice-firing cycle)
- D = Once-fired red stoneware
- E = Once-fired uncoloured stoneware
- F = Enamel works

(4) Potential for disposing of or recovering the sludge

(1) Mixing the sludge into the raw material for the substrate

This has already been done in a number of firms, particularly those which use the wet process for preparing raw materials for the substrate since this process means that the sludge is far better mixed in with the raw material. This is not possible with the dry process since the sludge has to be dried far more thoroughly.

Firing tests were carried out using clay blends made by adding up to 2% of various types of sludge with different lead contents to the Italian clay normally used for tiles and it was found that the variations which the addition of sludge caused in the sinter diagram because of the melting effect of certain components of the sludge were in almost all cases compatible with the industry's production requirements. It was also shown that the addition of pollutant substances to the substrate increased the concentration of these substances in flue gases before the sludge was cleaned, although there were no appreciable changes in the emission of pollutants after this cleaning stage. Since virtually all plants in Italy have a cleaning plant, this form of disposal should not cause significant environmental problems.

(11) Chemical stability of sludge

Tests carried out into the quantities of heavy metals deposited in the sludge showed that the uncontrolled tipping of sludge on the ground is dangerous and impracticable. Investigations were made into the possibility of stabilising them and making them chemically inert. Experiments have already been carried out with other types of solid residue into sintering and stabilising processes which use materials like cement, lime, bitumen, paraffin, organic polymers and the like as bonding agents. These methods seem potentially effective with sludge from the ceramics industry although they are relatively more expensive than the disposal system first suggested.

(111) Reuse of sludge in preparing enamels

The tests carried out showed that this can be done although at the present stage it is not yet possible to give generally applicable guidelines. The major obstacle is the fact that the composition of sludge varies considerably so that any kind of production planning with enamels containing sludge is practically impossible. Some firms have achieved interesting results in this field, chiefly

by checking the composition of sludge beforehand. In the enamelling departments, this can be done at the washing stage by recovering sludge from under the machine, keeping effluent from each individual batch separate as far as possible. After a suitable sedimentation process the enamel can be recovered directly and reused in production. This also substantially reduces the production of sludge from the washing process.

#### 4. CONCLUSIONS

The results of the survey indicate the following:

- (i) the amount of water consumed by the ceramics industry could be reduced substantially together with the environmental impact of discharges by recycling some or all of the effluent. The chemical and physical composition of the effluent means that it can be used for washing at certain stages in the process or the wet-grinding of raw materials for the substrate;
- xjj  
(ii) the chemical composition of this sludge varies according to the product, so that its quality must be expected to change even in the same plant. It was found however that up to 2% of sludge can generally be mixed with the raw material typically used for the production of the substrate, so that some effluent can be disposed of within the ceramics industry itself, particularly in view of the fact that firms already doing this are still not mixing any more than 0.5 to 0.7% sludge with their own raw materials;
- (iii) the reuse of sludge would appear to be the easiest and least expensive method of disposal, particularly by recovering the washing water from under the machines.

Contractor : Universite Libre de Bruxelles  
Laboratoire de Traitement des Eaux

Contract Nr. ENV-448 B

Project leader : Prof. R. WOLLAST

Title of the project : WASTEWATER TREATMENT BY COAGULATION  
WITH SODIUM ALUMINATE

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OBJECTIVE OF THE RESEARCH

Application of physico-chemical methods for wastewater treatments meets an increased interest because of their inherent advantages.

Among these methods, chemical coagulation and flocculation are used for the elimination of suspended solids of various sizes and composition, as well as for the removal of colloidal substances, phosphorus and heavy metals.

The objective of this research is to investigate the performance of a physico-chemical treatment using sodium aluminate as the main coagulant. This substance, which has sometimes been used as a coagulating agent in the production of drinking water, has never been used on a large scale as main reagent for wastewater purification.

The recent development of a new method for the production of sodium aluminate from the liquid effluents of the aluminium anodizing industry (S.WAJC, Pat. Pend.) could give a new interest to the competitive use of this product as coagulant in the field of wastewater treatment.

## MATERIALS AND METHODS

The experiments have been conducted both in the laboratory and on the field. The wastewater to be treated was supplied by the Brussels main sewer. This sewer covers an area of 4190 hectares with a population of 380.000 inhabitants and a 30% contribution of industrial wastewaters to the total polluting load. The mean composition of the test water is as follows :

suspended solids :	213	mg/l
B.O.D. :	290	mg/l
C.O.D. :	552	mg/l
Total N :	37	mgN/l
Total P :	10.2	mgP/l

The laboratory experiments were mainly conducted with a jar-test apparatus, according to a standardized procedure (rapid mixing for 2 minutes, slow mixing for 20 minutes, settling for 2 hours). Analysis of the C.O.D., suspended solids, nitrogen and phosphorus have been done according to the Standard Methods or with an Auto-Analyser. Heavy metals have been determined by atomic absorption spectrophotometry.

The field experiments have been realised on a pilot-scale unit continuously fed by the wastewater from the main sewer. This unit incorporates :

- a rapid mixing compartment (volume = 7 liters)
- a slow mixing compartment (volume = 7 liters)
- four flocculation compartment (volume = 15 liters each) with paddle agitators rotating at a decreasing speed (from 60 t/min in the first compartment to 12 t/min in the fourth one)
- a bottom-fed cylindrical settling tank (volume = 215 liters, surface = 0.29 m<sup>2</sup>)

Provisions are also made for the injection of coagulant and polyelectrolyte by means of dosing pumps. The sludge level in the settling tank is controlled by a photo-electric sludge detector commanding a sludge withdrawal pump. A volumetric variable speed pump is used to feed the pilot unit at a nominal

flow of 360 liters per hour.

## RESULTS

### Suspended solids removal

Jar-test experiments show that the use of sodium aluminate at a 20 mgAl/l concentration gives a 60% mean elimination of the suspended solids, as compared with 30 to 40% for settling without any coagulant addition.

Pilot-scale experiments show that the presence of a sludge blanket in the bottom of the settling tank still increases the efficiency of the method, with a removal superior to 70% on a 24 hours period (see Fig.1).

Addition of an anionic polyelectrolyte has no appreciable effect on the removal efficiency, but increases the size and the settling rate of the flocs, with an optimal dosage of 0.5 mg/l. The use of an anionic polyelectrolyte also allows to thicken the sludge (volume reduction of 60%) at a dosing of 13 mg polyelectrolyte / gram of sludge.

### Organic matter removal

C.O.D. elimination in the range 50%-70% have been obtained by use of sodium aluminate at a concentration of 20 mg Al/l. This has to be compared with the 30%-40% elimination measured on the same wastewater after settling without addition of coagulants, or with addition of classical reagents (alum, ferric chloride).

C.O.D. measurements on raw and filtered samples prior and after coagulation also show that almost all of the organic matter associated with particulate and colloidal material (diameter < 0.45  $\mu$ m) are removed by the coagulation/floculation process, which leaves the soluble fraction of the organic matter essentially unaffected.

For practical application, it is important to point out that no sign of floc destabilisation has been noticed at concentrations up to 60 mgAl/l.

#### Phosphorus removal

Addition of an aluminium salt leads to orthophosphate removal by precipitation of aluminium phosphate. In the case of sodium aluminate, the removal efficiency is generally superior to 90%, with residual concentration less than 2 mgP/l (see Fig.2), for water pH comprised between 7.5 and 8.0.

#### Heavy metals removal

Experiments conducted on artificially enriched wastewaters (5 to 100 mg/l Cu, Zn, Pb) show that the use of sodium aluminate leads to residual metal concentration inferior to 1 mg/l in all cases (typically : 0.5 mg/l for Zn; < 0.2 mg/l for Pb; 0.5 to 1 mg/l for Cu).

#### CONCLUSION

The reported results show that the coagulation/floculation by means of sodium aluminate can be an efficient method for phosphate and heavy metals removal. However, its use should not be limited to tertiary treatment only, because of its ability to seriously increase both suspended solids and organic matter removal. The best results are obtained at aluminium dosing as low as 20 mgAl/l, with the use of a sludge blanket clarifier. Addition of a coagulating aid (polyelectrolyte) is not required in the coagulation step, but enhances the sludge thickening.

This study has been partially supported by the Belgian Authorities, for whom the work is still in progress. Complete results of the research will be available end 1981.

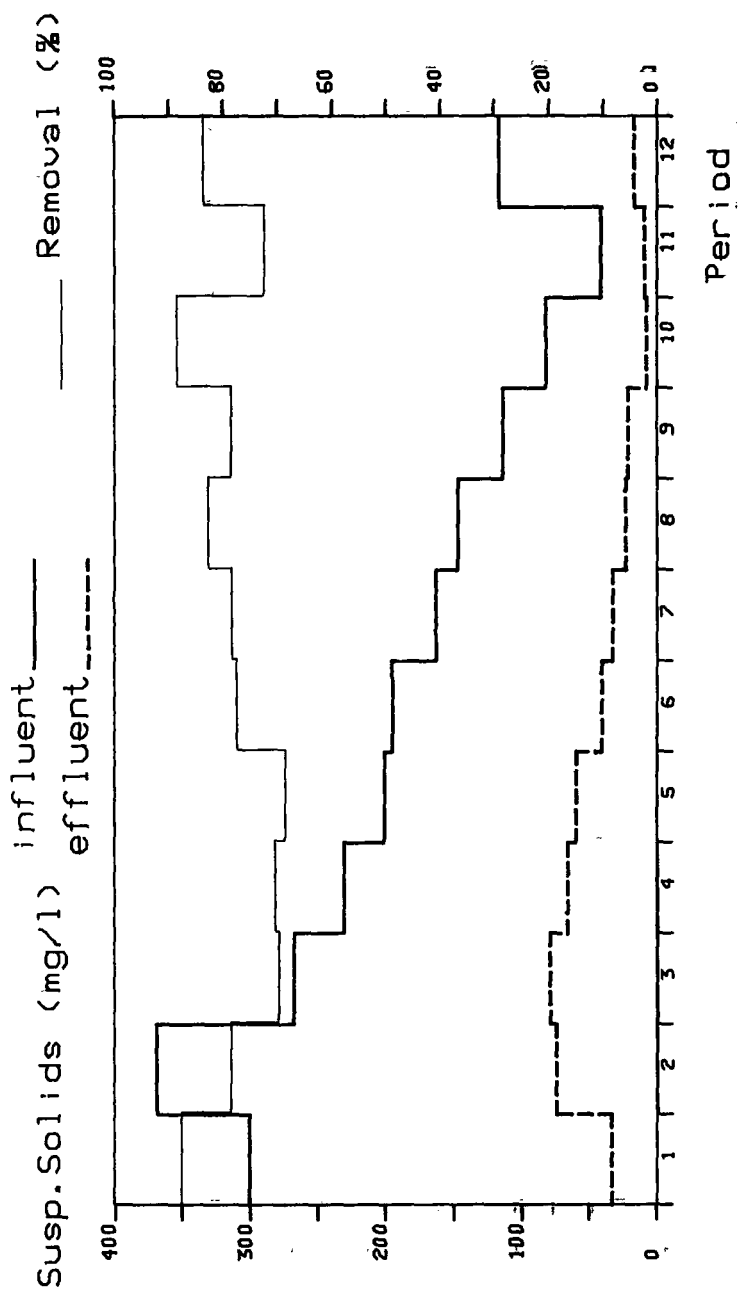


Figure 1: aluminate = 68 ppm ; polyel. = 0 ppm (%)



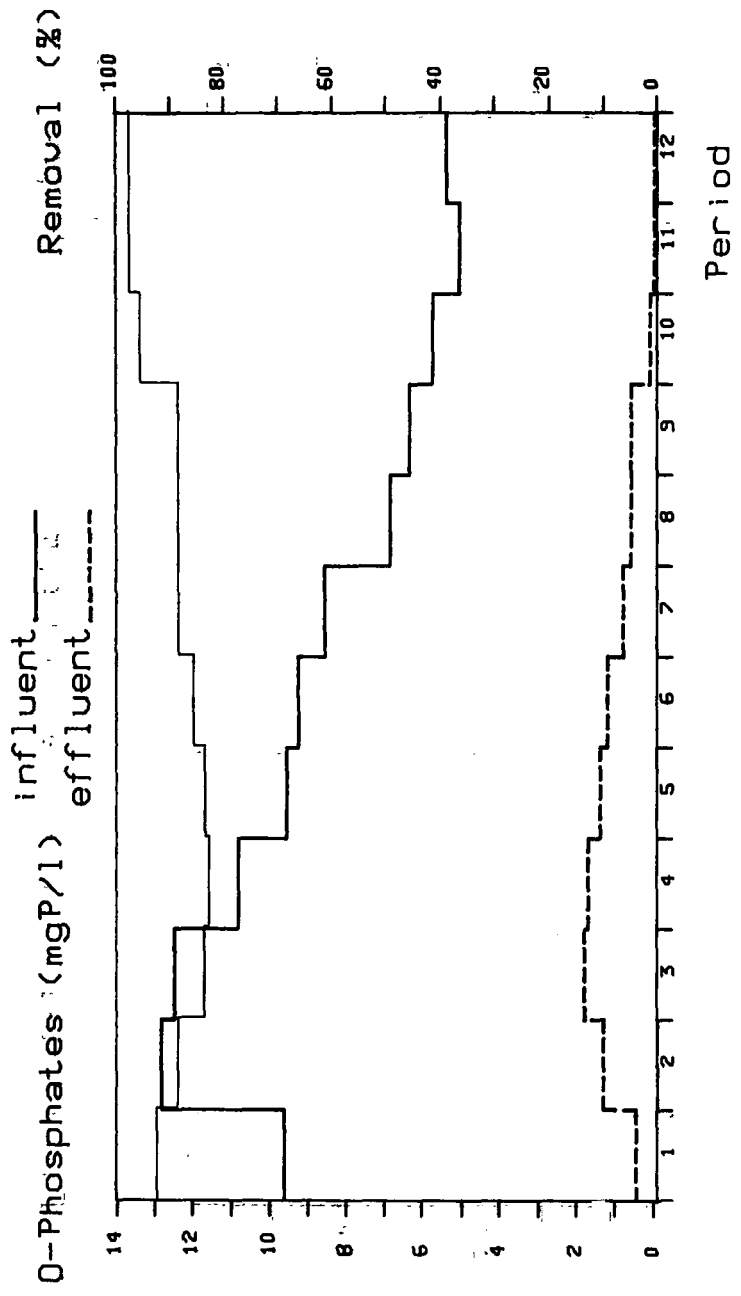


Figure 2 : aluminate = 68 ppm ; polyel. = 0 ppm

Contractor : UK DEPARTMENT OF THE ENVIRONMENT  
Contract n<sup>o</sup> : 312-78-1 ENV UK  
Project leader : MR G G WATSON (WITH MR N BANKS, WATER RESEARCH CENTRE)  
Title of project : FLOW AND LOAD BALANCING IN ADVANCED WASTEWATER TREATMENT

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#### OBJECTIVES OF THE RESEARCH

The principal objectives were to examine the effects of diurnal-flow balancing on primary sedimentation, on activated-sludge treatment with and without primary sedimentation, and on chemically-aided primary sedimentation, of municipal wastewater.

#### MATERIALS AND METHODS

The work was carried out by Water Research Centre at the Department of the Environment's large-scale Experimental Plant for sewage treatment situated at Coleshill near Birmingham.

Feed sewage was pumped from an outfall sewer discharging to the adjacent Coleshill Sewage Works which treats an average flow of 55 000 m<sup>3</sup>/d from a population of 250 000 living in an area of Birmingham containing little major industry. To avoid introducing an artificial relationship between flowrate and pollutant concentration, the diurnal pattern used was similar to that experienced at the Coleshill Sewage Works, giving flowrate variations of 0.33 to 1.7 times average. The corresponding diurnal variations in pollutant concentrations were similar in range.

The tank volume needed to balance-out the pattern of diurnal flowrate represented 18% of average daily flow. Two modes of balancing were used: in-line, in which all the sewage flow enters the balancing tank and a constant amount is continuously withdrawn, and side-line, in which only flow exceeding a preset rate enters the balancing tank (the rest passing directly to treatment), withdrawals being made later at times of low flow.

#### Experimental plant for primary sedimentation and activated-sludge treatment

A flow measurement and control system regulated a flow of screened and dewatered sewage to the required diurnal pattern. A similar system then divided this flow into two portions, each aggregating 167 m<sup>3</sup>/d, and these were passed to treatment - one by gravity to the diurnal flow plant, the other via a flow-balancing system to the constant (balanced) flow plant. A 4.5 m diameter balancing tank having a volume of 55 m<sup>3</sup> was used to accommodate the daily flow variation, nominally 30 m<sup>3</sup>. Settlement was obviated by drawing outgoing flow from the base of the tank, which was

fitted with a scraper. The diurnal-flow and balanced-flow plants each incorporated identical upward-flow sedimentation tanks 3 m in diameter, these and all other such tanks being of 3 m sidewall depth. The nominal hydraulic retention time was 3 hours (h) at average flow, ranging therefore from 1.8 h to 9.1 h under diurnal flow conditions.

Each plant incorporated two surface-aeration tanks 3.9 m X 3.9 m X 1.4 m effective depth, in series, giving a total nominal retention period of 5.5 h (sewage flow only). Each pair of aeration tanks was followed by a final settlement tank 3.67 m in diameter with a nominal retention period of 4.6 h. Mixed liquor was independently pumped at constant rate from the second aeration tank of each plant to its final settlement tank, diurnally-varying flowrates being accommodated by intermittent pumping at about three times average flowrate. Returned activated-sludge flowed by gravity on both plants at a constant  $167 \text{ m}^3/\text{d}$ .

#### Experimental plant for chemically-aided settlement

This was a single plant employing a separate flow of screened and degrittied sewage, flow in which was not divided. Experiments therefore involved sequential periods of operation with and without flow balancing.

##### Aluminium Sulphate

The average flowrate used was  $400 \text{ m}^3/\text{d}$ , the balancing volume required being amply provided by an in-line arrangement using two  $55 \text{ m}^3$  balancing-tanks. The chemical treatment stage incorporated a rapidly-stirred mixing tank with hydraulic retention period of 16 minutes, followed by a 56-minute retention flocculation tank fitted with slow stirring. The dosed sewage was passed to an upward-flow sedimentation tank 4.5 m in diameter giving a nominal hydraulic retention time of 2.8 h.

##### Lime

The average flowrate was  $450 \text{ m}^3/\text{d}$ , using a side-line balancing arrangement. Mixing-tank and flocculation-tank retention times of 12 and 40 minutes respectively were provided, followed by a 3.0 m diameter sedimentation tank which gave a nominal retention time of 1.1 h.

#### Sampling and analysis

Samples were taken proportionally to the flow throughout the experimentation and each day's samples were bulked for analysis. The results of averaging these daily analytical figures represent 'average daily performances', enabling the masses of pollutants applied to and removed by diurnal-flow and balanced-flow plants to be compared directly.

Additionally, occasional five-day survey periods were arranged during which hourly discrete samples were taken and analysed individually.

## RESULTS

### Balancing

Both systems were equally successful in achieving flow-balancing. The in-line system was the more effective in pollutant concentration smoothing. Concentration data from discrete hourly sampling were subjected to variance analysis with the results given below:

effluent samples taken	variance due to residual diurnal effects after balancing, as percentages of crude sewage variance				
	BOD	SS	Amn N	TOC	dissolved TOC
after in-line balancing	47.0	43.4	38.6	40.8	47.3
after side-line balancing	79.2	76.5	83.1	71.7	53.9

After side-line balancing, 70 - 80% of the observed variance due to diurnal concentration changes remains, whilst for the in-line arrangement the residual is only 40 - 50%. However, when a similar analysis was carried out on the effluents from primary sedimentation, with and without side-line balancing, residual values of 30 - 40% were found in each case. Similarly, for the effluents from the activated-sludge treatment of unsettled sewage with and without in-line balancing, residuals of about 9% were found.

Although both balancing arrangements effect differing and quantifiable degrees of concentration smoothing, neither produces an effect sufficiently marked to be observed after subsequent treatment processes. Careful consideration was given to the feasibility of experiments specifically designed to investigate load balancing, using currently available sensors, but on the basis of previous experience at Coleshill and elsewhere it was decided that a simple and reliable control system was not yet available.

Primary sedimentation (in-line balancing)

Average daily performance (75 days)	<u>diurnal flow</u>		<u>balanced flow</u>	
	<u>SS</u>	<u>BOD</u>	<u>SS</u>	<u>BOD</u>
feed (mg/l)	223	203	243	215
effluent (mg/l)	120	147	122	158
% removal	46	28	50	27

Periods of discrete hourly sampling  
(figures in brackets are upper 95 percentile  
values each derived from ~120 results)

effluent	144(208)	276(351)	149(178)	246(378)
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Activated-sludge treatment with primary sedimentation (in-line balancing)

Average daily performance

16 days with MLSS 1470/1480, sludge loading 0.49/0.49, sludge age 3.0/3.0*				
effluent	25	27	42	42
25 days with MLSS 1880/1830, sludge loading 0.31/0.34, sludge age 4.7/4.2*				
effluent	22	22	38	30
35 days with MLSS 4450/4030, sludge loading 0.14/0.18, sludge age 7.8/7.4*				
effluent	45	21	32	19

Activated-sludge treatment without primary sedimentation (in-line balancing)

Average daily performance

35 days with MLSS 4800/4500, BOD loading 0.25/0.26, sludge age 4.8/7.5*				
effluent	117	45	32	15
18 days with MLSS 2700/2600, BOD loading 0.37/0.38, sludge age 2.0/1.9*				
effluent	16	12	13	10

\*MLSS mg/l, sludge or BOD loading kg BOD/kg MLSS.d, sludge age d

Chemically-aided primary sedimentation

Average daily performance

26 mg/l Al <sub>2</sub> O <sub>3</sub> , constant dosing, 16/15 days	feed (mg/l)	250	236	221	223
	effluent (mg/l)	132	151	85	127
	% removal	47	36	62	43
26 mg/l Al <sub>2</sub> O <sub>3</sub> , dosing proptnl to sewage flow, 10/10 days	feed (mg/l)	234	213	-	-
	effluent (mg/l)	74	88	-	-
	% removal	68	59	-	-
lime to pH 9.5, 5/5 days	feed (mg/l)	290	83*	340	318
	effluent (mg/l)	62	67*	112	181
	% removal	79	19	67	43

\* TOC

		<u>diurnal flow</u>		<u>balanced flow</u>	
		<u>SS</u>	<u>BOD</u>	<u>SS</u>	<u>BOD</u>
lime to pH 11.3,	feed (mg/l)	299	347	278	297
5/5 days	effluent (mg/l)	67	176	24	140
	% removal	78	49	91	53

Remarks

For most of the experimental periods, conditions were chosen which resulted in daily variations ranging up to the maximum conventional design values in the diurnal-flow plant. Although (as noted above) retention in diurnal flow primary sedimentation varied between 9.1 and 1.8 h, whereas the balanced flow tank gave 3 h retention, resultant effluents showed no consistent differences either in respect of average daily performance or of the upper 95 percentile values derived from the survey periods of discrete sampling and analysis.

Similar remarks apply in the cases of both activated-sludge regimes. A five-day survey period was again undertaken with the following results (upper 95 percentile in brackets) representing about 110 samples

effluent TOC (mg/l)	24.3 (37.5)	20.6 (33.0)
· dissolved TOC (mg/l)	16.2 (22.0)	15.0 (23.0)

Passage through treatment processes smoothed-out concentrations in effluents from the diurnal-flow plant, so that little difference could be discerned between them and those from the balanced flow.

CONCLUSIONS

The satisfactory operation of in-line and side-line flow balancing was demonstrated and the former was shown to be the more effective in reducing diurnal variations in pollutant loads. No improvement was observed from diurnal-flow balancing in the performances of primary sedimentation, activated-sludge effluents being influenced more by non-diurnal operating characteristics such as aeration and final-settlement capacity, incipient denitrification and applied sludge loading. Such benefit as may have been given by diurnal flow balancing in chemically-aided primary sedimentation appeared to be obtainable by dosing chemical proportionately into a diurnally-varying flow.

As the work revealed no quantifiable benefits from flow-balancing, economic analysis was not thought justified; but examination of the economic consequences of reduction in power demands due to load balancing might be worthwhile.



**TOPIC 32 : WASTE DISPOSAL**



Contractor:      Forschungsinstitut für Edelmetalle und Metall-  
                  chemie, Schwäbisch Gmünd, West Germany  
Contract n<sup>o</sup>:     275-77 ENV D  
Project Leader:  Dr.Ch.J.Raub  
Title of Project: Influence of surroundings on behavior of  
                  Industrial sludge during storage on public  
                  dumps

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### 1) Objective of Research

Hydroxide sludges originating in the metal finishing industry contain  $\text{CN}^-$ ,  $\text{PO}_4^{3-}$ ,  $\text{CrO}_4^{2-}$ , mineral oils etc. in addition to various base metals. Up to now these sludges have to be deposited on various dumps, until new and economic recycling procedures have been developed.

In extension of earlier work, present investigations serve to study long time behavior of these sludges, stored together with other waste on public dumps e.g. together with household waste, and to find appropriate testing methods on its behavior. Special attention must be given to complex cyanides present in the deposited material.

Cyanides often are present as insoluble base metal cyanides or complex Fe-cyanides (relatively nonpoisonous). The amount of soluble alkali cyanides and of other complex cyanides is generally rather low. Nevertheless by various ways a dissolution of cyanides, as well as their decomposition can occur.

### 2) Materials and Methods

We studied typical hydroxide sludges from an electro - plating shop and a steel plant with a hardening division.

Experiments were made under following conditions:

- a) For laboratory tests we developed a setup, in which long term elution behavior depending upon various parameters can be measured, since it was observed that some of the standard methods yield rather unreliable results.
- b) A test, approximating real conditions, on an artificially built waste dump (60 l volume, mixture of hydroxide sludge and home waste) was established.
- c) Experiments on an official home waste dump in specially provided areas was undertaken. In this dump sludges of an electroplating plant and of two iron-working factories were deposited and the effluent waters tested.

Advantages and disadvantages of these methods are:

Laboratory tests enable the adjustment of various parameters and an accurate investigation of elution processes, since conditions on a real waste dump are difficult to reproduce and influence.

Tests on artificial small waste dump can be controlled easily and as in laboratory tests the kind of sludges can be varied without complications. Results do not always refer directly to the ones observed in practical behavior. Experiments on a real waste dump are difficult to be modified once they are set up and in addition are rather time consuming. Sludges with relatively higher cyanide concentrations were investigated this way.

We therefore decided that a combination of all methods would yield the most reliable and practical results.

### 3) Results

- a) Tests showed that elution behavior of the sludge is mostly depending upon the  $\text{CaCO}_3/\text{CO}_2$  equilibrium and the ammonia concentration of the water.

At pH  $\approx$  7.6 a strong dissolution of nickel occurs. There is only a small pH-range in which copper and nickel do not dissolve. Complications arise because of the oxidation of ammonia by bacteria, which forms nitrite and nitrate and causes a reduction in pH. In the presence of ammonia Zn redissolves at lower pH values at longer exposure times. Long time contact of Cr III containing sludge with water of pH  $\approx$  9 can cause dissolution of Cr VI.

Rain water is uncritical. Fig.1 shows the elution behavior of copper from an electroplating sludge with aerated, de-ionized water which was replaced after 55 days by ammonia containing water.

- b) Tests on the small experimental waste dump proved that copper and nickel are most critical elements. In addition to the composition of the sludge its method of segregation is of strong influence upon elution ( $\text{NaOH}$  vs.  $\text{Ca}(\text{OH})_2$  precipitation). Sludge with a better storage behavior is precipitated by  $\text{Ca}(\text{OH})_2$ .
- c) Cyanide. We have not found an appreciable dissolution of cyanide from the sludges investigated during long time storage. Only complex Fe-cyanides seem to dissolve in carbonated water of pH 10.

Sludges from iron hardening plants seem to give away in the beginning relative high concentrations of Fe-cyanides (up to 15 mg/l) and free  $\text{CN}^-$  (1-3 mg/l), but this values drop by a factor of 10 very soon. Artificial weathering of sludge during half a year did not cause any increase in the cy-

anide content of the eluate

- d) Public waste dump. Rain water only causes an elution comparable to laboratory tests. If water after passing through house waste reacts with the sludge more nickel and copper are eluated, but the Cr-VI-content in the eluate falls strongly.

4) Conclusion and additional comments

Of highest importance for dissolution of sludges are certain constituents of the leaching water e.g.  $\text{NH}_3$  or  $\text{NH}_4$  and  $\text{Ca}(\text{HCO}_3)_2$ . A high pH value is - in contrast to general opinion - of disadvantages to storage of nickel, copper and cyanide-containing sludges. Sludges not only give away certain elements during elution, but as well filter out certain critical constituents of the dump water. The public waste dump continues to be monitored.

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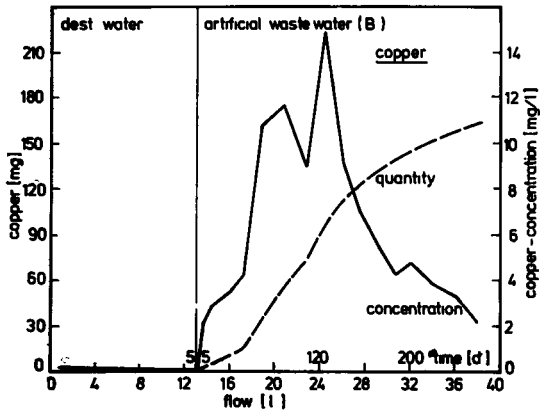


Fig.1 Elution of copper from an electroplating sludge

Contractor : INSTITUT DE RECHERCHES HYDROLOGIQUES

Contract N° ENV/378 F

Project leader : François COLIN

Title of Project : Development of methods for the study and the characterization of polluting liquid discharges and of solid wastes, in view of the evaluation of the consequences for the environment

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## **2. OBJECTIVE OF THE RESEARCH.**

The general objective of the research aims to prevent and solve the problems raised by the discharge of solid or liquid wastes into the natural environment.

The project comprises three distinct parts, which are conducted in a parallel and independent way, and which precisely relate to the following points :

- 2.1.** Definition of a method based on the global measurement of biomass, in order to detect the effects of polluting discharges and of chemicals on the aquatic microflora which is responsible of self-purification phenomena. Within these limits, the problem is to define a test allowing to reach a global and rapid response of the reaction of the considered system against the studied pollutants, in view to characterize the toxicity of industrial discharges and their influence on the aquatic environment or on biological treatment plants and the natural self-purification systems.
- 2.2.** Improvement of methods for the characterization and the study of solid wastes, in order to evaluate the consequences for the environment resulting from their dumping. This part consists to select and define parameters characterizing the evolution of wastes with time, and to look for a method for the utilization of the results, enabling to wholly forecast the risk due to dumping, in function of the peculiarities of the considered site.
- 2.3.** Gathering of basic data on the corrosivity and the scaling tendency of waters in function of their physical, physico-chemical and biological properties, in order to determine critical quality thresholds according to the conditions of utilization which determine the possibilities of recirculation or re-use of waters within the limits of proper technologies.

## II. MATERIALS AND METHODS.

Only the first part of the research contains an experimental part.

II.1. The principle of the experimental method consists to use the A.T.P. (Adenosine-Tri-Phosphate) concentration of a culture medium as a parameter for measuring the biomass which it contains. One then studies the evolution with time of the A.T.P. concentration of the culture, starting from the addition of a determined quantity of the studied chemical or polluting discharge.

Practically, each test consists to utilize a series of identical reactors containing an initial microbiological culture ; one of these reactors is used as a control ; at the initial moment, increasing doses of the studied chemical or discharge are added to the other ones.

According to the nature (bacterial or algal) of the culture, various steps are taken in order to maintain favourable conditions in the medium (stirring, additional substrate, illumination, blowing of oxygen, adjustment of salinity, etc.). Samples are taken during the whole experimental period (generally from 3 to 24 hours) with a decreasing frequency. The A.T.P. present in the samples is extracted by means of a method of chemical lysis with D.M.S.O. The A.T.P. thus solubilized is determined with the method of luciferine-luciferase bioluminescence.

This method has been applied successively to pure cultures of bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Serratia liquefaciens*), of algae (*Chlorella vulgaris*, *Scenedesmus subspicatus*), to mixed algal cultures and mixed bacterial cultures (activated sludges from urban treatment plants), in order to test the toxic action of chemicals (potassium bichromate, copper and zinc sulphates, phenol) and of effluents containing residues of chlorinated pesticides.

II.2. The second part of the research, comprising :

- the critical study of the general methods for estimating the influence of the dumping of a waste in a determined site,
  - the critical study of tests for defining the risk potential of wastes and evaluating their evolution after dumping,
  - the search for basic elements and the definition of an improved method for the evaluation of risks,
- is based on the collection of bibliographical and docu-

mentary data, as well as on the results of numerous studies and researches previously conducted in France and abroad.

- 11.3. The third part, concerning the collection of basic data about the corrosivity and scaling tendency of recirculated waters, within the limits of proper technologies, is based on the results of numerous studies and researches, effected not only on waters from natural origin, which differ in their mineralization and may be ranged in large groups, but also on waters derived therefrom, during their stay in the circuits under the action of temperature increases, salt concentration through evaporation, etc. The measured characteristics relate to scaling through the precipitation of calcium carbonate, generalized electrochemical corrosion, local corrosion through pitting and cracking, galvanic corrosion, selective corrosion. The considered metals are those usually utilized in industrial circuits (iron, galvanized steel, copper, aluminium, brass).

### III. RESULTS.

#### 111.1. Detection of the impact of polluting discharges on the aquatic microflora.

The method applied to mixed bacterial cultures (activated sludge), - the results being expressed as the percent residual A.T.P. relative to the control sample, - enables to evidence four typical behaviours :

- no action : this typical behaviour appears for non-biodegradable and non-toxic substances.
- global growth stimulation : relative to the control sample, the total A.T.P. concentration increases with time. This is the action of a substance which is at the same time biodegradable and non-toxic.
- non-reversing global inhibition : The A.T.P. percent relative to the control constantly decreases during the test, and reaches an asymptotic value which may be zero. This case happens when the substance is very toxic at the added quantities.
- reversible global inhibition : the A.T.P. concentration relative to the control first decreases, reaches a minimum, and then again increases, until an asymptotic level is obtained, which may be lower or equal to the initial value.



This type of representation, applied to the tests executed on mixed and pure cultures, still does not allow the establishment of a quantitative modelling, as it does not consider the evolution of the control samples with time. The representation using absolute A.T.P. values enabled the establishment of the following quantitative model :

- The addition of toxicants expresses itself with a very rapid decrease of A.T.P., which appears in a period of about thirty seconds for pure bacterial cultures, and of about ten minutes for activated sludge. This initial decrease may be more or less important, and its amplitude gives a first characteristic parameter of the toxic action. In some cases, an initial lowering of A.T.P. may be observed for the control sample itself, and results from a stress effect by the setting of the test.
- This phase of A.T.P. decrease is followed by an increase phase, more or less affected by the toxicant according to its nature and concentration. This increase is lower in the presence of a toxicant than in the control sample, but in any case it may be very satisfactorily modelled by a logistic function of the form :

$$A.T.P. = \frac{A.T.P. \text{ max}}{1 + e^{(\alpha t + \beta)}}$$

The determination of the numerical constants  $ATP_{max}$ ,  $\alpha$  and  $\beta$  which intervene in these functions enables to obtain other characteristic values of the action of the toxicant.

Finally, the proposed model correctly describes the various tests which we have conducted on pure cultures as well on mixed cultures. It enables to calculate the four parameters which quantitatively characterize the impact of the studied chemical or discharge on the considered culture.

The quantitative results obtained in the various studied cases are attached to the final report.

#### 111.2. Characterization of the wastes in order to evaluate the risks of dumping.

The critical examination of the general methods for the evaluation of risks, in particular those of PAVONI, HAGERTY, LEE and PHILLIPS, NATHWANI, has evidenced their positive items and their insufficiencies,

as well on the plane of the nature of utilized data as on the level of their application for reaching an operational decision.

Moreover 18 methods of tests and tentatives of global characterization of wastes and of their evolution have been examined.

An analysis of the phenomena involved in the surroundings of a dumping place was effected and distinguishes :

- the flow of considered water as a pollution vector (detailed study of the water balance and circulation)
- the production of soluble pollution by the waste, with the distinction of directly soluble forms of pollution and those which result from the evolution of the waste with time,
- the transport of the soluble pollution and the possibilities for its lowering, with the distinction of dilution, fixation mechanisms and destruction mechanisms,
- phenomena of a second order, such as the eventual influence of the carried pollution on transport phenomena (clogging of the subsoil).

The comparison of the available methods, the conclusions drawn from their critical examination and from the analysis of the involved phenomena enable to define an improved evaluation method.

### III.3. Collection of data about the scaling tendency and corrosivity of the re-utilized waters.

The gathered data have been synthetized in the form of detailed tables and of bibliographical lists concerning :

- the identification of the involved phenomena and the factors which influence them, and more specially those related to water quality,
- the available means of study for the establishment of diagnoses, the evaluation and observation of preventive and curative steps,
- the quality criteria of waters in relation to their interaction against materials (corrosion, scaling,

biological contamination). All data concerning this last item have been presented in the form of annotated tables, which constitute a basic tool for the management of water quality within the limits of proper technologies.

#### IV. CONCLUSIONS.

- IV.1. The descriptive model of the impact of toxic discharges or substances upon the cultures of microorganisms responsible of self-purification appears to be generally applicable, within the precision limits of the utilized experimental methods.

It enables to calculate numerical values which characterize the impact of substances or discharges against the purifying microflora, and therefore solves the raised problem, which was to define a quantitative evaluation method starting from a laboratory test.

- IV.2. An improved general method for evaluating the risks due to the dumping of wastes has been defined.

This work shall be continued with a research of an experimental character, which shall enable to make a selection among the available methods and tests for evaluating, in the laboratory, the behaviour of the wastes and for determining the detail of the method for integrating the experimental results into the defined methodological model.

- IV.3. As to the scaling tendency and the corrosivity of water, the whole of gathered data constitute a first tool, which should enable to determine the allowable limits in the field of re-use and recirculation of industrial water within the limits of the adoption of proper technologies this should make easier the adaptation of industrial processes to the conservation of the aquatic environment.

Contractor:	Water Research Centre
Contract No:	ENV-455 UK
Project Leader:	Dr. C Barber
Title of Project:	The treatment of leachate from domestic wastes in landfills (Phase 1)

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#### OBJECTIVES OF THE RESEARCH

The objectives of the research are to assess the benefits which might be obtained by recirculating leachate through the landfill from which it is derived, and to study the conditions necessary for treatment of leachate in a simple, aerobic biological treatment plant on a landfill site.

#### MATERIALS AND METHODS

##### LEACHATE RECIRCULATION STUDY

Recirculation of leachate as a method of leachate management is being investigated in a full-scale field trial at a landfill site near Scarborough, UK. The part of the landfill being used is 2.5 ha in area lined with a 3 mm thick heavy duty polyethylene membrane, and contains approximately 4 m depth of pulverised domestic wastes.

Leachate is recirculated by spraying over a 1 ha area of uncovered wastes in the lined site, from twelve 45 m lengths of 60 mm diameter 'heliflat' tubing. These tubes are perforated at 2.5 m intervals on their upper side with a 4 mm diameter hole, to give a series of fine jets of liquid 3-4 m high when spraying, which distribute the leachate evenly over the landfill surface. Leachate is pumped to the spray area from a storage lagoon through 200 m of 100 mm diameter tubing (fig. 1).

Another part of the lined area is being used as a control and has not received recirculated leachate. (fig. 1). Leachate from the wastes within the lined area is collected by four lines of porous tile drains (fig. 1), and flows to a sump from where it is pumped into a storage lagoon for disposal or for recirculation. Flows of leachate from the control and recirculation areas of the site are being measured using calibrated V-notch weirs fitted with ultrasonic measuring equipment. Flows are measured every hour and are recorded on magnetic tape using a data logger.

In order to provide a water balance for the site, daily rainfall data are being obtained for the site and the volume of leachate recirculated is measured by determination of the rate of flow of leachate through the supply tubing by dilution gauging, and by recording of the total time over which

spraying takes place. Water levels within the landfill are being recorded to determine the amount of water which is present within saturated parts of the site. Samples of leachate are taken from the 2 site drains (fig. 1) every 2 weeks for determination of TOC, BOD, COD, volatile acids, ammoniacal-N, oxidised-N, organic-N chloride and a range of metals, to determine changes with time in the strength and composition of leachate from the control and recirculation areas.

#### AEROBIC BIOLOGICAL TREATMENT OF LEACHATE

Batch aeration tests have been carried out at controlled temperatures of from 5°C-20°C, using 10 l capacity glass vessels. The effects of nutrient addition, seeding with microorganisms from sewage, and temperature on the efficiency of treatment were investigated using leachate samples collected from a number of landfill sites containing domestic wastes.

A series of continuous-flow experiments have been carried out to investigate treatment of a 'typical' leachate, (COD 5000 mg/l) in 20 l capacity complete-mixing aeration units. Nutrients were added, and initially reactors were operated at 10°C using a range of retention times of from 1-20 days. The effects on treatment efficiency of lowering the temperature to 5°C, and of increasing the concentration of ammonia in leachate from 80 mg/l to as high as 1000 mg/l, were also investigated in separate experiments using the same reactors.

### RESULTS

#### RECIRCULATION OF LEACHATE

Monitoring of flow and composition of leachate between March 1979 and September 1980, before recirculation of leachate commenced, indicated that on average the flow of leachate from the completed site was equivalent to 1 mm/d.

Leachate was unusually strong (maximum COD of 84000 mg/l which decreased to 40 000 mg/l by September 1980). In September 1980, recirculation of leachate commenced at an average rate of 4 mm/d on the 1 ha area, and continued until mid-November. A total of 500 m<sup>3</sup> of leachate (equivalent to 150 mm recirculation) was sprayed during this period. Because of heavy rainfall in October 1980, only limited spraying took place between November 1980 and January 1981. To February 1980, an additional 34 mm of leachate were sprayed on the landfill.

The spraying system worked well, and infiltration of liquids into the wastes was generally good although some ponding did occur on parts of the site where a thin clay soil cover was present.

As expected, the relatively small volume of leachate which has been recirculated has produced no effect on the volume or composition of leachate draining from the site, although recycled leachate and unusually heavy rainfall

combined to produce more extensive areas of saturation within the site than had previously been recorded. It is intended to continue monitoring of the site in phase 2 of the programme, to determine the effects of leachate recirculation until at least March 1982.

#### AEROBIC BIOLOGICAL TREATMENT

Batch leachate aeration experiments indicated that leachate from recently emplaced domestic wastes could be treated using aerobic biological processes at temperatures as low as 5°C. However, for successful treatment at these temperatures, nutrient (P) addition was usually necessary, to give a BOD:P ratio of less than 100:1. Seeding of aeration units with microorganisms from sewage was shown to be unnecessary.

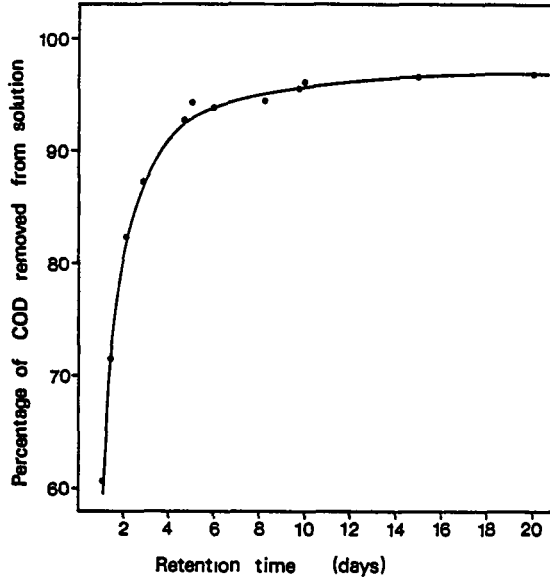
Experiments using continuous-flow aeration units showed that with a mean period of aeration of 5 days or more, greater than 90 per cent removal of COD from solution occurred at 10°C (fig. 2). Decreasing the temperature to 5°C for a period of 56 days caused a substantial increase in the COD of settled effluents, particularly for units with mean periods of aeration of 10 days or less (fig. 3). All units recovered when the temperature was returned to 10°C. These results indicated that at typical UK winter temperatures, a minimum retention time of 10 days in the aeration unit was necessary to provide substantial reductions in the BOD and COD of leachates.

No nitrification occurred in these experiments; removal of ammonia which took place was the result of conversion to organic nitrogen, or to a small extent volatilisation during aeration. Further investigations of ammonia removal by aeration of a leachate with a COD of 5000 mg/l, and initial concentrations of ammonia of from 80-1000 mg/l again showed no nitrification in units with a mean period of aeration of 10 days at 10°C. Although concentrations of ammonia up to 120 mg/l (as N) could be removed from leachate, by conversion to organic N - nitrogen during aeration, higher concentrations resulted in substantial amounts of ammonia in effluents, and also affected the settling properties of sludges which were produced.

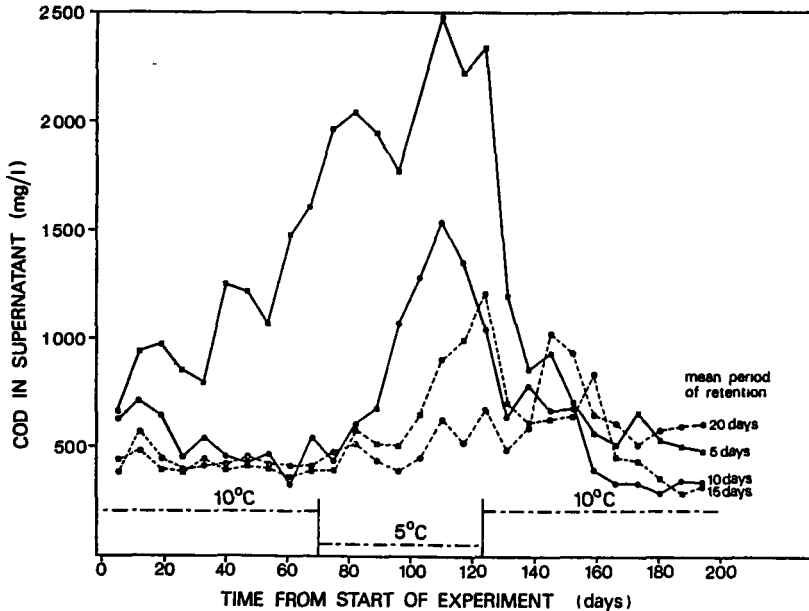
#### CONCLUSIONS

A simple system for recirculation of leachate by spray irrigation on part of a 2.5 ha lined landfill has worked well, although some ponding of leachate occurred in winter on small areas of the site where clay soil cover was present. The limited recirculation to date has shown no effect on the composition or volume of leachate compared with leachate from a control area, although there is a noticeable increase in storage of leachate within saturated parts of the site. Monitoring of this site will continue to at least March 1982 in phase 2 of the study.

**Figure 2** Percentage of COD removed from solution plotted against mean period of retention in days for complete mixing aeration units, operated at a temperature of 10°C without sludge recycling



**Figure 3** Effects of temperature on COD of settled effluent from units with various mean periods of retention



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Agrovej 8, DK-2630 Tåstrup, Denmark  
Contract no: 218-77-1 ENV DK  
Project leader: S.T. Jakobsen  
Title of project: Effects of fly ashes on nutrient uptake rates,  
growth rate and mineral content, including heavy  
metals of plants

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Objective of the research.

From a conviction that fly ashes deposited in concentrated amounts on small areas might give pollution problems, we believed that such problems might be less important by spreading the wastes in small concentrations on agricultural areas. But it seemed to us as the effect of incorporation of fly ashes on yield and quality of plants needed more investigations. Therefore, we performed experiments based on experiences obtained from previous experiments with solid wastes from a destrugas-sludge plant mixed in different soil types.

It was concluded from these experiments that the effects of the wastes was related to their influence on nutrient balances in the soil solutions, especially regarding the plant nutrient calcium. Changes of nutrient balances might give effect on growth rates, nutrient uptake rates and yields of crop.

Calcium in plants is not retranslocated from the top to the roots. As calcium is needed in the growing points of the roots that nutrient must be absorbed from the soil solution at the time it is needed. If calcium is withdrawn from the growing points even for a short time the cell division will stop, the membranes of the plastids will be destroyed, and the new roots will be brownish. The active nutrient uptake is then interrupted until new adventitious roots are developed a few days later.

Interaction between calcium and phosphate is of importance. Affinity between the two nutrients provides uptake of calcium when phosphate is absorbed by plant roots. But high concentrations of calcium in the soil solution or at the surfaces of the plant roots, may precipitate phosphates as sparingly soluble calcium phosphates. Therefore an acute stage of Ca-deficiency may occur. On the other hand,



not been fertilized with P for more than 10 years, and at the humic soil. The yield of grain was significantly decreased at these soils by application of fly ash 2.

Fly ash no. 3 had only small insignificant effects on growth rate, nutrient uptake rates and yield.

#### Supplementary pot experiment in the soil from Amosen

The large effect of fly ash 1 on the occurrence of Mn-deficiency could be explained either by a decreased availability of Mn in the soil, or by a decreased capability of the plant roots to absorb Mn from the soil. We thought that fly ash no. 1 had a disadvantageous influence on root development and root activity in this soil, which might be the cause of the yield depression. This assumption was confirmed in a supplementary pot experiment performed in a green house and it is described in report no. 4.

#### Pot experiment in 1978

Results from the experiment are described in reports no. 5 and 6. The following five alterations in the experimental plan influenced the results in relation to the results found in the first year:

1. The Ca-deficient soil from Sdr. Omme was included.
2. The humus soil from Amosen was heavy fertilized by a Mn-fertilizer.
3. Five different crops were grown.
4. Two NPK-fertilizers containing different amounts of P and K were used.
5. The establishment of the pot experiment was delayed until the 17th of May.

In the soil from Sdr. Omme a very pronounced Ca-deficiency occurred in control treatments and in treatments with applied fly ash no. 2. The growth of barley was stunted. The seedlings of lettuce, carrots and tomatoes died back. Application of fly ash no. 3 increased the growth of barley and some plants of other plant species survived. Application of fly ash no. 1 had a pronounced effect on that soil. The barley plants grew favourably throughout the season and the highest yield in the experiment was found in these treatments. Fly ash no. 1 had also a pronounced effect on growth of other-plant species.

Application of CaCO<sub>3</sub> also improved the early growth of barley.

But this improvement was not sustained throughout the season unless fly ash no. 1 was applied also.

In the humus soil from Amosen the fly ashes no. 1 and no. 3 had a positive effect on the yield of barley grain. This was opposite of the results found in 1977 and it might be because no Mn-deficiency was observed in 1978 as it was fertilized by a Mn-fertilizer.

In the second sandy soil and the loamy soil fly ash no. 1 reduced the early uptake of P and Ca and therefore also the growth rate. This was different from that found in 1977 and it is supposed to be caused by an interaction between a stunted root development owing to the low temperature in the spring and Ca-deficiency in roots. Later in the growing period the plants might grow faster where fly ash 1 was applied, but that could not surpass the early growth reduction in the sandy soil and the final yield was reduced.

Fly ash 2 reduced the uptake of P obviously because of precipitation of phosphates by the high content of Fe in fly ash no. 2. This had both effects on uptake of P and Ca and a reduced root activity.

#### Heavy metals

Fly ash no. 1 contained considerable amounts of Pb, Cd, Zn, Cu and Mn. It has been mentioned that the content of Mn was ineffective to remedy a Mn-deficiency at the soil from Amosen. The content of Cu made the concentration in the plant to increase but not to an undesirable level. The content of Pb in the plant was less affected by application of fly ash than by contamination from air pollution. Only in the uncultivated coarse sandy soil the watersoluble content of Cd reached considerable amounts. It was reduced to a quarter by application of  $\text{CaCO}_3$ . During the winter the concentration of Ca in the soil solution was reduced to very low concentration. The Cd was left in the soil as sparingly soluble Cd-salts. The content of Cd found in soil solution was reflected by the uptake in ryegrass.

#### Conclusion.

The effect of fly ashes on the chemical composition of soil solutions might be summarized by the following three rules:

1. A content of soluble anions, ex. chloride, sulfate or hydrogen-carbonate in fly ashes applied to a soil will give rise to an equivalent increase in contents of cations in the soil solution.



**TOPIC 34 : OIL SPILLS**  
**Collection and/or dispersion**

water tunnel using two-dimensional models of two scoop designs complete with their supports. Further tests in the same tunnel relate to a model undergoing forced vertical motion. In the tunnel tests, work-vessel speed was simulated by the velocity of the flow and the relative motion was represented by moving the point of suspension of the structure above the water.

The preliminary approach described above allowed the main problems associated with harmonic or random waves to be investigated. Here also, tests were carried out with two types of support.

### 2.3 Technological evaluation

In conjunction with the hydrodynamic studies described above, preliminary overall design evaluation were performed to assess the technological aspects of entire oil-skimming systems.

## RESULTS

### 3.1 Mathematical model

Based on data from the literature survey, the following results relating to a plate slipping across the free water surface were obtained :

- (a) Plate polar characteristic, i.e. lift coefficient in terms of drag coefficient ;
- (b) Position of point of application of resultant of hydrodynamic forces acting on plate in terms of angle of incidence ;
- (c) Position of stagnation point on plate in terms of incidence. This determine what fraction of the flow is scooped above the plate (the remainder flowing beneath the plate) ;
- (d) Thickness of scooped layer vs. depth of submersion in terms of incidence ;
- (e) Jet paths ahead of plate in terms of incidence at a velocity of 8 m/s ;
- (f) Relative motion between free surface and various points of attachment of scoop to work-vessel under Mediterranean wave conditions ;
- (g) As (f) for Atlantic conditions.

### 3.2 Physical models

Tests were carried out in a water tunnel using 1:6 scale models under Froude similarity conditions. Three types of test were performed for each of three scoop designs, viz. : flat plate, scraper-blade scoop, and a honeycomb scoop design.

The first type of test was carried out with the scoop immersed and at constant incidence and the hydrodynamic forces and moments exerted on the scoop were measured, viz. lift, drag and slip. Systematic study of the effects of depth of immersion and incidence led to a complete chart of the hydrodynamic force pattern around each scoop model. Once these applied forces were known, the attachments structures and the control system could be designed.

The second and third types of test were aimed at determining the behaviour of the oil scoops in waves and at estimating how closely the scoops must be made to follow the motion of the free surface. In the type 2 tests, scoop incidence was varied at constant depth to simulate incidence variation caused by waves. Under these conditions, the most effective scoop was the flat-plate.

In the type 3 tests, scoop immersion was varied at constant incidence to simulate wave-induced heave. The flat-plate was again the most effective design.

### 3.3 Technological studies

The aim of the technological studies was to dimension the structures required to secure the scoop and collector ducts to the hull of the work-vessel on the basis of the forces exerted on the scoops, structure design being such as to enable the scoops to be maintained at the free surface via an automatic control system.

After a number of exploratory studies, we are now looking into two main alternatives whose basic features are as follows :

- (a) Complete, independent, frame-mounted system that may be fitted to any vessel provided with appropriate attachment points ;
- (b) Minimum requirement for work-vessel modification, i.e. just reinforcement of anchor or attachment points ;
- (c) Scoop to fold down on deck or along the hull without protruding beyond the vessel's overall dimensions so that vessel can readily manoeuvre in harbour and proceed to spill ;
- (d) Rapid deployment of scoop into operating position when spill is reached.

#### 4. CONCLUSIONS

This research has provided the information needed to engineer a scoop-type marine skimming system. The tests carried out with scale models showed that the most effective scoop design in all situations is the flat-plate. Technological evaluation showed that a scoop-type skim system is feasible. The sketch below represents what we consider to be the most suitable design from amongst the various alternative designs we have assessed.

Contractor: Warren Spring Laboratory  
Contract No: ENV-402-UK  
Project Leader: Mr P R Morris  
Title of Project: "The Fate of Oil at Sea"

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#### Objectives of the Research

To study by means of controlled spills the relationship between the characteristics of the oil as it weathers and the prevailing physical environmental conditions, and to relate these findings to the effectiveness of dispersants on the spilled oil with time.

To undertake supporting work to provide other information on the use of dispersants using a laboratory scale tank test.

#### Materials and Methods

The sea trial experiments were conducted in an area of the North Sea in the region of 52° 10' N, 2° 40' E. Five separate controlled discharges of crude oil were undertaken, four of one tonne each, and one of 10 tonnes. As is customary any remaining oil was recovered or dispersed in each case on completion of the experimental work.

The viscosities of the samples were determined on board the observation vessel using a Ferranti portable rotary viscometer. Measurements were made as quickly as possible since the viscosities changed with time.

Samples recovered from the surface of the sea were examined for their response to chemical dispersants using a revolving flask technique, for compositional change using gas-liquid chromatography, and for water content in the emulsions.

The laboratory tank test work was conducted in a glass tank of dimensions 0.3m x 0.3 m x 1.5 m long fitted with a wave making device.

The tank was filled with a 3.5% NaCl solution and 50 mls of oil placed in a 25 cm diameter containment ring. The oil was treated with 2 mls dispersant added dropwise and the wave agitation started. Once the oil had been dispersed evenly into the water, the agitation was stopped and the tank water sampled



at mid water depth below the oil slick. The oil was extracted with chloroform and analysed spectrophotometrically.

## Results

### Fate Studies

#### (i) Evaporative Weight Loss

The results obtained show that the crude oils used lost between 23-26% by weight due to evaporation in the first two hours. After this initial rapid loss, due to evaporation of compounds with boiling ranges up to about the n-alkane Undecane (b pt 196°C) the rate decreases due to the lower vapour pressures of the remaining higher boiling point components. After four hours the evaporative losses had risen to between 27 and 30% by weight, and thereafter the evaporative loss rate is very slow. The evaporative losses occurred even though water in oil emulsion formation had occurred.

#### (ii) Emulsion Formation

Both the Kuwait and the Mixed N.Sea crude oils absorbed water extremely rapidly under the prevailing sea conditions (winds 15-20 knots), over 70% by volume in under an hour, rising to 79% after 4 hours. Ninian crude oil absorbed water less rapidly under similar sea conditions, achieving 66% water absorbed in 4 hours.

Forties crude oil, which was discharged under calmer conditions (wind speed 5 knots) absorbed water at a much slower rate, achieving 40% absorbed in 2.25 hours, with the water content remaining substantially constant up to 5 hours. The rate of water absorption by heavy fuel oil was extremely slow, reaching only 36% in 7.75 hours; this is believed to be due to the high viscosity of this bunker fuel.

#### (iii) Increase in Viscosity

The results of the viscosity determinations are given in Figures 1 and 2. It must be borne in mind that the time dependency of the change in viscosities is a function of the prevailing conditions at sea and that the curves for each oil represent only one such set of conditions.

The formation of water-on-oil emulsions is generally thought to be related to the asphaltene and crystalline wax content. The asphaltenes are considered to stabilize water-in-oil emulsions by forming a thick film around the water droplets within the oil phase and preventing them coalescing. The emulsion is believed to be further stabilized by adsorbed organic material and the products

of photo-oxidation.

#### (1v) Dispersion of Aged Oil

Samples were taken from the surface of the sea and submitted to a revolving flask emulsification test with a typical dispersant concentrate licensed for use by the Ministry of Agriculture, Fisheries and Food. The results showed that, after an apparent increase in efficiency probably due to the density increase of the oil, the dispersants eventually became ineffective. The decrease in efficiency is due to the viscosity increase in the oil; these sea tests bear out laboratory studies and show that above an oil viscosity limit of about 6000 cP at sea temperature, dispersants cease to be effective.

#### Laboratory Tank Tests

The rate of disappearance of the dispersed oil from the sample point, as it floated back to the water surface is a process which roughly follows first order kinetics, and constant for the rate of rise ( $-k$ ), together with the initial concentration of oil in the water  $(oil)_{t=0}$ , can be used as a measure of the efficiency of the dispersant.

A number of different dispersants were evaluated in the tank test using 50 ml of topped Kuwait crude and 2 ml of dispersant and the results used to put the dispersants in a ranking order. Spraying tests were subsequently conducted at sea using various oils sprayed onto the surface of the sea. These were then oversprayed with the dispersant and agitated with breaker boards. Both methods for evaluating dispersants gave the same ranking order.

Table 1 shows the effect of dispersant dose rate on efficiency. In these results the value of  $-k$  stays fairly constant whereas the oil concentration in the water immediately after agitation,  $(Oil)_{t=0}$  changes significantly with increasing dose rate of dispersant. It appears that for low treatment the droplet size of the oil carried into the water phase is similar to that for the high dose rates, but less oil is actually treated.

TABLE 1. - Effect of Dose Rate on Efficiency

Amount of Oil (ml)	Amount of Dispersant (ml)	k (min <sup>-1</sup> )	(Oil) <sub>t=0</sub> (ul l <sup>-1</sup> )
50	0.5	0.021	87
50	1	0.027	163
50	2	0.030	240
50	3	0.024	275
50	4	0.037	306
50	5	0.042	375

Conclusions

Fate Studies

It can be concluded that evaporation, accelerated by rapid spreading of the oil is the initial major process acting on crude oils at sea, and this may remove up to about 25% by weight of the oil in two hours under moderate wind conditions for the quantities split in the relatively small scale experiments conducted.

Evaporation and the formation of stable water-in-oil emulsions increase the viscosity of the weathered residues on the water to such an extent in several cases studied that they eventually become resistant to the effect of oil spill dispersants. The time in such cases in these small spills ranged from 75 mins to five hours, apparently reflecting the asphaltene content. The time taken for such changes to take place in larger spills is difficult to predict from these experiments, depending amongst other things upon sea state, water temperature and scale-up factor.

Laboratory Tank Tests

It can be concluded that:

- (i) In the tank test the rate of rise of the oil droplets after agitation might be used as a measure of the efficiency of the dispersant.

Results obtained with six dispersants correlated well with sea tests

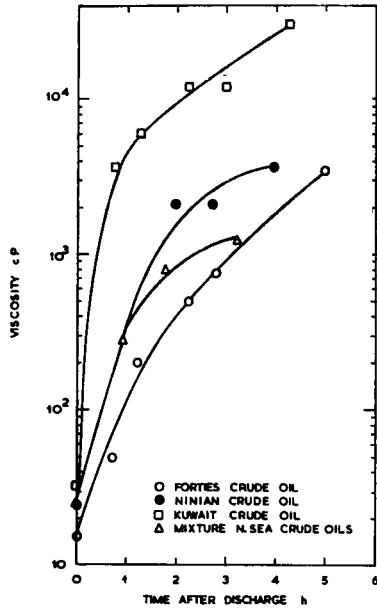
on oils of various viscosities. The tank test as conducted to date, however, has poor repeatability and the test requires further development.

- (ii) The effect of increasing the dose rate of a dispersant is to increase the amount of oil treated with dispersant and does not appear to significantly affect the size spectrum of the oil droplets formed.

Publications

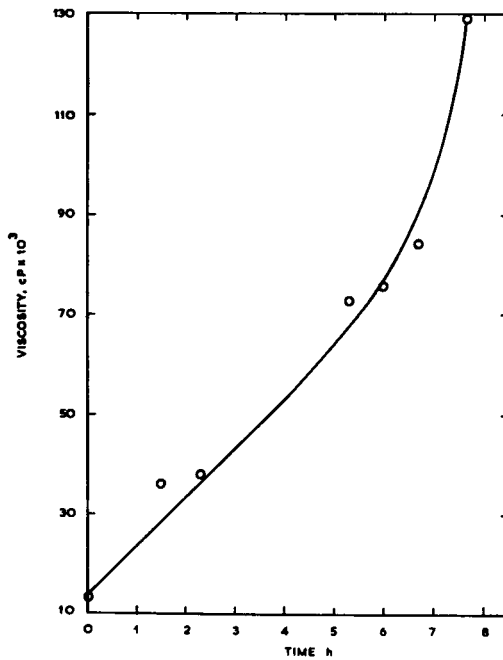
The Fate of Controlled Oil Spills at Sea. B.W.J.Lynch. Warren Spring Laboratory Report LR 390(OP).

The use of a Laboratory Wave Tank to Assess Oil Spill Dispersants. F N Martinelli. Warren Spring Laboratory Report LR391(OP)



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FIG 1. CHANGE IN VISCOSITY WITH TIME AFTER DISCHARGE AT SEA—CRUDE OILS



12923

FIG 2. CHANGE IN VISCOSITY OF HEAVY FUEL OIL WITH TIME AFTER DISCHARGE AT SEA

CONTRACTOR: Ministry of Agriculture, Fisheries and Food (MAFF)

CONTRACT NO: ENV 406 UK

PROJECT LEADER: R Lloyd BSc, FI Biol, FZS

TITLE OF PROJECT: Toxicity testing of oils in relation to dispersant testing

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Objective of the research

A list was prepared of the 11 oils most commonly transported through north-west European waters and the 9 oils produced in the major North Sea fields. Tests were made on each oil to determine:

- a) acute toxicity to brown shrimps (Crangon crangon) within a 96 h exposure period to obtain comparable toxicity curves and LC50 data;
- b) effect of three dispersants on the toxicity of each oil to brown shrimps under the conditions of the MAFF 'sea' test;
- c) acute toxicity to common limpets (Patella vulgata) under the conditions of the MAFF 'beach' test.

Materials and methods

Fresh samples were obtained of the 20 oils to be tested. Fresh Kuwait crude was included as a reference standard in all tests. Three dispersants were chosen to represent each of the major types of formulation currently available; these were the conventional (hydrocarbon solvent based) dispersant BP 1100X, the concentrate Synperonic OSD 20 and the 'self mix' concentrate "Corexit 9527".\*

Footnote

\* The sample of Corexit 9527 used for these experiments was different from the formulation in current production.

All 96 h tests were carried out in stirred tanks using adult brown shrimps (Franklin and Lloyd, in preparation). In each experiment a suitable range of concentrations of the test oil was used together with two concentrations of fresh Kuwait crude oil as a control on animal sensitivity. Dead animals were recorded and removed at frequent intervals and test solutions were renewed after 48 h. The median response times were then plotted against nominal oil concentrations to obtain toxicity curves. These tests were carried out between February 1979 and March 1981 and seasonal variations in sensitivity of the test animals were allowed for by rejecting data from those tests where the response to Kuwait oil was outside a narrow range and adjusting the data to take account of the variability of the response to tests with Kuwait oil within this range.

MAFF 'sea' tests using brown shrimps were carried out according to the methods described by Blackman et al, 1977. Each test included fresh Kuwait crude oil alone ( $1\ 000\ \mu\text{l l}^{-1}$ ), the test oil alone ( $1\ 000\ \mu\text{l l}^{-1}$ ) and the test oil ( $1\ 000\ \mu\text{l l}^{-1}$ ) with each of the three reference dispersants (equivalent to  $1\ 000\ \mu\text{l l}^{-1}$ ). Differences between mortalities (after 100 min exposure + 24 h recovery) occurring in the Kuwait and test oils, and in each of the three mixtures of dispersants and test oil, were determined by statistical analysis.

The standard MAFF 'beach' test (Blackman et al, 1977) was used to compare the toxicity of each test oil to limpets (6 h exposure + 72 h tidal recovery) with that of Kuwait.

### Results

The results of the three types of test on transported oils are summarised in Table 1 and on North Sea oils in Table 2.

It should be noted that the number of replicates used in the MAFF 'sea' test was less than the number now required (5) when a dispersant is submitted for Government approval under the Dumping at Sea Act 1974.

Conclusions

1. There was very little difference between the crude oils in their acute toxicity to shrimps.
2. Residual fuel oil was significantly less toxic than the crude oils.
3. There did not appear to be any relationship between the viscosity and toxicity of the crude oils.
4. The effect of each dispersant on the toxicity of the test oils (except Residual fuel) was similar to that of a mixture of dispersant and Kuwait oil; thus the toxicity of all the oils tested was affected in a similar way by each of the dispersants used.
5. Of the three dispersants tested Synperonic OSD 20 tended to have no effect on the toxicity of the oil (other than Residual fuel) whilst BP 1100X tended to reduce toxicity and "Corexit 9527" to increase it.
6. There was no significant relationship between the degree of toxicity of oil to shrimps and to limpets.

References

- Blackman, R.A.A., Franklin, F.L., Norton, M.G. and Wilson, K.W. (1977)  
New procedures for the toxicity testing of oil slick dispersants.  
Fish. Res. Tech. Rep., MAFF Direct. Fish. Res., Lowestoft, 39, 7pp.
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oils in relation to dispersant testing. Fish. Res. Tech. Rep.,  
MAFF Direct. Fish. Res., Lowestoft.

Oral Communications

- Toxicity testing of crude oil: presentation given by R Lloyd to the  
1st meeting of the Contact Group "Marine Pollution by Hydrocarbons",  
Aberdeen, Scotland, 10-11 November 1980.



Table 1 - The acute toxicity of 9 imported oils to shrimps and limpets

Oils (ranked in order of increasing viscosity)	LC(1)50% to Crangon crangon ( $\mu\text{l l}^{-1}$ ) (adjusted to take account of variations in sensitivity)			% mortality of Crangon in test test (mean of n replicates)			% mortality of Pecten yankovskii in beach test (mean of 5 replicates)				
	100 m	24 h	48 h	96 h	Oil alone			Test oil plus dispersant (n=3)			
		80	60	48	Kuwait (n = 4)	Test (n = 4)	SP 1100X	Synperonic OSD 20	"Corexit 9527"	Kuwait oil	Test oil
Abu Dhabi	650	80	60	48	88	90	67 <sup>-</sup> (b)	77 <sup>-</sup> (b)	93	45	59
Libyan	750	170	120	120	88	98 <sup>+</sup> (a)	63 <sup>-</sup> (b)	88 <sup>-</sup> (b)	93	46	47
Saudi Arabian	1 200	180	100	60	48	58	30 <sup>-</sup> (b)	50	73	45	48
Iran (light)	550	150	140	140	83	81	90	81	90	18	34 <sup>+</sup> (a)
Nigerian	900	65	54	50	33	64 <sup>+</sup> (a)	60	72	78	46	40
Kuwait	950	110	85	80	-	59	46	64	78 <sup>+</sup> (b)	-	-
Iraq	700	140	110	90	90	83	70	85	82	46	60 <sup>+</sup> (a)
Iran (heavy)	550	190	140	120	85	79	70	87	88	45	67 <sup>+</sup> (a)
Residual fuel	>4 000	>4 000	3 400	2 600	32	1 <sup>-</sup> (a)	0	0	2	27	10 <sup>-</sup> (a)

- = significantly lower mortality than oil control at 95% probability (p < 0.05)     a) Control = Kuwait oil     b) Control = test oil  
 + = " greater "

\*LC(1)50 is the median lethal concentration based on the nominal oil concentration present at the start of the test

Table 2 - The acute toxicity of 11 North Sea oils to shrimps and lipsets

Oils (ranked in order of increasing viscosity)	LC(1) <sub>50</sub> to Crangon crangon ( $\mu$ l l <sup>-1</sup> ) (adjusted to take account of variations in sensitivity)				% mortality of Crangon in 'sea' test (mean of n replicates)				% mortality of Patella vulgata in 'beach' test (mean of 5 replicates)		
	24 h	48 h	96 h	Oil alone	Test oil plus dispersant (n=3)		*Coraxit 95Z <sup>†</sup>	Kuwait oil	Test oil	Kuwait oil	Test oil
	100 m	500 m	1000 m	Kuwait (n = 4)	EP 1100X	Synparonic OSD 20					
Murchison	950	120	100	95	92	89	97	20	58 <sup>+</sup> (a)		58 <sup>+</sup> (a)
Thisile	700	150	130	64 <sup>+</sup> (a)	38	65	85 <sup>+</sup> (b)	45	69 <sup>+</sup> (a)		69 <sup>+</sup> (a)
Beryl	500	56	42	37	58	87	88	27	58 <sup>+</sup> (a)		58 <sup>+</sup> (a)
Montrose	650	110	88	70	67	85	95	46	62 <sup>+</sup> (a)		62 <sup>+</sup> (a)
Brent	1 100	57	36	32	18	57	83 <sup>+</sup> (b)	27	46 <sup>+</sup> (a)		46 <sup>+</sup> (a)
Piper	1 100	100	85	80	70	80	98 <sup>+</sup> (b)	45	78 <sup>+</sup> (a)		78 <sup>+</sup> (a)
Forties	1 100	110	92	85	68	80	93 <sup>+</sup> (b)	27	46 <sup>+</sup> (a)		46 <sup>+</sup> (a)
Ekofisk	650	85	60	50	78	86	92	44	43		43
Auk	1 100	85	75	70	19	65	62	44	33		33
Claymore	1 000	110	90	80	40	87	82	27	47 <sup>+</sup> (a)		47 <sup>+</sup> (a)
Argyll	800	150	100	80	73	78	87 <sup>+</sup> (b)	46	54		54

- = significantly lower mortality than oil control at 95% probability (p < 0.05)

+ = " greater " " " " " " " " " " " " " "

a) Control = Kuwait oil

b) Control = test oil

\*LC(1)<sub>50</sub> is the median lethal concentration based on the nominal oil concentration present at the start of the test

Contractor: The British Petroleum Company Limited  
Contract No: ENV-407-UK  
Project Leader: K.H. Bourne  
Title of Project: Disposal of Marine Oil Spill Residues by Application  
to Land

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A. PURPOSE OF THE STUDY

Following the spillage of crude oil at sea the residue that remains to be collected in subsequent clean-up operations off-or on-shore is often in the form of a highly stable, sea water-in-oil emulsion, commonly referred to as 'chocolate mousse'. Disposal of such a saline mousse presents a problem. Breaking the emulsion chemically is possible but expensive. Incineration is not possible without support fuel because of the high water content (up to 70 per cent) of the mousse and the material is not acceptable for addition to normal oil refinery process streams, because of its high salt and water content.

The general problem of the disposal of oil residues from refinery operations and land-based oil spills by sludge farming is currently under appraisal in Western Europe although it has been practised successfully for many years in the United States. As far as is known, however, disposal of saline oil emulsions by land farming methods has not been reported.

The research programme being carried out under EEC Contract No. ENV-407-UK(G) was designed to study the disposal of marine oil spill residues by application to land. The aims of the research were as follows:-

(1) To investigate in model laboratory systems the nature of the biodegradation of weathered crude oil by micro-organisms, the parameters affecting oil spill residue biodegradation and the isolation of factors or techniques which could be used to accelerate this degradation in soil;

(11) To follow the degradation of the same residue applied as a saline emulsion to agricultural soil in field plots and the effect of planting the oiled plots with selected plant species in the anticipation

that the combination of plants and micro-organisms would accelerate degradation on the minimum land area.

## B. RESEARCH WORK PROGRAMME AND RESULTS

The studies described in this report took place at two locations. The initial work on the preparation of the synthetic oil mousse and the experiments on the biodegradation of the oil blend used in the mousse, were carried out at the BP Research Centre, Sunbury-on-Thames, United Kingdom. The saline oil mousse was prepared in quantity at the Centre de Recherche, Lavéra Refinery, Société Française des Pétroles BP, in the south of France, for addition to soil plots prepared near the laboratories at Lavéra. Chemical analysis of oil biodegradation was carried out for both sites at Sunbury.

### 1. Composition and Preparation of the Saline Oil Emulsion (Mousse)

The investigations required a large quantity of a suitable synthetic saline mousse. The simulated weathered crude oil used was blended from 77% long atmospheric residue (BP  $\geq$  343°C) and 23% of a light gas oil cut (BP 232-243°C) both from Kuwait crude oil. The emulsion, containing 70% wt synthetic sea water, was prepared in a concrete mixer and had viscosity characteristics within the range of naturally produced emulsions.

### 2. Laboratory Studies at Sunbury

#### (1) Oil-soil Mixtures

Some preliminary experiments using a synthetic saline mousse applied to an aerated potting compost showed degradation of n-alkanes within six weeks. The addition of a commercially available preparation of hydrocarbon-utilising bacteria ("Petrobac") had little beneficial effect.

#### (11) Biodegradation of the Oil Blend in a Stirred Tank Reactor

The culture vessel, a one litre fermenter maintained at pH 7.0, 35°C, 2500 r.p.m. agitation with air flow rate of 6 litre/h, contained 600 mls of a mineral salts medium and 50 g of the oil blend component of the synthetic oil emulsion. The microbial inoculum used was

the "Petrobac" mixture or the predominant organism isolated from this population when actively degrading hydrocarbons in the fermenter.

Microbial activity during assimilation and/or co-oxidation of hydrocarbons was followed by gaseous exchange of the culture exit gases. The hydrocarbon degradation products were analysed by various methods using High Pressure Liquid Chromatography, Gas Chromatography, 19 x 19 Mass Spectrometry Matrix Analysis and High Resolution Low Volume Mass Spectrometry.

The following results were noted:-

- Initial breakdown of hydrocarbons was relatively rapid and was seen to accompany bursts of microbial respiratory activity between ca 20-70 hours, all the n-alkanes disappearing within 14 days. Degradation after this initial intense activity was then extremely slow.

- The total carbon lost as carbon dioxide from the oil mixture during the period of significant respiratory activity was ca 4 per cent by weight of the oil.

- At least 30 per cent of the oil was, however, oxidised to more polar derivatives, converted to biomass or lost as CO<sub>2</sub> during two weeks' biodegradation under optimal conditions.

- The order of degradation of oil constituents was approximately n-alkanes > isoalkanes > aromatics (up to C<sub>14</sub> and C<sub>32</sub> - C<sub>45</sub>) > naphthenes > middle range (C<sub>10</sub> - C<sub>30</sub>) aromatics. The latter appeared to be the most intractable compounds and included phenanthrenes, pyrenes, perylenes and larger fused ring structures.

- After the initial intense microbial activity which accompanied degradation of the more easily attacked components, the addition to the reactor of the dispersant BP1100x or n-hexadecane, caused further bursts of oxygen uptake and carbon dioxide evolution. It was not clear whether further oxidation of the original oil also occurred although assimilation was unlikely since carbon dioxide release was similar for the dispersant alone.

- At 45°C degradation of the oil residue was similar to that at 35°C. At 20°C, however, the course of degradation was considerably lengthened, with respiratory peaks occurring at approximately twice the times noted for other experimental runs.

- Sea water or 2 per cent NaCl in the culture medium did not inhibit the rate or extent of degradation indicated by gaseous exchange analysis.

- A changing bacterial flora was demonstrated during the course of oil degradation. The main respiratory peak was associated with the species Acinetobacter calcoaceticus which constituted 86 per cent of the organisms present in an active reactor population but is only a minor component of the "Petrobac" inoculum mixture. After 120 hours of degradation in the reactor, however, no viable organisms of this species could be isolated indicating a possible antibacterial action of some of the oil biodegradation products, eg short chain fatty acids.

- Growth of a monoculture of A. calcoaceticus in the oil blend resulted in similar respiratory changes to those seen with "Petrobac" as inoculum.

### 3. Soil Plot Experiments at Lavéra

The synthetic saline mousse was added to three of four plots measuring 3 m<sup>2</sup> of sandy agricultural soil. The oil emulsion was then tilled into the soil to give an oil-in-soil concentration of 5 per cent. One plot remained untreated as the control without oil. Two of the three treated plots were planted with leguminous species, one with broom and one with lucerne, in June. Climatic conditions were monitored throughout the year. Near drought conditions in the summer months necessitated heavy surface watering, especially of the planted plots, which led to waterlogging of the soil surface.

The microbial activity of the soil was examined regularly by gaseous exchange analysis on samples of soil from the plots incubated under controlled conditions in the laboratory. The amount of substrate carbon converted to CO<sub>2</sub> was estimated and soil samples were sent to Sunbury for analysis of ions and hydrocarbons using the methods mentioned in 2. (ii).

The following results were noted.

- Very little respiratory activity was apparent in the soil samples before late July (no samples taken between 6th June and 28th July). At the end of July, the control plots with and without oil addition still showed low activity while the two planted plots showed the effect of excessive watering in depressed microbial activity. Active disengagement of  $\text{CO}_2$  and mineralisation of carbon from all three oiled plots were, however, obtained in later samples taken in mid-September and mid-November. Slightly higher activity was indicated in the plot growing lucerne. The control plot without oil remained inactive throughout the year.

- Ion analyses showed that the added  $\text{Na}^+$  and  $\text{Cl}^-$  were washed out over the months.

- Substantial degradation of n-alkanes was noted during the period March to November with most activity after June, shorter chain n-alkanes (less than  $\text{C}_{18}$ ) being preferentially degraded. By November the unplanted plot had lost the greatest quantity of n-alkanes.

- Separation of the biodegraded oil into saturates, aromatics and residue fractions showed degradation of aromatics with an increase of polar residues apparently occurring at the same time as n-alkane degradation in the soil.

- More detailed analysis of the saturates and aromatics fractions based on a sample taken early in June (comparable results from the November samples are not available at the time of writing) indicate that, 13 weeks after oil addition, n-alkanes of  $> \text{C}_{18}$  were disappearing quite slowly, isoalkanes were untouched in contrast with the laboratory results, while the naphthenes and the lower and higher molecular weight aromatics had decreased, with the middle range aromatics unaffected, as in the laboratory studies.

### C. CONCLUSIONS

The biodegradation of the long atmospheric residue blend studied in the stirred tank reactor was similar to the patterns expected from reports in the literature. However, certain features of the work were

novel, ie use of optimal conditions, gaseous exchange analysis, and sensitive analytical techniques for examining the breakdown of the aromatics fraction. It was confirmed that oil biodegradation, after the initial breakdown of readily oxidisable hydrocarbons is extremely slow. Although at least 30 per cent of the oil was degraded within two weeks under optimal conditions, this process would be much slower in the field.

The results from the soil plots, although these were recognised as being only preliminary spot trials, supported the laboratory findings although approximately 21 weeks were required for the total extractable oil to fall by 30 per cent. However, whereas under optimal conditions attack on the different hydrocarbons fractions appeared to be sequential, in the slower conditions in the field, simultaneous attack on the saturates and the aromatics fractions seemed to be occurring.

No adverse effects in either the laboratory or field studies could be attributed to the presence of saline conditions.

No significant trends in oil degradation rate or pattern could be assigned to the presence of plants in the oil treated soil plots, in the period of the study reported.

In conclusion, it has been demonstrated that saline oil mousse, when applied to soil is slowly biodegraded to biomass, carbon dioxide and oxidised hydrocarbon derivatives. Laboratory studies have given insight into the pattern and stoichiometry of oil degradation and the influence of physical conditions on the microbial process. Longer-term studies would, however, be necessary to confirm subsequent total degradation of oils used in 'sludge farming' and ultimate rehabilitation of treated soils.





**RESEARCH AREA 4 : PROTECTION AND IMPROVEMENT OF THE NATURAL ENVIRONMENT**



**TOPIC 41a : ECOSYSTEM ECOLOGY - BIOGEOCHEMICAL CYCLES**

Contractor : Wildebiologische Gesellschaft München  
Postfach 170, D-8103 Oberammergau

Contract n° : ENV-336 D

Project leader : Ir. G. WIERSEMA

Title of project: Habitat use and potential distribution of the  
Ibex (Capra ibex L.) in the European Alps.  
An ecological evaluation for reproduction

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During the first research year (Sep 1980 - Aug 1981) of "PROJECT IBEX", substantial progress has been made in the achievement of the aims of the work (see research contract ENV-336-80-D (B)), being:

- The assessment of habitat suitability for Ibex in the European Alps, including the development and application of an appropriate methodology for a "wildlife rangeland survey".
- A delineation of the potential distribution of the Alpine Ibex.
- A well-defined strategy for (further) reintroduction, conservation and management.

The activities and results of the first research year have already been reported in three Quarterly Research Reports, comprising the following aspects:

- The 1st Quarterly Research Report (Sep - Nov 1980) describes the structural organisation, the international setting and the planning. Major aspects of the ecological requirements of the Alpine Ibex are discussed, together with habitat characteristics and habitat research methods. The tentative time schedule presented in this 1st report has been followed strictly, with the exception of field research in the Vanoise National Park which is now scheduled for Sep - Oct 1981.
- The 2nd Quarterly Research Report (Dec 1980 - Feb 1981) explains and illustrates the research work of "PROJECT IBEX" as a land evaluation for a particular land utilization type (being wildlife management and the conservation of Ibex with high alpine habitat). Furthermore this 2nd report reviews selected maps, references, aerial photography and satellite imagery. This report also includes a working paper on "The use of satellite imagery in the habitat research of "PROJECT IBEX".

- The 3rd Quarterly Research Report (Mar - May 1981) describes the preliminary appraisal of the interpretation of semi-detailed digitally processed satellite imagery, and presents some ideas on the logistics of the field work.

Major research innovations during the first project year have been achieved in:

- The development of an integrated "wildlife rangeland survey" methodology.
- The use of satellite MSS (multi spectral scanning) imagery.
- The elaboration of a scientific base for the management and conservation of high alpine habitat, illustrated especially on Ibx as a representative and almost ideal study object.

Next to the progress reports, 2 lectures and 1 publication have been prepared:

- "Rangeland research and the use of satellite imagery in the European Alps". Internal, technical consultation at the ITC (International Institute for Aerial Survey and Earth Sciences, Enschede, Netherlands). Date: 18 Mar 1981. "The conservation and management of Ibx in the European Alps". Intended to be published under the title "Die Erhaltung und Management von Steinbockkolonien in den Alpen" in the Yearbook 1982 of the "Verein zum Schutz der Bergwelt e.V. München".

Other publications will be completed during the second project year, and will treat the subjects that are mentioned under the "Introduction": ecological land survey in alpine environment, description and comparison of Ibx colonies in different climatic and geological zones, animal distribution and seasonal dependant habitat use, the use of satellite imagery and mapping methods for habitat requirements of Ibx. These publications are intended to serve as a base for the Ph.D thesis, which is planned for 1983. This thesis integrates the previous subjects and formulates a reintroduction and conservation strategy, together with a new approach a "wildlife rangeland survey".

Some aspects of the research work of "PROJECT IBEX" are planned to be presented at the "Third International Theriological Congress" in Helsinki, Aug 1982 under the section "Game Biology, Management and Protection of Endangered Species", and in the symposium entitled "Protection and management of large mammal populations".

The research of the first project year contributes to both species (ibex) orientated results, and to a more widely applicable scheme for "wildlife rangeland survey". For these reasons "PROJECT IBEX" responds adequately on the present-day needs for environmental conservation, as they are formulated e.g. in the Bern 1979 "Convention on the Conservation of European Wildlife and Natural Habitats".

Contractor : The Cévennes National Park

Contrat n° : 326-79-5 ENVF

Project leader : LEYNAUD E.

Title of Project : Research on the organisation and evolution of the ecological unities in the Cévennes National Park with a view to establishing the plan of management and control of this territory

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Preliminary note : The above mentioned contract is a follow-up to the contrat n° : 220-77-1 ENV F. The present paper describes the entire work that has been carried out in fulfilling these two successive contracts.

#### Objective of the Research.

The research concerned was of the type conducted with the aim of managing a national park. According to French legislation, a territory is classed "national park" when the conservation of fauna, flora, soil, waters and a in general a natural environment is of particular interest and it is important to protect this environment from all effects of natural degradation and preserve it from any artificial intervention liable to deteriorate its aspect, composition and evolution.

The mission of a national park does not therefore imply letting events take their course but, on the contrary, demands an active control capable of counteracting the consequences certain transformations might have on the environment under portection. A park's administrator should therefore be capable of foreseeing to what extent the natural riches he has the responsibility of preserving are subject to change.

To do this he must have access to that scientific information able to furnish replies to the following questions :

- which riches are to be protected ?
- what will become of these riches if this or that occur ?
- which measures should be taken to prevent or resist an evolution deemed harmful for the riches under protection ?

The aim of the research described in the following pages is to supply this scientific information in the particular case of the Cévennes National Park.

The methods used had therefore to enable discovery of the transformations and detection of their causes, mecanisms and consequences.

#### Materials and Methods.

It was necessary in order to succeed in this sort of research, to adopt a procedure permitting :



- the finest discernment of the complexity of the facts.
- discussions between the administrator and the scientists.
- the organisation of work in each branch.

To meet these three requirements researchers were led to :

- use the systemical approach as a common language for guiding reflection.
- plan work on the basis of a selection of sectoral studies to be undertaken.
- define a system of spatial unities which everyone was to use for collecting and treating data.
- adopt a research and management tool capable of inter-connecting data relative to the ecological and socio-economic systems : this was the triple computerised card-index - "owners" -, "farmers", "plots".

The system of spatial unities adopted is a system on three levels which are : the regional unity, the ecological unity and the plot. The Cévennes National Park (84 000 hectares) was thus divided into 4 regional unities which differ from each other as much by their physiographical as by their socio-economic and cultural characteristics. For the time being, only the Mont-Lozère - North Bougès regional unity has been studied. Each regional unity was split into ecological unities.

The ecological unity designates a certain geographical zone in which the geological nature of the basic rock, the mesoclimate and the aspect of the vegetation afford a certain degree of homogeneity. It is at the level of the ecological unity that the junction between the ecological type of approach and the socio-economic approach was made. Thus it is that in each ecological unity the inventory was made of :

- vertebrate communities and frondose anthropoid populations,
- socio-economic agents which have acted, are acting and are liable to act in the near future as well as the logic of their action.
- pastoral and forestry production potentialities and value as habitat for wild fauna.

The plot level permitted the linking up of the ecological unities with the decision unities, that is owners and cultivators.

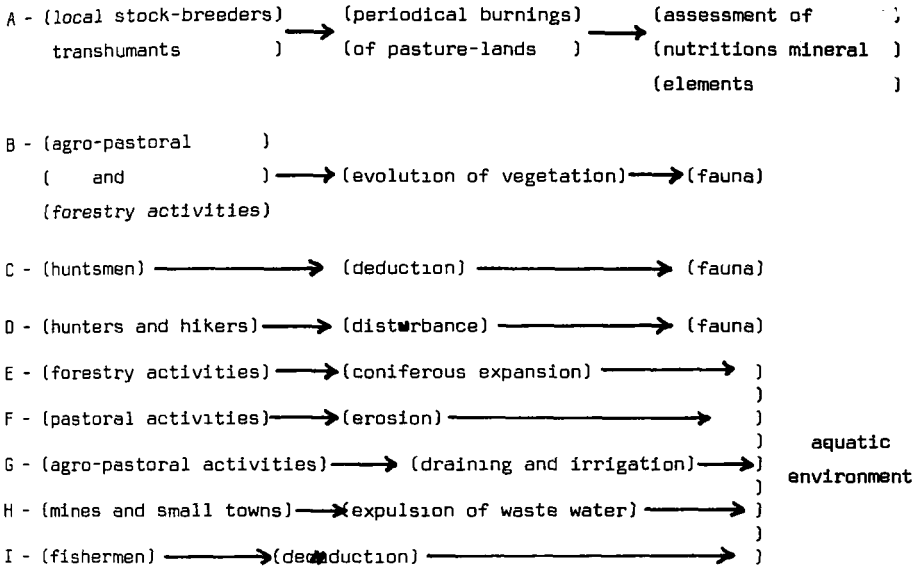
The sectoral studies to be undertaken were selected in the following way : on the basis of conservational objectives defined at the outset and information already acquired concerning the area, a set of schemas was established. The initial schemas showed the inter-relations between the main components of the systems under study, based on the knowledge of research-workers at the beginning of the study and the problems encountered. Afterwards, as results were progressively obtained, these problems were reconsidered and those which appeared without foundation were eliminated while, on the other hand, others, which had not been foreseen at the outset, were included. Thus the initial schemas have been rectified and refined.

The conservational aims chosen in order to elaborate the initial schemas were :

- to refrain from practices liable to upset the balance of nutrients mineral elements.

- to refrain from impoverishing fauna ; rather enrich it by reintroductions
- to maintain a good aquatic environment.
- to refrain from spoiling the aspect of landscapes considered remarkable as well as to prevent the disappearance of sites of scientific interest.

The main initial schemas which guided reflection are the following :



A modellisation of those parts of the schemas showing the functioning of the main mechanisms influencing conservational objectives was undertaken in order to simulate, following different scenarios, the possible ways the ecological unities will evolve during the next twenty years. The repercussions of these evolutions were evaluated and the scenarios classified according to whether they lead or not to evolutions compatible with conservational aims.

Simulation of the future evolution of the vegetation was effected using that simple model, transition matrices.

Simulation of the future evolution of potential values (pastoral and forestry production value as habitat for such and such a species of animal, desthetic value, possibilities of employment, etc.) was effected using "potentiality matrices".

This modellisation work used the data which was collected and treated by the triple computerised card-index.

#### Results.

The main results obtained concern the above B. H. and I schemas.

The forestry system now occupies half of the Mont-Lozere - North Bougès regional unity, the agro-pastoral system a quarter and the remainder is, for the time being, left wild.

The agro-pastoral system is independent of the forestry system, except as regards bilberry-picking.

Sheep-grazing, no longer a result of transhumance only, and insufficient cattle-grazing maintain the pastoral value of the runways on a level decidedly lower than it might be ; if past pastoral activities have led to a gradual impoverishment of the biological productivity of the region, it is not sure that present practices, for other reasons, be without risk ; at the same time, the study of the effects of the periodical burnings of under-grazed plant formations on the nutrients mineral elements situation must be pursued for several years before any conclusions may be drawn.

The results of the research already permit, however, the proposal of agro-pastoral practices more consistent with the maintenance, on the runways, of a biological productivity as close as possible to its maximum. The resulting herbaceous formations would not be of a lasting fundamental value for wild fauna, but the agro-pastoral system which adopted these practices could play the part of a genetic conservatory for certain rustic breeds of cattle.

As a general rule, the action of the forestry system represents a biological recovery compared with the situation inherited from the past (impoverishment owing to excessive clearing and sheep-grazing).

Silviculture leads to a scheduled transformation of the aspect of the forest lands, which have stemmed half from plantations on naked soil and half from an enriching of stunted beech-woods, which represented the remains of the original forest standard evolution processes concerning forest-lands under the effect of silviculture were described and the different stages composing them assessed as regards their value as habitat for wild fauna. On an average, in the long run, this value would seem to diminish in comparison with the present situation.

The desertion of a quarter of the territory has led to the formation of moors, whose typology has been established. The study of the spatial-temporal distribution of fauna has shown the great importance of these complex plant formations for the survival of a diversified wild fauna which finds food and shelter there. These formations constitute, however, only a transitory stage in the evolution of the vegetation ; their future depends on the decisions which will be taken by the agro-pastoral and forestry systems. On account of their importance for fauna, it would be fitting that the National Park accord them particular attention and it is not unlikely that this organisation be brought to intervene, at least with regard to some of them, in order to assure their maintenance by a treatment allowing them to evolve no longer in a linear but in a cyclic fashion.

No obvious relation was able to be established between the evolution of terrestrial ecological unities and the aquatic environment as far as the above E and F schemas are concerned. Measures taken within the scope of schema A should afford supplementary information on this subject in a few years time. Rivers are greatly exploited by anglers (schema) and captures constitute an important percentage of the net fishing production : nevertheless, trout stocks seem as yet unthreatened.

Generally speaking, the watercourses show no traces of pollution (schema H) but the possible opening of uranium mines would profoundly modify this state of affairs.

#### Conclusion.

This work was above all intended to test a research procedure admitting of an administrator-scientist dialogue as well as a real interrelation between branches. It seems to have been successful for the systematical approach compelled research workers to adopt an attitude of mind that led them to :

- give prior importance to the description of the relations between elements, rather than the description of the elements themselves.
- try and ascertain the order of importance of all the main data, rather than try and gauge the precise degree of importance of a few data easily measurable.

The result for this research was an improved efficiency of the means employed as well as a certain ease in achieving a synthesis, in order to arrive at an overall explanation of the studied phenomena.

At the same time, all possible ways of using the triple computerised index-card have not yet been explored.

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#### List of publications made within the scope of the present research

- BRISEBARE A-M, 1977 - Evolution of Transhumance on the Mont-Lozère,
- CAMUS D., Sept. 1977 - Agricultural Production Systems in the Mont-Lozère - North Bougès Region.
- CURMI J., Oct. 1978 - Estimation of the Agricultural Revenue of the Mont-Lozère - North Bougès Production Systems.
- DEJEAN R., May 1978, - Reflection on the Elaboration of a Method for the rapid Determination of the Productivity of Forest lands.
- DEMAISON A, April 1979 - Study of the Entomo-fauna and "hypergaion" arachnids in the ecological unities of the Mont-Lozère - North Bougès Regional Unity.
- DEMAISON A., Nov. 1980 - Preliminary Study for the Protection of the Entomo-fauna in the Mont-Lozère - North Bougès Regional Unity.

- DEVES B., Oct. 1978 - Study of River Fishing in the Mont-Lozère - North Bougès Regional
- FONTANEL and NEGRE, 1978 - Cartography and Ecological Inventory of the Humid Zones in the Cévennes National Park.
- GRANIER P., Oct. 1978 - Study of the Fodder value of Pasture Lands on the Mont-Lozère and the Socio-economic Consequences.
- GRANIER P., June 1980 - Study of the Influence of Agro-pastoral Practices on the Production and Evolution of the Ecological Unities in the Mont-Lozère - North Bougès Regional Unity.
- KERMABON J. (de), JAFFUEL R., DUBRAY D., July 1980 - Studies of Nesting Bird Populations in the Mont-Lozère - North Bougès Regional Unity.
- KERMABON J. (de) and BONNET J., April 1979 - Diurnal Birds of Prey in the Mont-Lozère - North Bougès Regional Unity.
- KERMABON J. (de) and the above, April 1979 - Ecological Approach to Mammal Density in the Mont-Lozère - North Bougès Regional Unity.
- LAMY and MIKOLASEK, Oct. 1978 - Hydrobiological Study of the Watercourses in the Mont-Lozère - North Bougès Regional Unity.
- LAMY G., April 1980 - Study of Impact on the watercourses following a Plan for Opening an Uranium Mine in the "Bondons" Commune.
- LAMY G., April 1980 - Conifer Plantations, Forestry Work and Quality of River Fishing.
- MACCAGNO Y., Oct. 1978 - Inventory of Remarkable Botanical Sites in the Mont-Lozère - North Bougès Regional Unity.
- MAIGNE Ph., Oct. 1977 - Socio-economic Study of the Mont-Lozère - North Bougès Regional Unity.
- PILLET Ph., June 1979 - Research on the Organisation and Evolution of the Ecological Unities in the Cévennes National Park with a View to establishing the Plan of Management and Control of this Territory. (Final report of the 1st phase - for the period 1st May to 30 th April 1979).
- PILLET Ph., February 1981 - idem.  
(Final report of 2nd phase - for the period 1st May 1979 to 30 th December 1980).
- PLAIGE V., Oct. 1978 - Phyto-ecological Contribution to the Pastoral Study of the Mont-Lozère - North Bougès Regional Unity.
- PLAIGE V.; May 1980 - Elaboration of a Typology of low-lying compound Moors in the Mont-Lozère - North Bougès Regional Unity.

CONTRACTOR: Istituto di Anatomia Comparata, Università di Siena,  
53100 Siena, ITALY

CONTRACT n° ENV/390 I

PROJECT LEADER: Prof. Aristeo Renzoni

TITLE OF PROJECT: The biogeochemical mercury cycle and the im-  
portance of the Hg/Se interaction for an assessment  
of Hg pollution in the Mediterranean

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#### OBJECTIVE OF THE RESEARCH

The aim of this program which is, in part, an extension of the "Comparative Studies on Heavy Metal Pollution and Balance in Selected Regions of European Seas" Contract n° 173, 77, 1, Contract Leader Prof. Nürnberg K.F.A. Jühlich begun under the first phase of the Environmental Research Program, was to:

- a) identify the source of the high mercury content observed in certain marine organisms of the Mediterranean Sea;
- b) evaluate the amount of mercury (and other heavy metals) in the sediments of various Tyrrhenian areas for a better knowledge of the biogeochemical cycle of the mercury in an area rich in cinnabar deposits;
- c) investigate whether high Hg concentrations are correlated to high Selenium content, and whether the controversial hypothesis that Se counteracts the toxicity of mercury could be proved.

#### MATERIAL AND METHODS

The material consists of:

- a) marine organisms (benthic and pelagic) collected in several areas of the Tyrrhenian Sea; i.e. the Mussel (Mytilus galloprovincialis), Norwegian lobster (Nephrops norvegicus), Striped mullet (Mullus barbatus), Anchovy (Engraulis encrasicolus), Swordfish (Xiphias gladius) and Red Tuna (Thunnus thynnus).
- b) samples of surficial sediments collected from coastal areas close to the inland cinnabar deposits and from areas at variable distance from natural and/or anthropogenic mercury

contamination.

All the animals were analyzed for mercury according to the usual technique (method reported in G.U. Italian Republic 1971) and after positive intercalibration exercise with the International Laboratory in Monaco.

The Striped Mullet and Norwegian Lobster were also analyzed for Selenium with an AAS (Perkin Elmer 300 S) equipped with the attachment for hydride generation and thermal decomposition (MES-1) following the procedure suggested by the AAS firm.

Total mercury in the surficial sediments was evaluated according to the procedure suggested by Agemian and Chau 1976 and reported in Baldi and Bargagli 1981.

Mercury fractions, ("leachable and non-leachable", according to the terminology introduced by Chester and Stoner 1975) were determined following the procedures reported in Baldi and Bargagli 1981.

## RESULTS

### Fauna:

Mussel - Specimens of this bivalve were collected in various localities along the Tyrrhenian coast. The mercury content was found to be slightly over the "background" level at all stations.

In one group of specimens from the station closest to the area with many cinnabar deposits, the mercury residues are always higher than in nearby (30-40 metres apart) groups. The fact, difficult to explain at present, seems to show that this species is quite useful for very restricted areas only.

Norwegian Lobster - The specimens were collected at three stations at various depths (100 and 300 metres) and at different distances from the coast and from the cinnabar deposits.

Mercury residues (from 0.2 to 3.1 ppm) were very similar in specimens of the same size and sex from all stations; all values obtained plotted against the body size show quite clearly that;

- a) the mercury level is positively correlated with the body weight;
- b) the correlation is quite different in the two sexes, the level in the female being higher than that in the male of the same weight class.

The many analyses performed have also indicated that there is no evident seasonal variation and that the selenium content of the muscle does not appear to be correlated with the mercury concentration.

Striped Mullet - Specimens obtained at three Tyrrhenian stations show a high variability of mercury levels (from 0.1 to 8 ppm) due to the difference between specimens of different age and sex and between specimens caught at various depths. In fact, mercury residues are higher in the males than in the females of the same size class; a positive correlation between mercury residue and depth of the fishing ground has been demonstrated at least in an area where specimens were available at various depths and distances from the coast and where anthropogenic mercury contamination could be excluded.

The first data on selenium content do not seem to show the typical molar ratio to mercury found in other top-predator fish species and in some marine mammals by other authors. Analyses of this element are continuing for this as well as for other marine organisms.

Swordfish - Mercury residues in this species are quite high (up to 4.4 ppm) for specimens of about 50 Kilos, and a positive correlation between mercury concentration and body weight exists.

Tuna - Specimens of Tuna (from very small to very large ones) obtained from various localities within the western Mediterranean basin show a wide range of values (from 0.1 to 4.2 ppm). The data obtained and processed showed that within the large size group there exist two populations: one with a high body burden of mercury and positive correlation with the length (and/or weight) and another with a lower concentration of mercury and no evident correlation with the size of the animals. The mercury content of Tuna collected in the Atlantic (just before entering the Mediterranean through the Strait of Gibraltar) instead is relatively low and is in the same range of values of those of the second population caught in the Mediterranean Sea i.e: the population with low levels; this finding suggests that the two groups present in the Mediterranean during the reproductive season are 1) specimens that have spent their entire life in this sea, and 2) those that live in the Atlantic Ocean and enter in the Mediterranean only for genetic migration.

Shore-birds - Preliminary observations in the eggs of the herring gull Larus argentatus and little tern Sterna albifrons albifrons from two quite different areas of the Tyrrhenian



Sea indicate high concentrations of mercury in both species, especially in those of the tern (average 13 ppm wet weight) feeding in a lagoon near an industrialized area. In tern eggs a positive correlation on a molar basis between this metal and selenium has also been found.

### Sediments

The study of the mercury concentration in the surficial sediments of a large coastal area of the Tyrrhenian Sea has shown that:

- a) high concentrations of this metal were found in a limited area close to the effluent of a chlor-alkali plant;
- b) high concentrations were also found in a large marine area off the mouths of 3 rivers draining several hills and the Amiata Mountain, all rich in cinnabar deposits.

Despite this fact, fish (especially Striped Mullet) caught in this last area, show much lower mercury residues than those fished at a greater depth and distance from the coast.

The possibility that the inorganic mercury deriving from the weathering of the cinnabar deposits of the Amiata region and the many mining and extracting processes is not immediately bioavailable has been considered likely; many other physical and chemical parameters however are being measured (surface area of samples with different grain size) pH, EH, organic matter content, organic mercury fractions, etc.).

Organic and inorganic mercury forms, and leachable and non-leachable mercury and its fractions, are now being studied and their bioavailability evaluated.

### Comments

Most of the fish caught in the Tyrrhenian Sea, and in several other areas of the Mediterranean Sea have high mercury residues.

Sediments close to anthropogenic sources of mercury and near rivers draining areas with rich cinnabar deposits also have high mercury concentrations.

Data obtained so far show that anthropogenic sources (even very substantial) affect limited areas only and that the high residues found in Mediterranean marine animals are predominantly due to natural sources (cinnabar deposits).

Besides these geochemical anomalies, the distance from the coast, from the natural source, the depth of the other fishing grounds, the forms of the mercury and many other physico-chemical parameters also evidently play a role of great importance in the dynamic interchange of the metal between air, water, sediment and biota.

Fig. 1 - Correlation between total mercury ( $\mu\text{g.g}^{-1}$ ; w.w.) and body weight in Nephrops norvegicus. - Open circles = females; solid circles = males; vertical lines = 95% confidence interval; large circles with vertical lines = average values; small circles = single values.

Fig. 2 - Map of one study area in the Tyrrhenian Sea showing the concentration of total mercury in the surficial sediments ( $\mu\text{g.g}^{-1}$ ; d.w.).

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Fig. 1

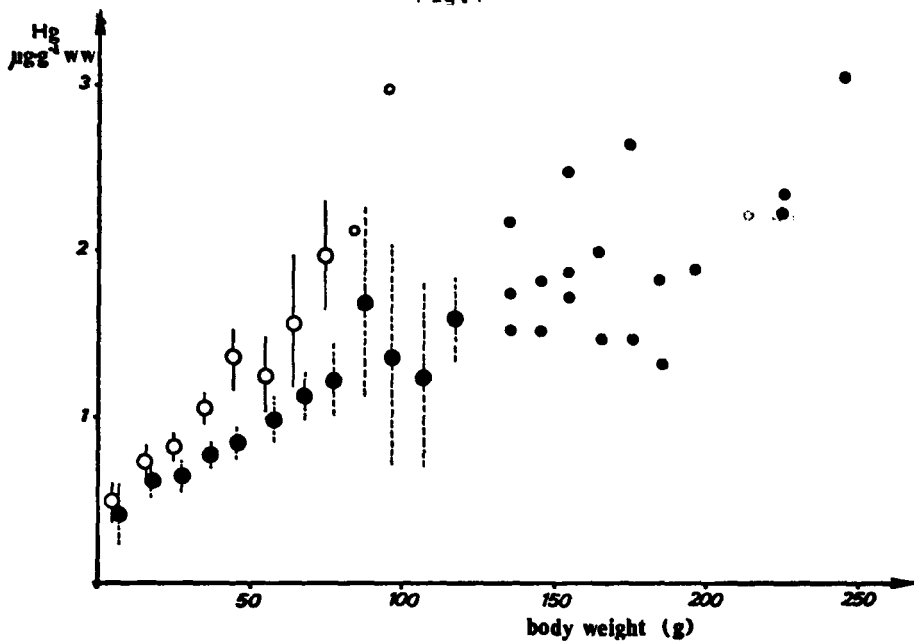
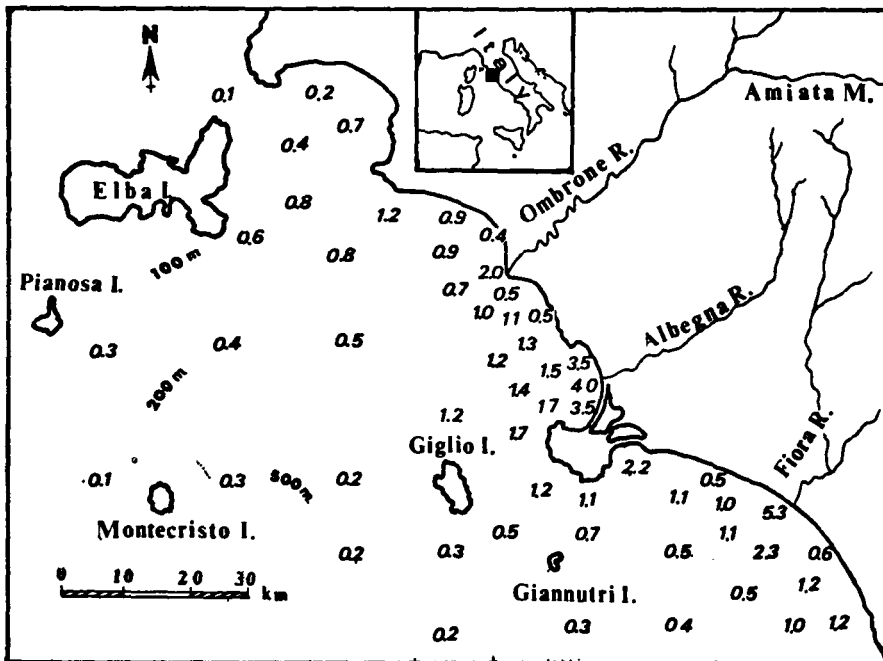


Fig. 2



Contractor De Staat der Nederlanden:  
Contract no. 331-79-1 ENV N  
Project leader. Dr. M.H. den Boer  
Title of project: Causes of the fluctuations in the population of harbour  
seals in the Dutch Wadden Sea

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#### Objective of the research

The harbour seal population in the Dutch Wadden Sea decreased significantly during the last decades. Calculations based on bounty data revealed that the population decreased from about 2700 in 1950 to about 900 in 1959. Annual aerial surveys - which were carried out since that time - showed a slight increase due to the stop of hunting. However, after 1964 a new decline occurred and since 1974 the population stays at a level of about 450 specimens.

During 1974 to 1978 the population dynamics of the population have been studied. Frequent aerial surveys provided data on the size of the population. These data have been used in a simulation model and the birth rate and the initial juvenile mortality were calculated. During boat trips an indication of the age composition was obtained by measuring track widths. By comparing these results with similar results from a stable population in Schleswig Holstein it appeared 1) that juvenile mortality in the first weeks in the Dutch population is higher than that in Schleswig Holstein but the overall mortality of pups in both areas in their first three months of life is of the same order; 2) that pup production in the Dutch population is low compared to the population in Schleswig Holstein; 3) that apparently immigration from elsewhere occurs.

To investigate the contribution of environmental pollution to the decline of the Dutch seal population tissues of dead, stranded animals originating from Schleswig Holstein, Denmark and The Netherlands were collected and analysed for PCB's, o,p'-DDT, p,p'-(DDT, DDE, TDE), dieldrin, aldrin, endrin, endosulfan,  $\alpha, \beta, \gamma$ -HCH, HCB, QCB (pentachlorobenzene), HEPO, total mercury, methylmercury, selenium and bromium.

Tests on possible differences in residue levels between Schleswig Holstein plus Denmark on one hand and The Netherlands on the other revealed that especially PCB levels were significantly higher in Dutch adult seals. Considering epidemiological and experimental data on the effects of PCB's on mammalian reproduction, strong support is obtained

for the hypothesis that PCB's are responsible for the decreased reproduction in seals from the Dutch Wadden Sea. Several investigations are carried out to test this hypothesis during 1979 and 1980.

- a) Aerial surveys are carried out to count the number of seals and to follow the reproduction and the population structure.
- b) Experiments are started of two different types
  - 1) To test whether the quality of the food is important; two groups of seals are kept. One of these groups will be fed with the normal diet of fish from the Dutch Wadden Sea, the other group will be fed with relatively 'clean' fish from the Atlantic.  
From both groups the reproductive performance will be studied in detail. Especially the levels of oestradiol and progesteron will be followed during the reproductive cycle.
  - 2) Another type of experiments is carried out with mink. In these experiments minks are fed with contaminated fish from the Wadden Sea. Other minks are fed with different components of this food.
- c) The behaviour of seals is observed during 1979 and 1980 in areas with much and with little disturbance.
- d) Wild seals are followed using telemetry equipment to study their wintering grounds.

### Results

The total number of seals present in 1979 amounted to 533 specimens and the maximum number of pups observed was 55. The number of pups was less than the foregoing year. As the figure for the total number was higher, the number of subadults and adults has increased, apparently due to immigration from adjacent areas. During 1980 the maximum number counted was 514 of which 63 pups.

The experiments using seals started at the end of 1980, at which time we were able to collect a number of seals at the East Coast of England. The first preliminary results of the experiments with mink revealed that the toxicity of Clophen A 60 was higher compared to Clophen A 30. The experiments will be extended during 1981 and 1982.

During 1979 and 1980 the behaviour of seals was observed concerning the effect of disturbance on the survival of pups. Time budget studies revealed information on a.o. the time the seals stayed on sandbanks during low water periods and the number and the total time of nursing

bouts. Up to now the results are published in internal reports.

During 1979 a number of seals were caught to study the dispersal and the activity pattern. A heavier type of nylon netting was used to prevent injuries to the head and flippers. Seals were caught on August 16 and November 1. On this last day two seals were marked with paint. A few days later they were seen on the same sandbank.

On August 16 an adult female was caught, provided with a transmitter and released. Details of the transmitter are published by Broekhuizen et. al. 1979. The first night she stayed in the neighbourhood. The second day she was tracked just over the German border, about 10 km to the east where she was also seen on August 19 and 20. On August 22 she was tracked again on the site where she was caught staying there in the neighbourhood until September 9. After that she shifted about 20 km to the north, 10 km north of Borkum. On September 16 she was seen near the island of Juist, 10 km to the east where she was found dead on September 29.

Automatic registration of the signal was achieved by positioning a whip antenna on a high chimney on the nearby coast. Since the transmitter is only on the air when the seal is on the surface, it can be seen from the type of registration when the seal is on a sandbank, swimming under water and coming up to breath, or swimming in shallow water. This year's individual rested on sandbanks for periods of 2.5 hours on the average (n=21).

#### Conclusions and comments

The results are preliminary especially regarding the experiments with seals and mink as these experiments just started during 1980. They will be continued under our own research programme. Regarding the field observations on the impact of the disturbance of seals we must conclude that the Dutch Wadden Sea must be regarded as an area with a rather high grade of disturbance. Observations are needed in a really undisturbed area to compare our situation.

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Oral communications

1979

- A meeting of the Marine and Estuarine Contact Group of the Commission of the European Communities in Southampton, April 26-27, was attended by Reijnders and a report on this project was presented.
- The annual meeting of the Marine Mammals Committee of the ICES in Warsaw, September 30-October 6 has been attended by Reijnders and two reports concerning the present study were presented.
- A meeting of the European Seal group, Göteborg, November 19-21 was attended by Van Haaften and Reijnders.

1980

- A report was presented by Reijnders in Manchester (March) to the European Association for Aquatic Mammals.
- The meeting of the Marine and Estuarine Contact Group on Texel (May) was attended by Den Boer who presented a report and Reijnders.
- The meeting of ICES in Copenhagen was attended by Van Haaften.
- A meeting of the European Seal Group in Cambridge was attended by Reijnders and Van Haaften.

Contractor: University of Copenhagen

Contract n<sup>o</sup> 251-78-1 ENV DK

Project leader: F.E.Eckardt

Title of project: *Use and Conservation of the Plant Cover in Greenland*

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A - Objective of the research

The objective of the project, as stated in the contract between the University of Copenhagen and the European Community, is to provide criteria for good management of the plant cover in Greenland. To achieve this goal, research was carried out in ecologically well differentiated types of vegetation in areas with continental and coastal climate, and comprised 1) the determination of CO<sub>2</sub>-exchange of the selected vegetation at leaf, canopy and ecosystem level as a function of various weather parameters, 2) the evaluation of change in standing biomass during the growing period, 3) the assessment of the effects of removal of green biomass on CO<sub>2</sub>-exchange, 4) the identification of limiting factors, climatic, genetic and nutritional, nitrogen fixation included, in plant dry matter production and 5) the testing of new techniques, such as remote sensing, as a means to generalize experimental results to larger land surfaces.

B - Materials and methods

Research work was carried out in three sites, Kangerlussuaq, Qeqertarsuaq and Upernaviarsuk. The Kangerlussuaq site, situated just above the arctic circle, about 30 km from the icecap, has a typical continental climate. The type of plant cover varies greatly with slope and azimuth, the southern slopes being characterized by a steppe-like vegetation. Level ground is covered with various dwarf bushes, with patches of grasses. The vegetation is grazed by muskox and reindeer.

The Qeqertarsuaq site, on the island of Qeqertarsuaq has a typical coastal climate. Extensive surfaces are covered by moors with dwarfshrubs. Lichens abound both among the dwarfshrubs and on the bare rock. Reindeer, introduced in other parts of the island, seem to prosper.

The Upernaviarsuk site, situated about 700 km to the south of Kangerlussuaq has a coastal climate too. Five types of plant cover can be distinguished: an overgrazed vegetation, a well fertilized hayfield, a dwarfbush vegetation at the foot of the hills protected against grazing, a dense her-



baceous vegetation on the hill sides, and a lichen and moss vegetation on the top of the hills, the latter two being grazed only occasionally. Upernaviar-suk is a well managed agricultural research center. The main grazing animal is sheep.

The study of the photosynthetic production has essentially been based - on the measurement of  $\text{CO}_2$ -exchange in preference to that of standing crop. This was to take account of the large variations in weather conditions from day to day and year to year which characterizes the climate in Greenland. Whereas it is difficult to assess the response of the above-ground biomass to the various climatic factors, due to the long relaxation times of the growth processes involved, the response of photosynthesis and respiration to, for example, irradiance and temperature, is almost immediate.

Among the several parameters measured within the context of the project can also be mentioned: nitrogen fixation, ammonification and nitrification, as well as the content of certain chemical components in the plant and soil material. Equipment for measuring  $\text{CO}_2$ -exchange included micrometeorological sensors and controlled-environment plant-chambers of different sizes. The choice of experimental plots was preceded by a detailed vegetation analysis.

### C - Results

Results of the project can be grouped into four areas of interest, the environment, the physiological behaviour of the plants, the effects of grazing and various human activities on the vegetation, and the generalization of experimental data for larger land surfaces.

The environment. - The total amount of solar energy received in Greenland in the middle of summer, over a 24 hour period, is of the same order of magnitude as that received in, for example, the temperate regions of the northern hemisphere. The difference is first of all in the length of the summer. The microclimate varies greatly with azimuth and slope, in particular in areas with continental climate. This is also true for the temperature conditions in the soil which are, furthermore, strongly affected by the plant cover. The plants create, so to say, their own edaphic climate, a fact which may contribute to explaining the mosaic structure of the vegetation. A major role in heat insulating the soil is played by the litter which often accumulates over several years, due to the slow rate of decomposition.

Nitrogen and phosphorus seem to be rather abundant in the soil, suggesting a small leaching. The pool of nitrogen is replenished thanks to abio-

tic fixation in the atmosphere and biotic fixation by, *inter alia*, blue green bacteria in symbiosis with lichens.

The physiological behaviour. - The photosynthetic capacity of the higher plants seems not to differ significantly from that of the temperate regions, and here, as there, the lowest values are found in sclerophyllous species. Plant production is intimately linked with the weather situations, with the brevity of the summer constituting the major limiting factor. In the region with continental climate, leafing and hence photosynthetic activity, in many species, does not start before summer solstice. On the other hand, maximum leaf area, mainly in grasses, is first attained when the length of the day is rapidly on the decline.

In a mature dwarfbush community, with blue green willow (*Salix glauca*) as the dominant species,  $\text{CO}_2$  absorbed and  $\text{CO}_2$  given off by the ecosystem during the summer period seemed almost to balance, the carbon surplus being barely sufficient to ensure the building up of next years leaves. Stem respiration, in spite of the low temperatures, was found to be an important item in the energy budget of the plant. It was small, however, if one takes into account the advantages of woody stems, which is, in addition to securing a good exposure of leaves to the sun's rays, to ensure maximum photosynthesis at an advanced date, due to early and rapid leafing. Stomata closed during the night, but not during daytime in spite of the dry climate. Closure of the stomata would have seriously strained the energy budget. One is here, clearly, in the presence of an ecosystem close to maturity. The vegetation resembles to some extent an old forest, *en miniature*, with the stems deeply buried in their own dead leaves, a fact which is consistent with the very high soil respiration rates observed.

A different behaviour was found in a dense grass vegetation in southern Greenland, where  $\text{CO}_2$ -absorption strongly exceeded that of  $\text{CO}_2$ -release. This gave rise to a translocation from the above ground biomass to the roots that was so strong that it took place even during regrowth few days after cutting. In the absence of cutting, on the other hand, above ground biomass increased to such an extent that, due to mutual shading of the leaves, net  $\text{CO}_2$ -absorption of the ecosystem became nil or negative when solar radiation was reduced by clouds in the afternoon. High leaf area index was an advantage only at high irradiancies. In plots overgrazed by sheep, in the same area, net production was much lower, due to the small leaf area, but at low irradiancies, solar radiation was then, in terms of photosynthesis, rather well exploited.

Lichens are exceptional in their capacity to sustain prolonged drought and to resume normal photosynthesis almost immediately after wetting. Their net  $\text{CO}_2$ -uptake, *i.e.* their dry matter production, is, however, much lower than that of higher plants. Some are capable of fixing atmospheric dinitrogen thanks to symbiosis with bluegreen bacteria, and may play some role in replenishing the reserves of nitrogen in the soil. The amount of nitrogen supplied to the ecosystem this way, in the area studied, proved to be of the same order of magnitude as that received by precipitation, or only a little higher. For one thing, nitrogen fixation is strongly dependent on temperature and water stress, for the other, nitrogen fixing lichens occupy only a small fraction of the vegetation covered areas. The nitrogen content of the soil under nitrogen fixing lichens seems not to exceed that found under lichens lacking bluegreen bacteria. The nitrogen fixing lichens apparently use the nitrogen for their own growth, so that the nitrogen first will be added to soil after their death. This behaviour is easy to understand considering the high energy requirements of the nitrogen fixing process.

Effects of grazing. - A fifty percent reduction in the leaf area, by clipping, caused a much higher reduction of  $\text{CO}_2$ -absorption in grasses, immediately after cutting, than in the dwarfbush. In grasses, however, as distinct from the dwarfbush,  $\text{CO}_2$ -absorption of the remaining leaves, in particular if the cutting was performed close to the soil surface, could increase drastically within a week. If cutting was performed level with the ground, it caused, however, before photosynthesis resumed, some respiratory loss of  $\text{CO}_2$  from the ecosystem, and this for a day or two. A long-term effect of heavy grazing can be a considerable change in the species structure of the plant cover.

In the Kangerlussuaq region the dwarfbush is highly sensible to human intervention and, if destroyed, it is often replaced by grasses, such as meadow grass (*Poa pratensis*). This transformation of the plant cover is due, apparently, to the incapability of the mature ecosystem to restore itself, at least for a considerable span of years. The transformation seems not to be caused by a change in the nutrient status of the soil, brought about by the release of chemical substances from the decaying willows. On the one hand, the amount of nutrients contained in the aboveground biomass is small in comparison with that of the soil, on the other, the difference in chemical composition of the soil under the two types of plant cover is insignificant. In southern Greenland, where the summer is longer, the same willow species tolerates heavy grazing for extended periods of time.

One of the effects of overgrazing in southern Greenland is a much reduced dry matter production. This is due, apparently, to the reduction in the photosynthetically active biomass as photosynthesis per unit above-ground biomass remains of the same order of magnitude in the overgrazed area as in the hayfield. It is, therefore, not to be expected that dry matter production will substantially increase by merely adding fertilizers as, for example, in the hayfield. Some protection against grazing is also necessary. Overgrazing seemed not, on the other hand, to be accompanied by any major increase in leaching, nor in soil erosion. There is some evidence to support the idea, however, that the microbiological activity in the soil, which makes nutrients available to the plants, can be strongly inhibited, a point which ought to be studied more in detail.

Generalization of data. - Attempts to use Landsat data for generalizing experimental field data for larger land surfaces were not entirely successful, as experimental plots could not be identified with sufficient certainty. In working on the computer, with an Interactive Digital Image Manipulation System software packet, it became clear, however, that image manipulation, on the basis of satellite data, does actually constitute a most important source of inspiration, which may give valuable ideas as to how the plant cover can be classified and mapped.

There can be little doubt that analyses can be brought much further than realized in the framework of the present project, and that many experimental productivity data can be generalized for larger land areas, in particular when imagery from future satellites, with higher resolution and better selected spectral sensitivity bands, become available. It became also clear, though, that this new technique involves a certain risk and that, without frequent verifications in the field, such analyses may be misleading and give rise to considerable errors. A particular difficulty is to eliminate the effects of shadows. Vegetation mapping is clearly an iterative process, where field observations must go hand in hand with computer work. The enthusiasm for the new technique should not obscure the fact that field studies will continue to constitute the major part of the work for a long time still.

#### D - Conclusions

The major limiting factor in plant production in Greenland is the radiant energy input, which determines temperature conditions and the rate of photosynthesis. Photosynthetic capacity in the higher plants is of the same order of magnitude as that found in temperate regions, but as the summer is

short, annual dry matter production is small. Dry matter production, day by day, varies strongly with the various weather situations.

Mature dwarfbush ecosystems, in some areas, are highly sensible to human intervention, and the destruction of the plants can entail their replacement by a grass vegetation. This change, it is true, disregarding aestetical consequences, may sometimes be rather beneficial. In the case studied, the grasses constitute an essential food source for the reindeer population, whereas the dwarfbush is only just occasionally eaten. The longterm effects, however, of this change, are unknown.

Translocation between root and shoot plays an overriding role for survival in the higher plants. In grasses, in particular, it endows the plant with an extraordinary capacity to resume photosynthesis shortly after removal of the above-ground biomass, whether by mowing or by grazing. This translocation presupposes, however, the existence of a well developed root system with high storage capacity, a fact which suggests that recently established grass crops may be quite vulnerable to grazing.

Overgrazing is followed by a strong reduction in plant production, due to inefficient use of the solar energy, in particular at high irradiances. Plant production can, therefore, not be expected to increase by mere fertilizing if grazing is not restricted. Overgrazing seems not, on the other hand, to give rise to any alarming increase in leaching or erosion, as the nitrogen and phosphorus content of the soil remains rather high, but some effect on the microbiological activity in the soil, thanks to which nutrients are made available to the plants, is likely. It is a question that is still left open.

Photosynthetic capacity in lichens is much smaller than in higher plants. At the same time, photosynthesis depends, as also nitrogen fixation, on a constellation of factors, among which the water content of the plant, plays a prominent role. CO<sub>2</sub>-absorption, as well as nitrogen fixation, is, therefore, limited to short periods of time, a fact which explains the very low dry matter production in lichens and their vulnerability when exposed to grazing.

An important problem in research in Greenland is the generalization, for larger land surfaces, of results from plant eco-physiological studies carried out in small experimental field plots. Here remote sensing from airborne and satelliteborne platforms, if intimately linked with vegetation analysis and plant eco-physiological work, may offer a good solution in the futur.

Publications. Eckardt, F.E. (1978) - Solen og den fotosyntetiske produktion - Forskning i Grønland, no 1 p.8-11. Eckardt, F.E. (1980) - Application of Landsat imagery to vegetation analysis in Greenland - mimeographed, 15 pp. Eckardt, F.E., Heerfordt, L., Jørgensen, H.M. og Vaag, P. (1980) - Photosynthetic production in Greenland as related to climate, plant cover and grazing pressure - manuscript.

**TOPIC 41b : OZONE SHIELD DEPLETION**

CONTRACTOR : Université de Paris Sud  
CONTRACT N° : 299-77 ENV F  
PROJECT LEADER : Paul-Marie Guyon  
TITLE OF PROJECT : Relaxation of Atmospheric Molecules excited by  
Photon Impact

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I. Molecules, excited by 40 ev photons, i.e. 200 to 300 Å range present a number of quasistationary states in addition to the various neutral dissociation and ionization continua. These quasi bound states also called prioritized or predissociated states can decay non radiatively to produce neutral and ionized products in various energy states. These products are precursors of further collisions (reactions) which lead to the final physicochemical equilibrium of the system.

The knowledge of the spectroscopy of these molecules in that energy range as well as the determination of the branching ratios into final decay channels is thus necessary for an accurate description of atmospheric physicochemical processes. We proposed an experimental approach of these problems which may also stimulate new progress in the theory of excited molecular states dynamics.

II. Experimental procedure :

Synchrotron radiation dispersed by various vacuum ultra violet monochromators is used to excite the molecules. The excited states decay is studied by several techniques :

- 1°. Time resolved fluorescence spectroscopy
- 2°. Fluorescence excitation spectroscopy
- 3°. Photoionization mass spectrometry
- 4°. Photoelectron spectroscopy

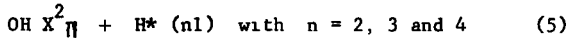
These experiments are performed on an ensemble of molecules at low pressure in collision free conditions.

### III. Results Photodissociation of an isolated molecule

$H_2$  - using the highest resolution achievable with the monochromators (i.e; 0.04 Å) it was possible to excite selectively the rovibronic levels of  $H_2$  (single rotational levels of excited electronic and vibrational states), both below and above neutral dissociation limits  $H^* (n1) + H (1s)$ ,  $n = 2, 3$  and 4 and ionization limits  $H_2^+ + e^-$  ( $v' = 0, 1, 2$  etc.).

We observed the competition between the radiative fluorescence decay and the non radiative decay processes : ionization and dissociation. The fundamental study leads to a better understanding of the couplings between discrete and continuum states. (1, 2, 3, 4)

$H_2O - H_2O$  was excited between 10 to 30 e v, where ionization should be the dominant process We observed several dissociative continua and predissociated states and identified some of the decay channels :



$N_2O$ ,  $COS$  and  $CS_2$  (7,8). Excitation of these molecules above the first ionization limit yields excited neutral dissociation fragments  $N_2$ ,  $NO$ ,  $CO$  and  $CS$  whose emissions can be distinguished from that of excited ions using appropriate filters It is observed that dissociative continua compete with ionization continua to relax the excited neutral Rydberg states Interaction of both continua results in a profound modification of the final ion state distribution from that expected for a direct ionization process. This mechanism called Resonance Autoionization resulting from an efficient conversion of electronic energy stored in the ion core into vibrational energy produces highly excited vibrational levels of molecular ions such as  $N_2O^+$ ,  $COS^+$  and  $CS_2^+$  whose reactivity may differ significantly from that of ground vibrational states of the ions These distributions are measured in the following experiment.



CH<sub>3</sub>ONO CD<sub>3</sub>ONO C<sub>2</sub>H<sub>5</sub>ONO C<sub>3</sub>H<sub>7</sub>ONO and C<sub>1</sub>NO (9, 10, 11).

With these larger R - ONO molecules which have a preferential dissociation path : RO + NO we wanted to test how the radical structure R affected the fragmentation quantum yield..

It appears that the quantum yield decreases with the size of R but the reaction remains non statistical i e. non complete randomization of energy.

#### PHOTOIONIZATION OF AN ISOLATED MOLECULE

The decay of excited molecules into the ionization channels results in the production of ions in various states of internal and kinetic energy . The branching ratio among these final states varies sharply with excitation energy when one encounters resonance states (preionized states). Using photo electron spectroscopy, we attempted to measure these branching ratios . Vibrational distributions of the final ion states was thus determined as a function of incident photo energy (12, 13). An alternative approach was to measure partial photoionization cross sections for radiative states of molecular ions by fluorescence excitation spectroscopy (14, 15).

#### 1° Photoelectron Spectroscopy studies

A new photoelectron spectroscopy was developed using the time of flight analysis of photoelectrons kinetic energy (16) This method specially sensitive to low energy electrons was applied to triatomic molecules such as N<sub>2</sub>O, COS and CS<sub>2</sub>. It resulted in the discovery of a new photoionization process : resonant autoionization It was observed that these molecules, excited in well defined regions of the absorption spectrum, Franck Condon Gaps, actually produced highly vibrationally excited ion states with a distribution sharply peaked at the exciting photon energy :  $E(\text{ion}) = h\nu$  thus yielding low energy photoelectrons. This process, was shown by photodissociation experiments to be associated with excitation of dissociative neutral states.

The same technique applied to diatomic molecules such as  $O_2$  showed a fast variation of ion vibrational distribution when one excited the molecule over a resonance state. These experiments lead to a better understanding of the autoionization process and of shape resonances.

## 2. Fluorescence excitation Spectroscopy (FES)

The analysis of the Fluorescence yield from excited ion states as a function of incident photon energy was done for  $O_2^+$ ,  $b^4\Sigma_g^-$  and  $A^2\Pi$ ,  $N_2O^+$   $A^2\Sigma^+$ ,  $N_2^+$   $B^2\Sigma^+$  and  $A^2\Pi$  and several other polyatomic ions. This method which is very sensitive yielded a number of informations about the threshold photoionization cross sections, and consequently on the Autoionization processes.

## 3. Photoelectron photoion coincidence experiments

After being formed by photon absorption a molecular ion such as  $O_2^+$  or  $N_2O^+$  can undergo further dissociations process. If one measures in a delayed coincidence experiment the fragment ion and the photoelectron, both kinetic energy analysed, one determines the dissociation branching ratio into final fragment states for energy selected parent ion states.

In the case of  $O_2^+$  all the electronic states observed by photoelectron spectroscopy beyond the  $O^+$  ( $^4S$ ) +  $O$  ( $^3P$ ) dissociation limit are seen to be predissociated mostly to higher dissociation limits of  $O^+$  +  $O$  yielding electronically excited fragments. In the case of  $N_2O^+$  two different predissociation mechanisms have been observed for  $N_2O^+$  ions having almost the same internal excitation energy depending whether the parent ion was initially formed in the electronically excited state  $A^2\Sigma^+$  or in the vibrationally excited ground state. In the first case  $NO^+$  +  $N$  are main products whereas in the second case  $O^+$  +  $N_2$  are essentially produced.

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  - b) Non Franck Condon transitions in autoionization of  $N_2O$  présenté par P.M. Guyon
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Contractor : Faculté de Sciences, Université de Reims, F.  
Contract n° : 300-77 ENV F  
Project Leader : Prof. P. JOUVE  
Title of project : Construction of a Fourier-transform spectrometer  
with a path difference of 3 metres

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#### CHARACTERISTICS OF THE INTERFEROMETER

Maximum path difference - 3 metres; resolution -  $3.5 \cdot 10^{-3} \text{ cm}^{-1}$ .

Diameter of the optical system - 120 mm.

Precision of frequency measurements - to within  $10^{-9}$ .

Minimum recording period - 10 minutes.

Method of carriage displacement : in steps or continuous movement.

This apparatus is a slightly modified copy of the interferometers constructed at the Aimé Cotton Laboratory by Pierre Connes and Guy Michel and their co-workers.

This interferometer consists of five main parts :

1. The illuminating optical system and fixed cat's-eye, separator and mixer, optical detection system and IR detector;
2. A carriage supporting the movable cat's-eye and the carriage slave mechanism.
3. A laser-beam interferometer producing the fringe signal required by the slave mechanism.
4. A device for the recording and acquisition of the interferogram which has been sampled.
5. A computer for the real-time and batch processing of the interferogram's Fourier transform.

#### STRUCTURE OF THE FOURIER TRANSFORM SPECTROMETER

The spectrometer structure is divided into two parts, i.e. an interferometer which produces an interferogram and a computing system which interprets the interferogram in the form of a spectrum. To these two parts we shall add a third, which will adapt the interferometer to an optical system for recording solar spectra.

## I. Interferometer

The interferometer is of the Michelson type. We have chosen an optical system with a diameter of 120 mm. This optical system is much too large for solar spectra, but we chose it to be able to record the thermal emission spectra of constituents of the stratosphere.

### 1. Optical system

#### (a) Mirrors

The interferometer is of the "folded-arm" type. The illuminating and focusing mirrors on the detectors are spherical. The optical systems on each arm are of the "cat's-eye" type, i.e. an afocal system consisting of a concave mirror and a convex mirror having coincident centres. These systems are such that the reflected beam always remains parallel to the incident beam when the entire system is displaced.

#### (b) Separator

The separator works on the transmission and reflection principle. We intend to cover the 0.8-20 microns waveband.

We have provided for three separators, namely, one of Infrasil covering 0.8 to 3 microns, one of  $\text{CaF}_2$  covering 2.5 to 9 microns and one of ZnSe covering 8 to 18 microns.

Each separator consists of two semi-circular plates with a degree of planeness (flatness) equal to  $\lambda/5$  and a degree of parallelism equal to  $\lambda/2$ . The two plates are placed in the same vertical plane but separated by distance of  $[n - 1/n] t$ , where  $n$  is the mean index of the working range and  $t$  the thickness of the plate. The path taken by the light beams is shown in Fig. 1.

#### (c) Mobile carriage

The movable cat's-eye is mounted on a moving carriage of the "slave" type. The cat's-eye is supported on an articulated parallelogram which is given the movements of a pantograph by a low amplitude



linear motor. The articulations of the parallelogram are held in place by flexible steel blades. The weight of the parallelogram and the mirrors and the tension in the springs are adjusted to make the system astatic, i.e., the return force when the parallelogram is moved relatively to the carriage is negligible. The position of the parallelogram in relation to the carriage is measured with a travel sensor which has a working range of  $\pm 0.25$  mm, accurate to within one micron.

The carriage itself supporting the parallelogram in this way travels on roller bearings over a track formed by a stabilized 46 aluminium beam. A direct-current motor pulls the carriage through two cables. The speed of travel is measured by a tachymetric dynamo and the position is indicated by a 10-turn potentiometer.

## 2. Electronic equipment

### (a) Slave-mechanism and measuring laser

The laser used is a 500 microwatt He-Ne laser, stabilized on the saturated absorption line of iodine. This laser was built in our laboratory to drawings supplied by Mr. Cerez of the Atomic Clock Laboratory at Orsay, Paris. The frequency of the laser is stable to within  $10^{-9}$ . This laser is used for measuring the path difference in the interferometer. Two detectors are used for picking up the fringes obtained when the path difference varies in the interferometer.

### (b) Movable cat's-eye slave mechanism

The slave mechanism electronic equipment is the most complicated part of the system. It works on the principle that the detectors of the fringes obtained in laser light emit a signal with a frequency proportional to the path difference in the interferometer (frequency A).

We create electronically a signal with a programmable frequency. The sampling theory tells us before each spectrum the interferogram pattern to use. The signal frequency is then proportional to the required path difference (frequency B).

A comparison of the frequencies A and B gives a signal which is extinguished when these frequencies are equal, i.e., when the required path difference is equal to the actual path difference. This signal is called an "error signal". The low-frequency component of this error signal actuates the linear motor and the high frequency component the slave-mechanism ceramics. When this signal is extinguished internal modulation is applied, the infrared flux through the instrument is recorded and we proceed to the next pattern. The interferogram is recorded thus, pattern by pattern.

(c) Electronic measuring equipment

This consists of a synchronous detection amplifier and a voltmeter system for putting the data in a numerical form.

II. Data acquisition and spectrum calculation system

This very important part of the instrument is completely bought-in. It consists of a PDP 11/34 computer, a printer, an alphanumeric screen and a plotting table.

1. Data acquisition system

Through an interface, the PDP 11/34 computer receives the data transformed by the voltmeter. These data are memorized in real time on a hard 5 Mega octet disk.

(a) Spectrum calculation

The spectrum is calculated and plotted by batch processing. The capacity of the central memory system of the PDP 11/34 computer has been raised to 256 k octets and we have added a floating processor which reduces the calculation time in the central memory by a factor of 5.

The points of the interferogram are transferred in blocks to the memory disk and, after the calculation process has been performed, the calculated spectrum is preserved on another hard 5 Mega octet disk. The plotting of the spectrum takes place in a third stage.

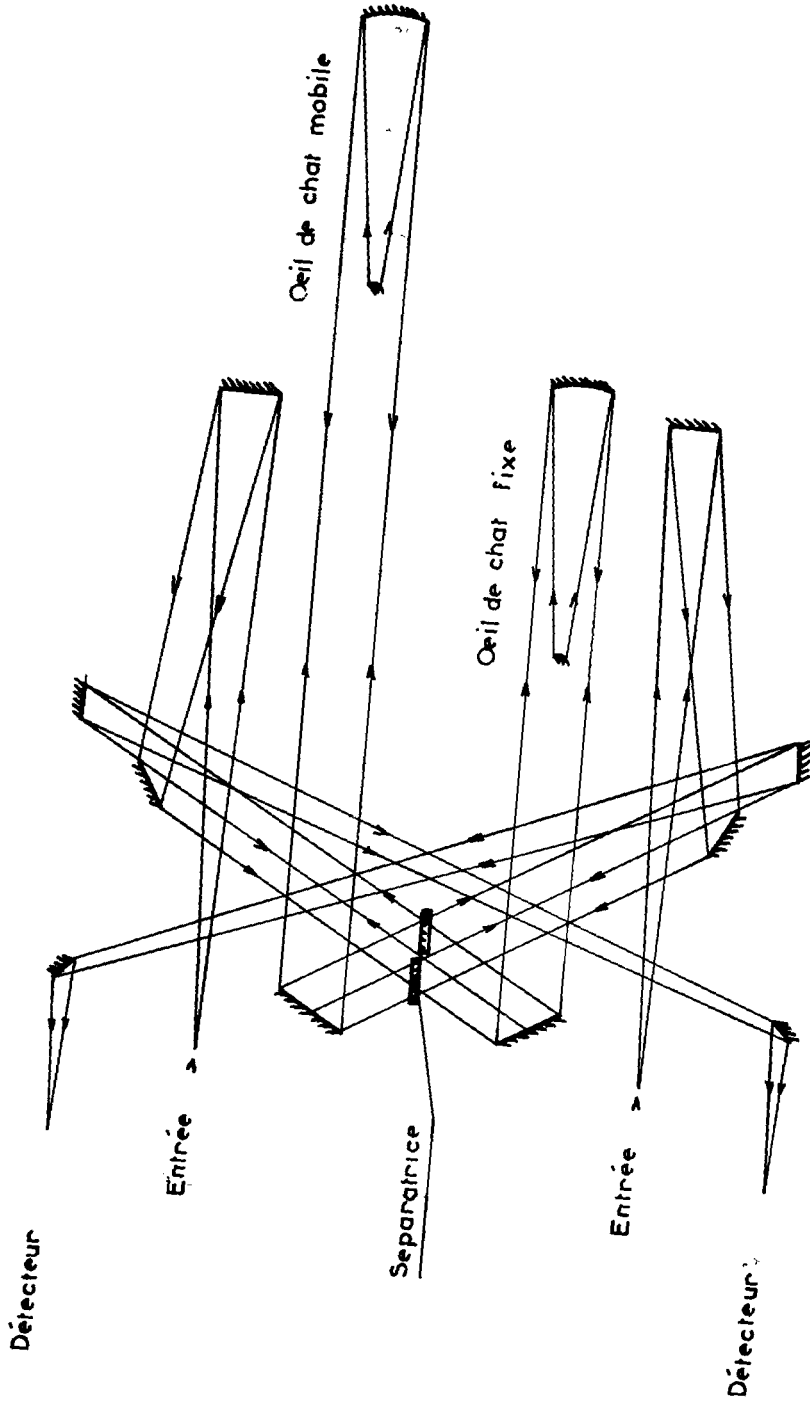


Fig. 1

Contractor: Università degli Studi dell'Aquila

Contract: n° ENV/393 1

Project Leader: Guido VISCONTI

Title of Project: **Geophysical consequences due to the variation  
of atmosphere chemical composition**

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## 1 - Objective of the research

The research which is being carried out contains three main aspects.

### a) Studies of photochemical feedback mechanism.

The temperature structure of the Earth's atmosphere is influenced by a number of components, the most important being carbon dioxide ( $\text{CO}_2$ ), water vapor ( $\text{H}_2\text{O}$ ) and ozone ( $\text{O}_3$ ). Major perturbations in the concentrations of these gases could result in a change of the atmospheric temperature that in turn would influence reaction rates relevant to photochemical processes. The most obvious examples correspond to the increase of carbon dioxide. In this case to an increase of the tropospheric temperature will correspond a much larger decrease of the temperature of the stratosphere; a colder stratosphere will have the effect of slowing down the destruction rate of ozone leading to a large increase of the concentration of this gas above 40 Km.

There are a number of mechanisms of this kind related to  $\text{N}_2\text{O}$ , CO and chlorine compounds. Variation of  $\text{N}_2\text{O}$  and CO are related to use of fertilizer and combustion of fossil fuels. An increase in  $\text{N}_2\text{O}$  concentration would affect both the  $\text{NO}_x$  mixing ratio and the greenhouse effect. Also increase of CO would bring a decrease of OH radicals and a corresponding increase in methane ( $\text{CH}_4$ ) which in turns could also effect the greenhouse and indirectly the temperature of the stratosphere due to the ozone reduction.

Our study is intended to clarify some of these mechanisms by simulating the relevant perturbations with a one-dimensional radiative-convective model of the atmosphere.

b) Two-dimensional distribution of trace gases in the atmosphere.

Modeling of the diffusion and transport of trace gases in the atmosphere by a two-dimensional model could give useful informations about the methods used at present time to parameterize transport processes in the atmosphere (i.e. eddy diffusion coefficients). In particular such studies carried out on chlorofluorocarbons like  $\text{CFCl}_3$ , could give indications on the real lifetime of such a compound against known loss processes. A precise knowledge of the lifetime is essential in order to assess the possible existence of the other loss mechanisms. The same model is being used to study production of stratospheric aerosols subsequent to catastrophic volcanic eruption like Mt. St. Helen. This requires the introduction of the appropriate sulfur chemistry and heterogeneous processes. Comparison of the model results with observed stratospheric aerosol profiles could give useful informations about the processes responsible for aerosol formation and transport.

c) Transport mechanisms for ozone in the stratosphere.

In order to improve simulation of the ozone meteorology in recent years a number of proposals have been made indicating planetary waves as responsible for the ozone transport. Such models start by considering how Rossby waves of long wavelength propagate into the stratosphere; a linearized wave model is then constructed coupled with the ozone photochemistry. Most of the propagation studies for Rossby waves assume the Newtonian cooling approximation to calculate the radiative sampling. A preliminary aim of our research is to study the effect of including in such a model radiative damping as calculated by detailed radiative code which takes into account contribution from  $\text{CO}_2$ ,  $\text{O}_3$  and  $\text{H}_2\text{O}$ .

## 2 - Methods

For the research outlined above we use a number of computer models.

- a) Perturbations in the chemical composition of the atmosphere of the Earth are studied with a one-dimensional radiative-convective-photochemical model. Such model include a radiative-convective numerical code able to evaluate the thermal structure of the atmosphere of the Earth between 0 and 70 Km and a photochemical-transport code.

The radiative code evaluates the radiative flux divergence due to different gases and uses radiative equilibrium in the stratosphere. A convective adjustment is used for the troposphere.

Photochemistry and transport are taken into account with a model which solves the continuity-diffusion equation with a time dependend scheme.

- b) The two-dimensional calculations are performed with a photochemical-transport model extending from 85°N to 85°S and from 0 to 50 Km. The time dependend continuity diffusion equation is solved in two dimensions. Transport terms include both eddy diffusion and meridional and vertical winds. The model takes into account seasonal variations by changing the dynamic as well the radiation field and the distribution of temperature.
- c) As preliminary step to study vertical propagation of Rossby waves a numerical method it is used to solve the potential vorticity equation. The numerical code was kindly provided by Dr. R. Garcia of the National Center for Atmospheric Research. In this case the equation it is solved with a semi-implicit scheme to give both the vertical amplitude and the phase speed of the mode.

Independently we have developed a method which assumed a distribution of the form:

$$\psi(x,z,t) = \psi(z)\exp(z/2H) \exp_1(Kx-ct)$$

so to reduce the solution of the equation to an eigenvalue problem in  $c$ .

### 3 - Results

During the time elapsed since the signature of the contract a number of progress have been made.

- a) The existing one-dimensional model has been improved in a number of points. In particular we have included the radiative contribution of  $N_2O$  and  $CH_4$  following the usual absorptance method outlined by Ramanathan.

A complete updating of the reaction rates and photodissociation coefficients was also made and a reference case is being run at the present time.

- b) A calculation of the diffusion and transport for  $CF11(CF1_3)$  has been performed with a two dimensional model Results of these calculation are given in Fig. 1 as mixing ratio as a function of latitude and altitude. For these calculations we have assumed a point source located at  $45^\circ N$  with an intensity based on the data released by the Chemical Manufacturing Association. Integration was started on the year 1960 and continued for 20 years. Fig. 2 compares the increase in global mixing ratio calculated with our model (which assume photolysis as the only sink) and the same quantity calculated with assumed lifetimes.

For the sulfur chemistry we have modified the 2D model to include the relevant reactions and prediction equation are solved for  $H_2SO_4$ ,  $SO_x$  and COS. We are in the process of including the prediction equation for the aerosol.

c) The method used to solve the potential vorticity equation has been tested for a number of wavelength and basic condition of the atmosphere structure (i.e. temperature and wind profile). Fig. 3 illustrates some of the results obtained so far. At present time we are running the model to provide the references cases of phase velocity versus wavenumber and amplitude versus wavenumber. These cases will be compared later with those obtained by calculating exactly the radiative damping.

#### 4 - Conclusions

The research has advanced on all the major objective in the short time since the signature of the contract. We are confident that all the basic calculations could be performed by the time the contract expires.

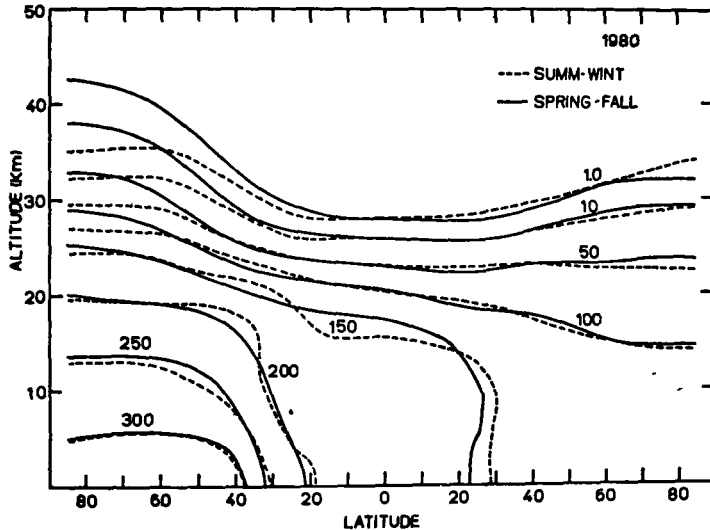


Fig. 1 - Mixing ratios of CF-11 in units of  $10^{-12}$  by volume as a function of latitude and season for the year 1980.



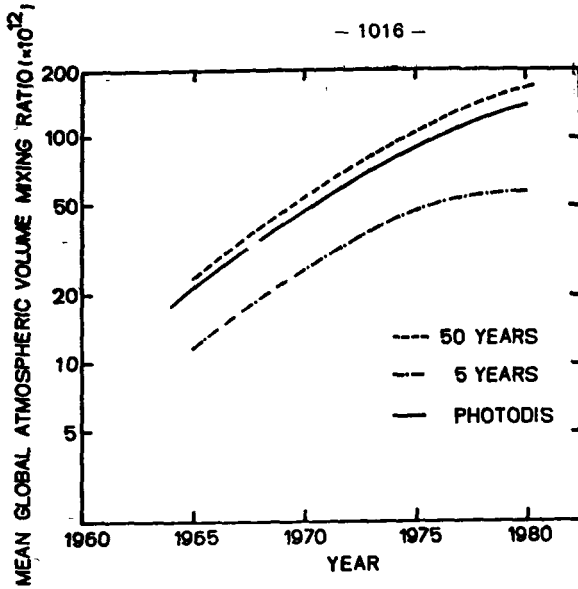


Fig. 2 - Mean global mixing ratio of CF-11 as a function of the year compared with the expected mixing ratio obtained with a constant lifetime.

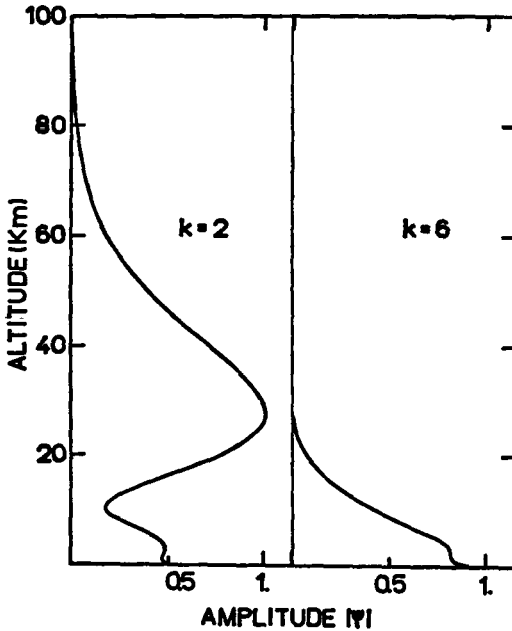


Fig. 3 - Amplitude of the quasigeostrophic streamfunction  $\Psi$  as a function of altitude for two different zonal wavenumber,  $K = 2$  (left) and  $K = 6$  (right).

Contractor: Institut d'Aéronomie Spatiale de Belgique

Contract n° : 303-78-f ENVB

Project leader : Paul C. SIMON

Title of project : Measurements of absorption cross section for halocarbon photochemical reactions

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### OBJECTIVES OF THE RESEARCH

This research deals with laboratory measurements of absorption cross sections in the 175-240 wavelength range for halocarbon of industrial interest, belonging in the methane, ethane and ethylene families. Photodissociation of these gases in the stratosphere leads to the release of atomic chlorine which in turn initiates a catalytic destruction cycle for ozone. In order to evaluate correctly the chlorine production in the stratosphere, absorption cross sections which can exhibit a temperature dependence, must be measured in a temperature range corresponding to stratospheric conditions.

### MATERIALS AND METHODS

The ultraviolet absorption cross sections of halocarbons have been measured as a function of temperature with a classical single beam equipment including a McPherson monochromator model 225 of 1 m focal length, working under vacuum of the order of  $10^{-6}$  torr obtained by means of a turbomolecular pump and an absorption cell of 2 m optical path which can be evacuated down to  $10^{-8}$  torr by an ionic pump. The light source is a deuterium lamp and the signal at the exit of the absorption cell is recorded via a solar blind photomultiplier tube type EMP 542 P-09-18 with a RbTe photocathode. A complete description of the experimental device has been given previously by Wisenberg and Vanlaethem (1978).

Determination of the absorption cross section is made after several sequential recordings of the incident and absorbed fluxes measured in the same temperature conditions.

In all cases, measurements were performed in a wide pressure range, and the validity of Beer's Law was confirmed. At ambient temperature, the quoted accuracy is of the order of  $\pm 2\%$ , but at lower temperatures, the uncertainty factor on T results on overall absorption cross sections values which are to be considered with a  $\pm 4\%$  confidence interval.

All samples were of analytical grade and were used after vacuum distillation and thorough degassing.

### RESULTS

Absorption cross-section measurements have been carried out for chloromethanes, chlorofluoromethanes and chlorofluoroethanes. Results have also been obtained for the methylchloroform and the methyl bromide.

#### Chloro- and chlorofluoromethanes :

a - Ambient temperature.

Chloro- and chlorofluoromethanes display continuous absorption in the region 180-240 nm, with absorption cross sections ranging roughly from  $10^{-18}$  to  $2.10^{-18}$  cm<sup>2</sup>.molecule<sup>-1</sup>. The progressive substitution of the H atoms of the

basic methane entity by chlorine atoms leads to increased absorption and extension of the absorption region towards higher wavelengths. On the contrary, the presence of fluorine atoms tends to stabilize the molecule whose absorption spectrum is depressed and shifted towards lower wavelengths.

Our results have proved to be in excellent agreement with quantitative values recently published for most of the studied compounds. A detailed comparison has already been published (Vanlaethem-Meurée *et al.*, 1978a-b) and only relevant references are given for comparison in fig. 1,2 and 3. In the case of methylene chloride,  $\text{CH}_2\text{Cl}_2$ , no quantitative measurements were previously available, if one excepts the data of Gordus and Bernstein (1954) limited to a narrow wavelength range (205-220 nm). Values obtained in this work for  $\text{CF}_3\text{Cl}$  are significantly lower in the region of weak absorption (up to 30% depending on the wavelength) than the ones obtained by Chou *et al.* (1978) with a 10 cm absorption cell. It must be pointed out that the use of a 2 m absorption cell allows accurate determinations even in the region of low absorption, so that measurements have generally been extended towards smaller absorption cross sections than usually considered until now. An exponential decrease of the absorption cross section with wavelength is observed in this region. These results substantiate the hypothesis according to which tropospheric photodissociation is negligible compared to the stratospheric contribution even in the case of the most absorbing species ( $\text{CCl}_4$ ), the absorption cross section does not exceed to  $10^{-20}$   $\text{cm}^2$  in the 300 nm region (Rebbert and Ausloos, 1977)

#### b - Low temperatures

For each compound, absorption cross sections have been measured for different temperatures in the largest range afforded by vapor pressure conditions which limit the maximum working pressure

As can be seen on fig. 4,5 and 6 absorption cross sections decrease with temperature by a factor which depends also on the wavelength and chemical nature of the compound itself, the effect is the most important at low temperatures, in the vicinity of the absorption threshold and in the case of highly chlorinated substances, but vanishes progressively in the region of high absorption. Temperature effect, if any, was too small to be detected in our experimental conditions in the case of  $\text{CF}_3\text{Cl}$ . For all compounds, the analysis of the absorption cross section vs. temperature relationship for a given wavelength an exponential decrease in the wavelength and temperature ranges considered, so that extrapolation down to 210 K appears to be valid. In the region of high absorption, reasonable values of absorption cross sections have been assumed in order to provide a smooth transition with the temperature independent part of the absorption spectrum. Similar measurements have already been made in the particular case of  $\text{CF}_2\text{Cl}_2$  and  $\text{CFCl}_3$  by Bass and Ledford (1976) who used optical paths up to 10 m ( $T = 223$  K) and Chou *et al.* (1977) with a 10 cm absorption cell ( $T$  from 212 to 296 K). Intercomparison including our own data, as given on fig 7, shows a reasonable agreement between the different sets of measurements even if a slight dispersion of the experimental points becomes noticeable in the region of low absorption in the whole temperature range. However, one has to keep in mind that the overall accuracy of the measurements is considerably lowered by the difficulty to define the actual temperature of the absorbing gas. Low temperature (208 K) absorption cross sections for  $\text{CH}_3\text{Cl}$ ,  $\text{CFCl}_3$ ,  $\text{CF}_2\text{Cl}_2$ ,  $\text{CHF}_2\text{Cl}$  and  $\text{CHFCl}_2$  have also been recently proposed by Hubrich *et al.* (1977) : their results confirm qualitatively the general tendencies above mentioned. However, a critical analysis of the experimental conditions adopted in their work reveals that a good lot of the measurements have been performed in unfavourable conditions of very high transmission, which greatly affects the validity of the results from a quantitative point of view.

HEAD CAPTION  
Chlorofluoroethanes :

The absorption cross sections of three chlorofluoroethanes  $C_2F_3Cl_3$ ,  $C_2F_4Cl_2$  and  $C_2F_5Cl$  have been measured in the wavelength interval 170-230 nm. Results are given in fig. 8 and are compared with the recent measurements showing a good agreement except in the case of  $C_2F_5Cl$  for which our results are significantly lower than those published by Chou et al. (1978) but in fair agreement with those reported by Robbins (1976c). Fig. 9 exhibits the absorption cross section values obtained from low temperature measurements. The same conclusions as for the chlorofluoromethanes already discussed can be drawn. Temperature effect is not detectable in the case of  $C_2F_5Cl$ .

Methylchloroform :

At ambient temperature, measurements have been performed at working pressures ranging from 0.1 to 30 torr, and Beer's law was found to hold in all cases. In such conditions, appreciable absorption (more than 10%) is easily observed for wavelengths between 182 and 240 nm, so that absorption cross sections can be determined with good accuracy ( $\pm 2\%$ ).

As can be seen on fig. 10, our results are in fair agreement with those reported by Robbins (1976), but significantly lower than the values proposed by Rowland (unpublished data, 1977). Determinations have been extended to low temperature conditions (272 K, 252 K, 220 K).

A detailed discussion of these results has been published by Vanlaethem-Meuree et al. (1979).

Methyl Bromide :

The absorption cross sections of methyl bromide have been measured between 180 and 260 nm and are represented on Fig 11. The agreement with the values proposed by Robbins (1976c) is excellent. The temperature effect appears beyond 220 nm and becomes significant for the lower values of the cross sections as it is illustrated in Fig. 12.

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**FIGURE CAPTIONS**

- Fig. 1 : Absorption cross sections of chloromethane vs. wavelength.
- Fig. 2 : Absorption cross sections of chlorofluoromethanes vs. wavelength. The measurements of Rowland and Molina (1975) and Bass and Ledford (1976) are not represented here for the sake of clarity.
- Fig. 3 : Absorption cross sections of the dichloromonofluoromethane and the monochlorodifluoromethane vs. wavelength. The chloroform spectrum is given for comparison.
- Fig. 4 : Absorption cross sections of chloromethane vs. wavelength, as a function of temperature.
- Fig. 5 : Absorption cross sections of chlorofluoromethane vs. wavelength, as a function of temperature.
- Fig. 6 : Absorption cross sections of the dichloromonofluoromethane and the monochlorodifluoromethane vs. wavelength, as a function of temperature.
- Fig. 7 : Temperature dependance of absorption cross section of the dichlorodifluoromethane at two wavelengths.
- Fig. 8 : Absorption cross sections of chlorofluoroethanes vs. wavelength.
- Fig. 9 : Absorption cross sections of chlorofluoroethanes vs. wavelength, as a function of temperature.
- Fig. 10 : Absorption cross sections of the methylchloroform vs. wavelength, as a function of temperature.
- Fig. 11 : Absorption cross sections of the methyl bromide vs. wavelength.
- Fig. 12 : Absorption cross sections of the methyl bromide vs. wavelength, as a function of temperature.

Fig.1

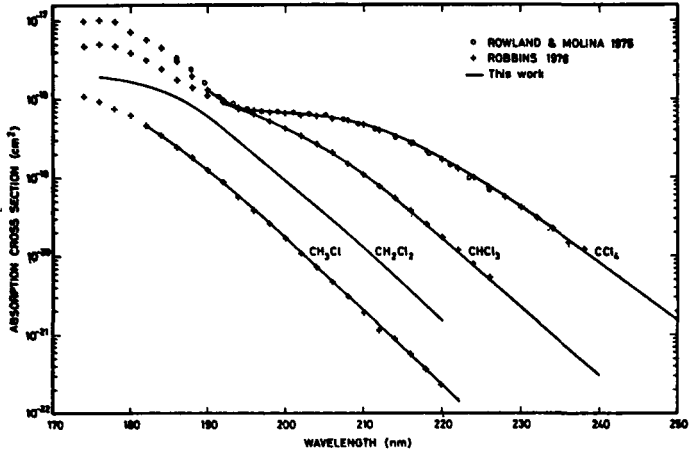


Fig.2

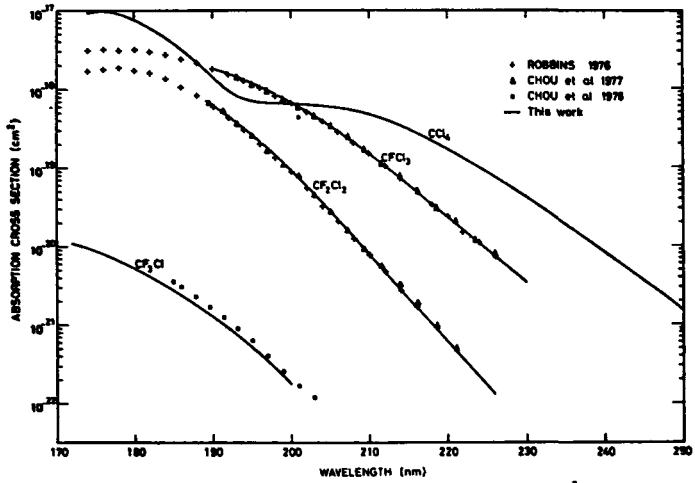


Fig.3

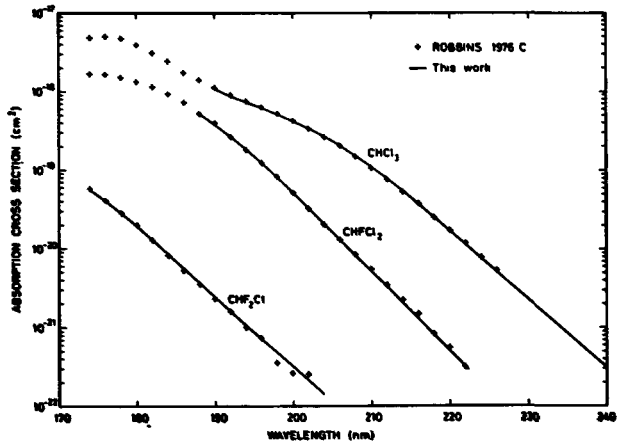


Fig. 4

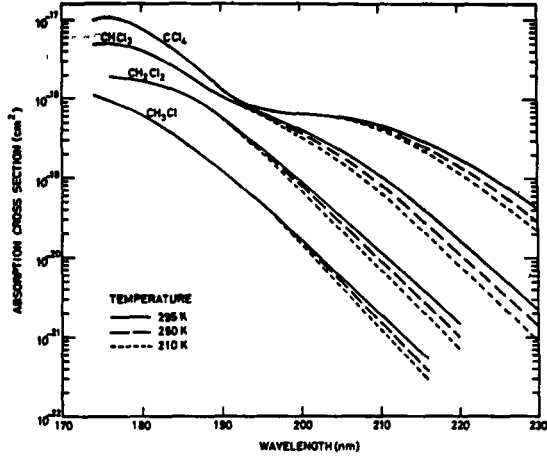


Fig. 5

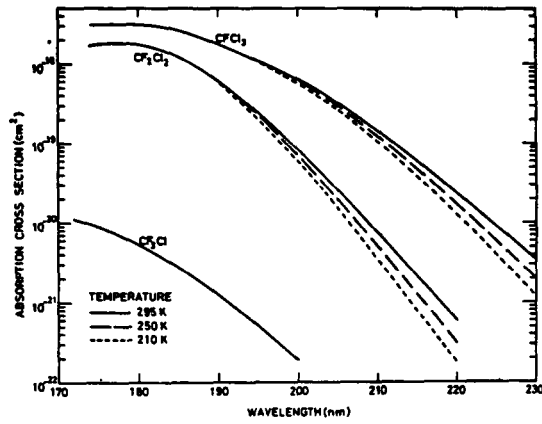


Fig. 6

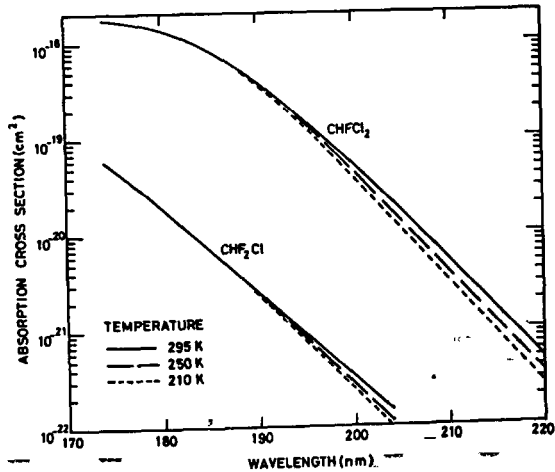




Fig.7

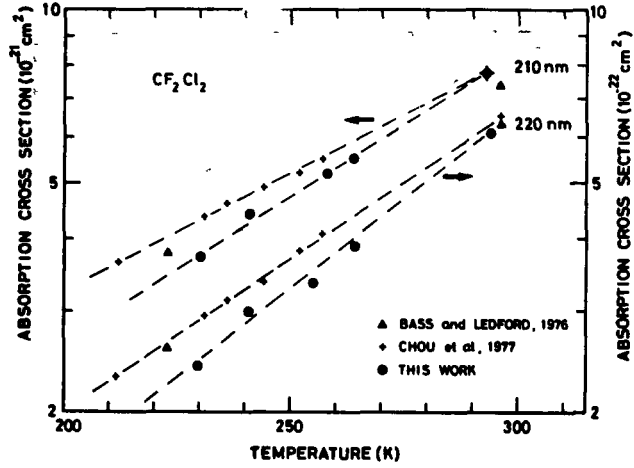


Fig.8

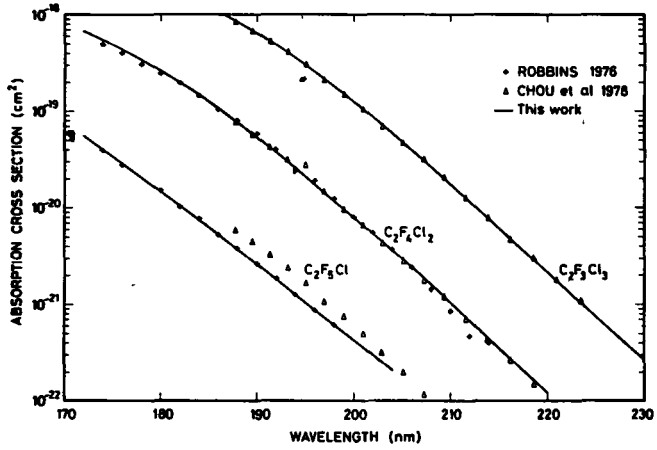
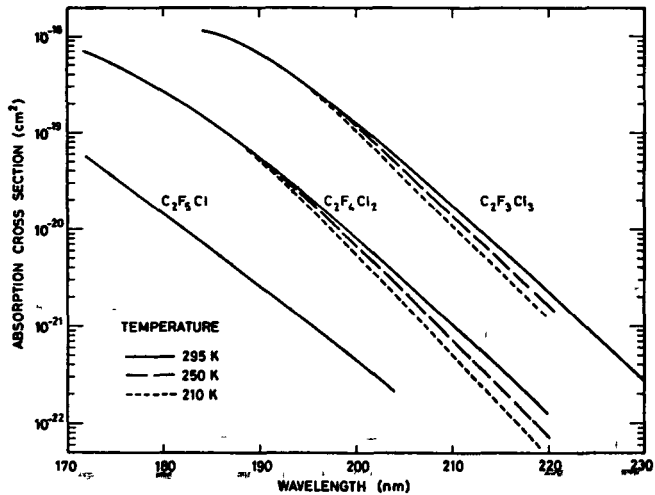


Fig.9



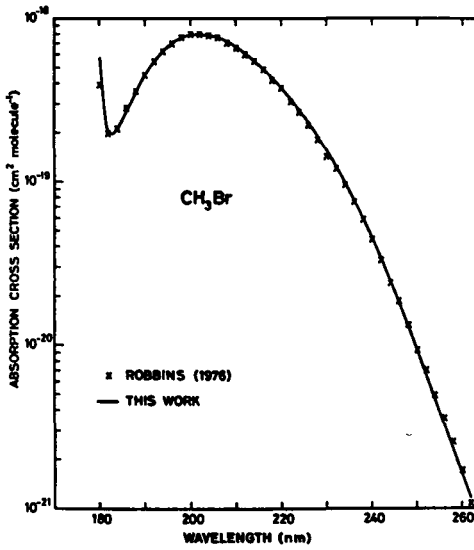
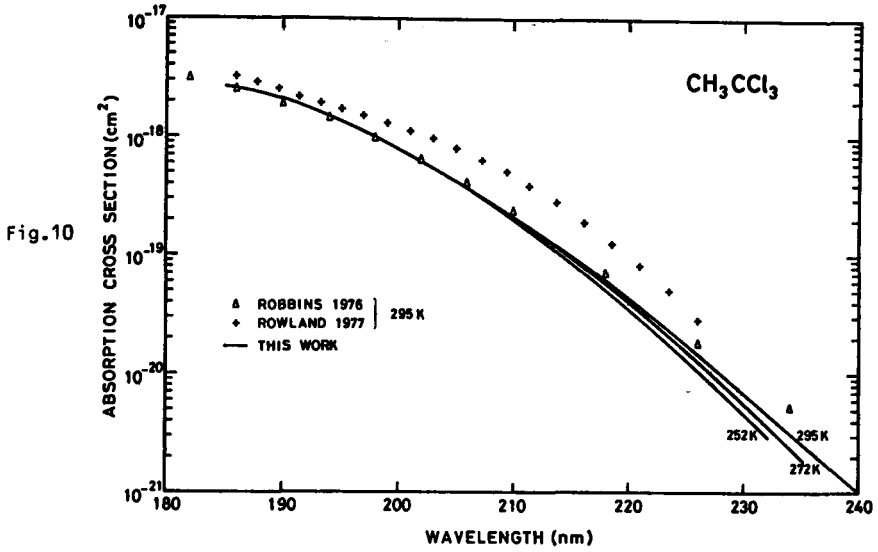


Fig.11

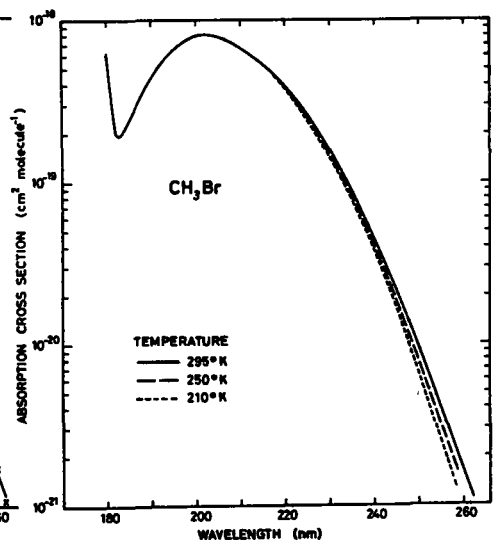


Fig.12

Contractor : Environmental and Medical Sciences Division  
AERE, Harwell, Didcot, Oxfordshire

Contract n° : ENV/454-80 UK

Project leader : A.E.J. EGGLETON

Title of project : Kinetic studies of reactions of importance  
for modelling stratospheric ozone

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## 1. Objectives

The objective of the present study is to determine kinetic parameters for elementary reactions relevant to the atmospheric chemistry of ozone, using the molecular modulation technique. In particular two areas of study have been proposed viz.

- (a) To examine the kinetics and determine the rate coefficient for the reaction of OH with HO<sub>2</sub> as a function of temperature, using the photolysis of O<sub>3</sub> in the presence of H<sub>2</sub>O as a source of the radicals.
- (b) To examine the kinetics of reactions of the BrO radical, using U.V. absorption near 339.0 nm to monitor this species. The main reactions of interest are the disproportionation of 2 BrO radicals, and reaction with NO<sub>2</sub>, OH and HO<sub>2</sub>.

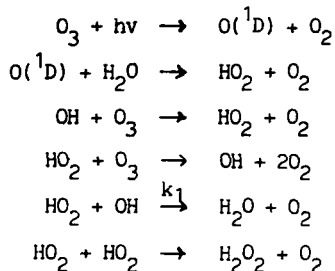
## 2. Methods

The kinetics studies have been made using Molecular Modulation Ultra-violet Spectroscopy which enables direct observation and monitoring of active species such as OH, HO<sub>2</sub> and BrO in photochemical systems. For the present work a quartz reaction cell 86 cm long, surrounded by a water jacket for temperature control, was illuminated at 253.7 nm with up to six low pressure mercury lamps, square wave modulated at frequencies between 0.1 and 25 Hz. The radicals HO<sub>2</sub> and BrO were monitored by measurement of the in-phase and in-quadrature components of absorption at selected wavelengths on a beam from a Deuterium lamp using a digital lock-in detector. OH was monitored similarly by absorption of resonance radiation obtained from a microwave powered discharge in moist Argon. The absorption components which reflect the concentration-time behaviour of the radical during a photolysis cycle can be used to determine rate coefficients and absorption cross sections of the reacting species. Data acquisition and treatment was conducted on line using a minicomputer. Computer modelling techniques were used to fit the data to system chemistry.

### 3. Results.

#### 3.1 The reaction of OH with HO<sub>2</sub> (reaction 1)

This reaction has been studied in the photolysis of O<sub>3</sub>+O<sub>2</sub>+H<sub>2</sub>O together with a diluent gas (He or N<sub>2</sub>) when the following reactions, constituting a short chain reaction for ozone decomposition, occur:



This system offers one of the few experimental systems which can provide kinetic information on reaction (1). This is because the reaction of OH with O<sub>3</sub> is relatively slow allowing high values of the concentration ratio [OH]/[HO<sub>2</sub>] to be obtained in steady state photolysis. The observed kinetic behaviour of HO<sub>2</sub> and OH, over a wide range of O<sub>3</sub>, H<sub>2</sub>O and diluent gas composition and light intensity, was consistent with the above mechanism. The rate coefficient for reaction of OH with HO<sub>2</sub> was determined by computer simulation of the results using an optimisation routine to obtain best fit values. The mean value obtained was

$$k_1 = (6.2^{+4.0}_{-2.0}) \times 10^{-11} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$$

invariant with temperature in the range 288-358 K. This is intermediate between previous measurements at low pressure in a discharge flow system and those at high pressure obtained by flash and steady-state photolysis. The differences seem to point to a complex mechanism for this reaction, possibly involving poly-oxide HO<sub>x</sub> complexes. No direct evidence for species of this type was found in the present study but some difficulty was encountered in interpretation of the kinetic details. In future work on this system recording of the entire concentration-time profile of the radicals will be attempted using a multichannel signal averaging technique.

### 3.2 Kinetics of BrO radical

Preliminary experiments have been conducted which have demonstrated the feasibility of production and monitoring BrO using molecular modulation. Part of the  $A^2\Pi \leftarrow X^2\Pi$  spectrum of BrO has been recorded at a resolution of 0.22 nm by observation of modulated absorption in the photolysis of  $O_3$ -HBr- $O_2$  mixtures and  $O_3$ -Br $_2$ - $O_2$  mixtures. BrO, monitored at 338.3 nm (the band head of the  $v'=4 \leftarrow v''=0$  transition), exhibited second order kinetics in these systems, indicating decay by the reaction



A value of  $3.1 \times 10^{-13} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$  was obtained for the overall decay rate coefficient.

### 4. Conclusions

The rate coefficient for the reaction of OH with  $HO_2$  has been measured in a study of the photolysis of  $O_3 + H_2O + O_2$  mixtures, satisfying the primary objective of part (a) of this study. Preliminary studies have demonstrated that the molecular modulation technique is appropriate for the investigation of BrO kinetics. A discussion of the significance of the results of both parts of this study for atmospheric chemistry will be deferred until the study is complete.

### 5. Future Work

Work on BrO Spectra and Kinetics is continuing. Using the system  $O_3 - O_2 - Br_2$  with photolysis at 254 nm it is proposed to

- (a) Record the entire spectrum for the  $A^2\Pi \leftarrow X^2\Pi$  transitions of BrO in the region 290-365 nm, at sufficiently high resolution to determine the true band shape.
- (b) Determine the absorption cross-section for BrO at 338.3 nm and using results from (a) calculate the rate coefficient for photodissociation of BrO in the atmosphere.
- (c) Determine the absolute rate coefficients and branching ratios for the two channels in the disproportionation of BrO as a function of temperature.
- (d) To investigate the feasibility of measuring the rate coefficient for the reaction  $\text{BrO} + \text{HO}_2 \rightarrow \text{HOBr} + \text{O}_2$  by the addition of  $H_2$  to the  $O_3$ - $O_2$ - $Br_2$  photolysis system.

### Publications

A paper has been prepared describing the experimental determination of the OH +  $HO_2$  rate coefficient and has been submitted for publication in J.Photochem.

Contractor: University of Copenhagen  
Contract no: 307-77-1 ENV DK  
Project Leader: Prof. Ole Buchardt  
Title of Project: Halocarbons in the Environment

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### I. Objective of the Research

$\text{CFCl}_3$ ,  $\text{CF}_2\text{Cl}_2$ ,  $\text{CFCl}_2\text{CFCl}_2$ ,  $\text{CHCl}_2\text{CFCl}_2$  and methylchloroform ( $\text{CCl}_3\text{CH}_3$ ) were subjected to solar or artificial irradiation under various circumstances in order to examine their photochemical degradation.

### II. Materials and Methods

The chlorofluorocarbons,  $\text{CFCl}_3$ ,  $\text{CF}_2\text{Cl}_2$  and  $\text{CFCl}_2\text{CFCl}_2$  (Du Pont) were standard quality and used without further purification. Trichloroethylene ( $\text{CCl}_2=\text{CHCl}$ ),  $\text{CHCl}_2\text{CFCl}_2$  and methylchloroform were distilled twice before use.

Long-time irradiation studies were carried out by placing series of Pyrex-glass ampoules containing the halocarbon sample and air outdoor and subjecting these to natural solar irradiation. After the irradiation period, the samples were analyzed by freezing the content out with liquid nitrogen, breaking the ampoule and adding water. The extent of the degradation was found by analyzing the pH and the concentration of chloride and fluoride ions electrometrically.

The short-term studies were carried out in two ways. First by taking similar ampoules and subjecting them to artificial irradiation (300 nm) of greater intensity than the natural solar light and analyzing by the same methods as above. Secondly, by preparing samples of halocarbons and air in 500 ml flasks equipped with a rubber septum. They were irradiated in a Rayonet reactor at 300 nm. The reaction was followed by taking samples with a gas syringe through the septum, and analyzing with gas-chromatography or combined gas-chromatography/mass spectrometry.

### III. Results

Neither the long term studies nor the artificial degradation of  $\text{CFCl}_3$ ,  $\text{CF}_2\text{Cl}_2$  and  $(\text{CFCl}_2)_2$  showed any measurable signs of any degradation.

Trichloroethylene, which due to its fast photodegradation was used as a test substance to check our analytical system, photodegrades in a silica-catalyzed reaction to give dichloroacetyl chloride. Furthermore, we found that the photooxidation had an induction period, where no reaction could be detected. After some time it suddenly reacts very fast with oxygen to generate dichloroacetyl chloride. This product is itself slowly photolyzed and gives phosgene. We also found that addition of dichloroacetyl chloride to a sample prior to the irradiation eliminates the induction period. The length of the induction period was found to be independent of the partial pressure of trichloroethylene in the range from 0.1 to 10 mm Hg but it was inversely proportional to the number of irradiating lamps used. The induction period was greatly prolonged by the presence of water, but not completely suppressed. We therefore conclude that there are two different mechanisms operating in these experiments. In the first phase during the induction period the trichloroethylene is reacting on the silica glass surface of the container, and in the second phase the oxidation is sensitized by the preformed dichloroacetyl chloride, giving an autocatalytic reaction.

Methylchloroform was subjected to similar degradation experiments both under natural solar irradiation and artificial irradiation at 300 nm. The degradation in sealed Pyrex ampoules under solar irradiation was found to be complete in a period of three weeks and, like trichloroethylene, to be subject to an induction period. Under artificial irradiation at 300 nm in a Rayonet reactor, the degradation is completed in two days with an induction period of 20 hours, followed by a 10 hour period of rapid degradation, and a 20 hour period that completes the degradation.

Finally we subjected  $\text{CHCl}_2\text{CFCl}_2$  to the same type of experiments, and observed that this compound also showed degradation after an

induction period. By combined gas-chromatography-mass spectrometry we identified the primary photoproduct as dichlorofluoroacetyl chloride, which itself is further degraded to phosgene or carbonyl chloride fluoride.

#### IV. Conclusions

From the long term studies it can be concluded that the perhalogenated methanes and ethanes are completely stable under normal tropospheric conditions, whereas the unsaturated and the hydrogen containing halocarbons are degradable under tropospheric conditions and that they are subject to catalysis by glass (silica) surfaces. The magnitude of this sink in the atmosphere cannot be assessed at present.

The observed catalytic effect of the primary photoproducts indicates that excitation transfer may take place in some of these compounds. However, such a process is not expected to have any importance under true atmospheric conditions.





**TOPIC 41:  $\text{pCO}_2$  ACCUMULATION**

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Contractor: Prof. Dr. Egon T. Degens, SCOPE/UNEP International  
Carbon Unit, Geological and Paleontological Institute,  
University of Hamburg

Contract n<sup>o</sup>: ENV 373-80 D

Project leader: Dr. Stephan Kempe

Title of project: CO<sub>2</sub>-pressure and carbon loads calculated from  
long-term record of major rivers, e.g. of River  
Rhine

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#### Objective of the research

Fossil fuel burning, deforestation and accelerated soil oxidation by agriculture have increased the concentration of CO<sub>2</sub> in the air by 10 % within the last 30 years. Due to its molecular structure, CO<sub>2</sub> is an infrared absorber and its concentration potentially can increase global temperatures. Current models call for a two to three degree increase at doubled CO<sub>2</sub> concentration expected for the first half of the 21st century. The exact rate of CO<sub>2</sub> increase, however, is not only depending on energy consumption and deforestation rates, but also on the redistribution of CO<sub>2</sub> within the biogeochemical carbon cycle. Even today part of the anthropogenic carbon is disappearing from the air without being traced by the scientific community (the so-called missing carbon, some 1-3 10<sup>9</sup> t per year).

At present we believe that this missing carbon is transferred in rather small lots into various parts of the environment, into the biosphere, the freshwater and the coastal seas. Studies of these ecological compartments in terms of their carbon budget are therefore pressing.

The International Carbon Center has started a program to investigate into this direction. It involves the study of existing hydrochemical records of rivers and the monitoring of some of the largest rivers of the world within the international cooperative program "Carbon Transport of Major World Rivers". Within the scope of the study for the second environment research program of the EG the River Rhine and its largest tributary, the River Mosel, were investigated for variations in CO<sub>2</sub> pressure and for their total carbon loads discharged in order to establish long-term means and filter out trends.

### Materials and methods

Hydrochemical data of the Rivers Rhine and Mosel have been published by the International Commission for the Protection of the River Rhine since 1963. However, not all the samples analysed could be evaluated, due to gaps in the data series of critical parameters (e.g. pH). The following stations and years could be used for the calculation: Stein am Rhein 74-77, Oehningen 79, Kembs 74-77, Maxau 79, Seltz 74-77, Mainz 79, Braubach 74-77 and 78-79, Koblenz/Mosel 63-78, Bad Honnef 79, Lobith 63-78 and 78-79, and Kampen, Vreeswijk, Gorinchem 63-78. The data were processed by a computerized electrolyte model of natural waters yielding  $\text{CO}_2$  pressure, mineral saturation indices of calcite, dolomite, and gypsum and transport parameters. Integration of concentrations over time and discharge yielded absolute transport figures. Standard statistical tests were used to prove the significance of discovered trends.

### Results

$\text{CO}_2$  pressure values of the Niederrhein reach, due to excess respiration of organic material within the water, annual means between 2000 and almost 10 000 ppm, i.e. on average more than 10 times the atmospheric  $\text{CO}_2$  partial pressure (340 ppm). Slow kinetics of  $\text{CO}_2$  degassing to air kept up these high values. On the other hand, in the Bodensee, due to overfertilized photosynthesis, the  $\text{CO}_2$  pressure in summer is depressed below that of atmospheric value. In the lake, lowest  $\text{CO}_2$  values are reached in summer, while the river experiences its lowest value in April and highest respiration values in October. This switch-over between lake and river biochemical characteristics is reached down-river of station Kembs near Basel. These annual variations (4 and 16 years means) and the differences in mean height of  $\text{CO}_2$  pressure is displayed in Figure 1.

The 16 years  $\text{CO}_2$  pressure records of the Niederrhein stations in Germany and the Netherlands experience a statistically significant increase until about 1972 and a much steeper decrease since that time (Fig. 2 displays negative log. of  $\text{PCO}_2$ , i.e. increase in pressure is decrease in curve). This can be connected to intensified building of sewage plants to keep back organic waste.

In the long-term record, highest  $\text{CO}_2$  values and hence strongest respiration is reached at station Braubach, below which the pressure relaxes towards the sea (Fig. 3, unbroken line). Within the last years (78-79), however, this trend reversed and pressure rises steeply at station Lobith, indicating advancing pollution from the Ruhr region.

High correlation significance of  $\text{CO}_2$  pressure with total organic carbon, biological oxygen demand and  $\text{KMnO}_4$  usage prove its role as a very sensitive indicator of the concentration of degradable organic substance. Correlation with  $\text{PO}_4$  is inconclusive, but  $\text{NO}_3$  and  $\text{O}_2$  are consumed during respiration yielding negative correlations of their content with  $\text{CO}_2$  pressure.

The Rhine transports (16 years mean of station Lobith)  $2.1 \cdot 10^6$  t C as  $\text{HCO}_3^-$ /a,  $0.19 \cdot 10^6$  t C/a as free  $\text{CO}_2$  and  $0.65$  total org. C  $10^6$  t/a. Annual figures are listed in Table 1 and displayed in Figure 4. The water volume amounts to  $70 \text{ km}^3$ /a which make the Rhine the 36st largest river in the world. Its load in  $\text{NO}_3\text{-N}$ ,  $0.67 \cdot 10^6$  t/a and  $\text{PO}_4\text{-P}$   $0.017 \cdot 10^6$  t/a would, however, give it a much higher rank. Delivering this mass to the North Sea and considering the C/N/P ratios of marine organisms, more than double of the inorganic carbon load of the river could be fixed as organic carbon.  $\text{NO}_3\text{-N}$  load does not show a significant trend with time, but  $\text{PO}_4\text{-P}$  increased from 8000 t in 1963 to over 25 000 t in 1978 showing that the pollution of the Rhine is by no means under control.

#### Conclusions and additional comments

In view of the carbon cycle the present study reveals several possible sinks and sources:

- 1) Rivers with 10-30 times higher  $\text{PCO}_2$  than air act as an additional  $\text{CO}_2$  source as they are constantly degassing  $\text{CO}_2$ . The size of this source can be approximated by developing a simple box model with the results of this study.
- 2) Rivers with high organic pollution transport a significant quantity of free  $\text{CO}_2$  in addition to the carbonate-C load. In case of the Rhine this amounts to some 10 % of carbonate-C comparative in size to the transport of the total carbonate-C of the River Mosel. This free  $\text{CO}_2$  discharged to the sea forms a very small additional sink.

3) Charged by very high concentrations of  $\text{NO}_3\text{-N}$  and  $\text{PO}_4\text{-P}$ , the Rhine can induce phytoplankton growth in the North Sea binding more than half the amount of inorganic carbon as its own load in form of organic carbon. This is a sizable sink for carbon, especially if sedimentation of the organic matter occurs.

4) The author feels, that in addition the mixing of river water charged with excess  $\text{CO}_2$  and calcite saturated seawater will cause extensive mixing corrosion at its mouth on sedimentated marine carbonates, thereby binding free  $\text{CO}_2$  in ionic form. This mechanism could prove to be another one of the looked for sinks of  $\text{CO}_2$  in coastal environments.

Table 1  
Discharge, carbon, nitrogen, phosphorus, and oxygen  
transport of River Rhine at station Lobich 1963-1978

year	disch. $\text{km}^3/\text{a}$	$\text{HCO}_3\text{-C}$ $10^6 \text{ t/a}$	$\text{CO}_2\text{-C}$ $10^6 \text{ t/a}$	TOC-C $10^6 \text{ t/a}$	$\text{NO}_3\text{-N}$ $10^6 \text{ t/a}$	$\text{PO}_4\text{-P}$ $10^6 \text{ t/a}$	$\text{O}_2\text{-O}$ $10^6 \text{ t/a}$
1963	56.9860	1.8751	0.1362	0.0000	0.1785	0.0080	0.3880
1964	47.1150	1.5086	0.1245	0.0000	0.1542	0.0096	0.2975
1965	99.9330	2.8594	0.2373	0.0000	0.2698	0.0170	0.5945
1966	98.1080	2.5554	0.1667	0.0000	0.1726	0.0168	0.5564
1967	79.4710	2.3590	0.1715	0.0000	0.1740	0.0157	0.5041
1968	91.4540	2.5403	0.2018	0.0000	0.2040	0.0145	0.5339
1969	74.1100	2.9009	0.3175	0.0000	0.2463	0.0183	0.5370
1970	93.3380	3.2250	0.2434	0.0000	0.3061	0.0190	0.7756
1971	47.3040	1.6924	0.1838	0.0000	0.1362	0.0146	0.2623
1972	49.8270	1.7456	0.2564	0.0000	0.1407	0.0175	0.3296
1973	54.5570	1.5619	0.2152	0.0000	0.1456	0.0156	0.2961
1974	69.3790	1.6617	0.1881	0.0000	0.1672	0.0190	0.3052
1975	65.9100	2.0839	0.1945	0.6918	0.2097	0.0248	0.4342
1976	41.3120	1.2304	0.1195	0.5514	0.1508	0.0173	0.2613
1977	67.7390	2.0620	0.1825	0.7468	0.2641	0.0255	0.5049
1978	79.0920	2.1342	0.1484	0.6158	0.2404	0.0249	0.4838
N	16.0000	16.0000	16.0000	4.0000	16.0000	16.0000	16.0000
M	69.7272	2.1247	0.1930	0.6515	0.1980	0.0174	0.4416
s	19.2837	0.5701	0.0525	0.0856	0.0534	0.0049	0.1452
s <sup>2</sup>	371.8604	0.3250	0.0028	0.0073	0.0029	0.0000	0.0211
vX	27.6559	26.8321	27.1832	13.1459	26.9745	27.9406	32.8790
r with disch.		<u>0.873</u>	0.321	<u>0.530</u>	<u>0.667</u>	0.246	<u>0.873</u>
r with time		0.317	0.022	0.049	0.088	<u>0.802</u>	0.210

Regressions.  $\text{DIC } 10^6 \text{ t/a} = 0.0258 \times \text{disch. km}^3/\text{a} + 0.325$   
 $\text{TOC } 10^6 \text{ t/a} = 0.00285 \times \text{disch. km}^3/\text{a} + 0.470$

Figure 1

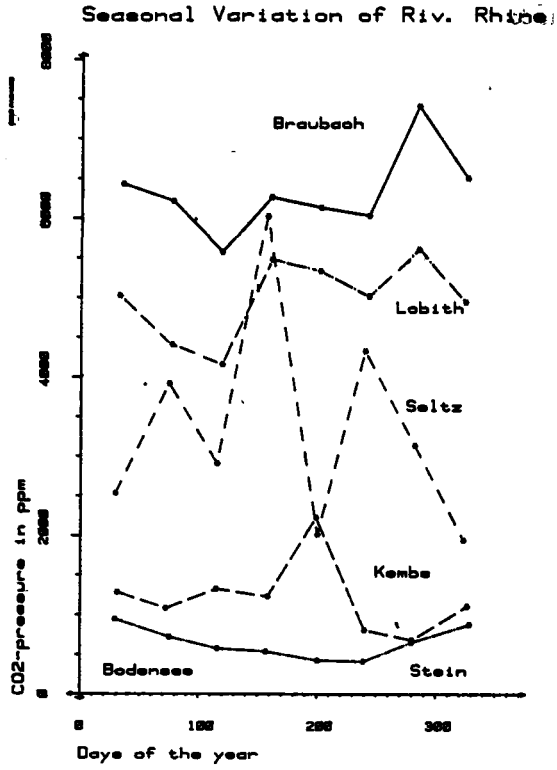


Figure 2

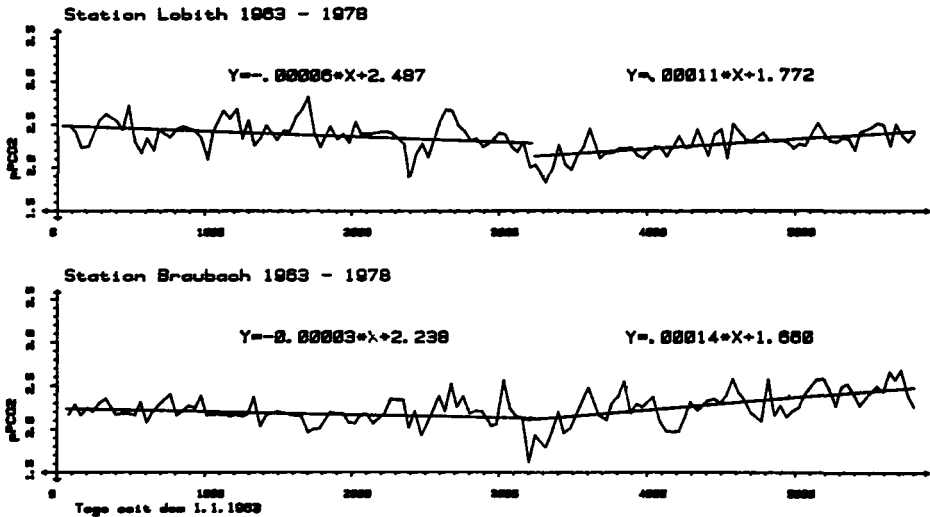


Figure 3

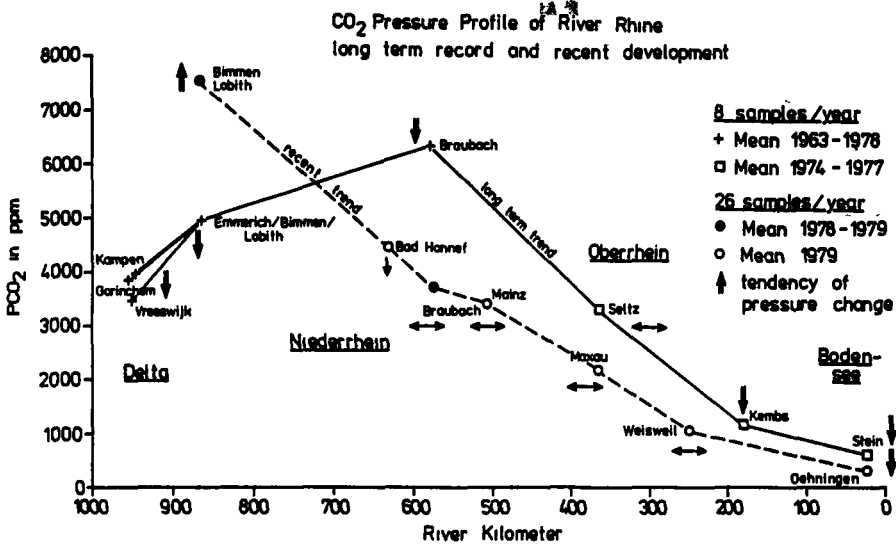
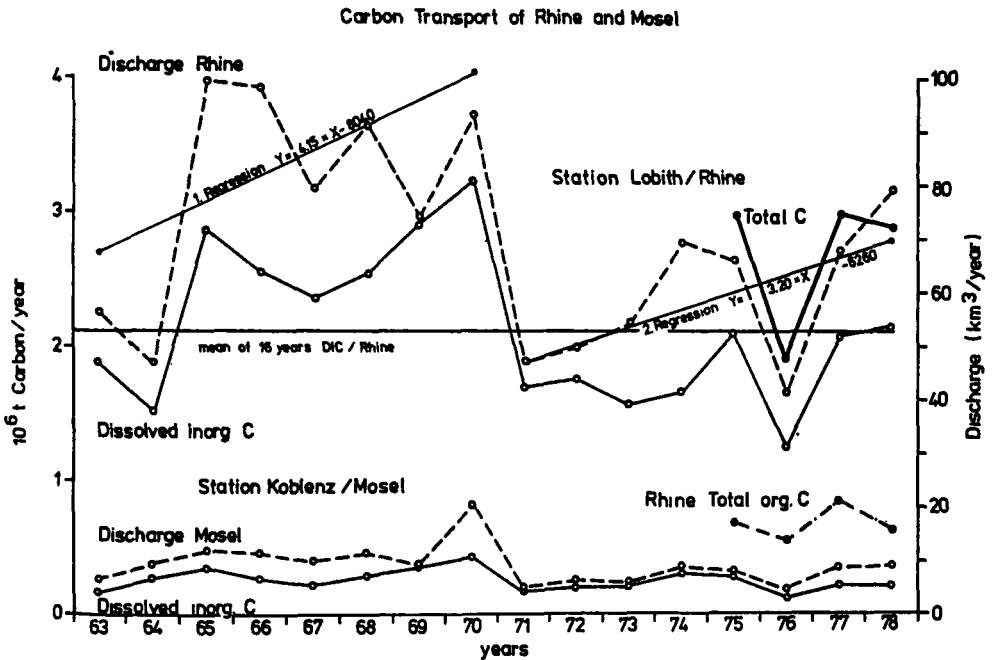


Figure 4





Contractor : Institute of Atmospheric Chemistry  
Nuclear Research Centre, 5170 Jülich, FRG

Contract n° : ENV/420-80 D

Project leader : H.D. FREYER

Title of project :  $^{13}\text{C}$  record in tree rings during the past half  
millenium fluctuations and anthropogenic impact

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Fossil fuel  $\text{CO}_2$  and biospheric respiration  $\text{CO}_2$  are marked by a  $\delta^{13}\text{C}$  deficit of about  $-218 \text{ }^{\circ}/\text{oo}$  (PDB scale) with respect to atmospheric background  $\text{CO}_2$ . Thus, parallel to the long term increase in concentration of atmospheric  $\text{CO}_2$ , its carbon isotope ratio decreases with time, a fact which is recognizable in the record of carbon fixed in the wood of tree rings. Existing measurements leave no doubt that the  $^{13}\text{C}$  content in the cellulose fraction of modern wood has indeed decreased significantly. However, an uncertainty exists in the interpretation of decreasing  $^{13}\text{C}$  data in tree rings since 1850 AD, mainly because several environmental and climatic effects are known or suspected to disturb the  $^{13}\text{C}$  trend in wood. Information on the natural  $^{13}\text{C}$  variability in tree rings within the preindustrial period, which is still unknown, will minimize this uncertainty. In this investigation  $^{13}\text{C}$  data for five Scotts pine trees from extremely isolated mire complexes of Northern Sweden are reported. The data cover the past half millenium. From these data the general trend of past atmospheric  $^{13}\text{C}$  levels as well as climatic fluctuations can be recognized. Climatic fluctuations, however, became noticeable only by small but significant superpositions on the general trend, as for example during the Little Ice Age of the 17th century or the temperature maximum during the first half of this century. Otherwise,  $^{13}\text{C}$  in wood in the preindustrial period over more than 300 years are remarkably constant. Then, from about 1850 AD up to date, a mean  $^{13}\text{C}$  decrease is found with a total of about  $2 \text{ }^{\circ}/\text{oo}$  ( $\delta^{13}\text{C}$ ), which confirms similar records on younger trees. This decrease reflects mainly the increasing atmospheric  $\text{CO}_2$  levels. Judging from its magnitude and form the trend must have been caused not only by fossil fuel burning, but also by deforestation and change of land biota.

Contractor: University of Liège - Prof. E. BETZ, Rector

Contract n° ENV-446 B

Project leader : Prof. A. DISTECHE - Oceanology (Univ.Liège)

Title of project : Buffer capacity of seawater to regulate atmospheric  
CO<sub>2</sub> content (in situ and laboratory measurements)

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AD INTERIM PROGRESS REPORT - Oct.1,1980 - March 31,1981

GENERAL INITIAL OBJECTIVES OF THE RESEARCH (4 years plan submitted in 1979-  
summary)

1. *Theoretical : as a function of temperature, pressure, saturation level of carbonates :*
  - 1.1. *Speciation of carbonic acid in sea water*
  - 1.2. *Solubility studies on particulate biogenic carbonates*
  - 1.3. *Determination of the buffer capacity of seawater, at constant alkalinity and constant carbonate regime.*
2. *In situ measurements : pH, ECO<sub>2</sub>, alkalinity, STD, profiles in the mixed layer ; evaluation of CO<sub>2</sub> exchanges with atmosphere under various conditions (site : Mediterranean Sea, Calvi, Corsica - Oceanographic Station, University of Liège).*

MATERIAL AND METHODS (initial project)

- *pH and pNa glass electrodes for in situ and laboratory work including Ca-Mg CO<sub>3</sub> saturometry.*
- *General techniques used in chemical oceanography to measure alkalinity, ECO<sub>2</sub>.*
- *Enzymatical approach to the determination of the effect of exogene enzymes on the rate of solubilization of biogenic carbonates (chitinase) ; surface chemistry of carbonates (Auger spectroscopy).*
- *Correlation of in situ CO<sub>2</sub> measurements with physical air-sea interactions conditions (currentmeter moorings, meteorological oceanographic buoy ; STD profiles).*

RESULTS

The initial research proposals have been reduced to fit as best in the frame of a one year contract starting in October 1980 that is at the end of the active marine photosynthetic activity period, a major factor in the understanding of CO<sub>2</sub> changes in the ocean. Fortunately a series of continuous pH records at 8m depth, above the Posidonia seagrass bed of the bay of Calvi as well as various profiles up to the surface had been recorded during 48 hrs, respectively on the 16-18/6/80, 17-19/7/80, 31/8-2/9/80 and 9-11/10/80 with the purpose of testing our instrumentation. Temperature, salinity, oxygen and light intensity data were available for most of these periods.

Laboratory tests are now well under way to verify the validity of the results and to try and interpret the observed facts. Similar series will be repeated this year.

The site above the seagrass bed was chosen because it generates a very

regular CO<sub>2</sub> signal linked to the photoperiod as shown in fig.1. CO<sub>2</sub> is taken up during the day by photosynthesis and CO<sub>2</sub> production because of respiration is visible during the night. This regularity is of importance to make interpretations more easy, avoiding the problems linked to the patchiness of phytoplankton, f.ex., requiring much longer time series and averaging to evaluate CO<sub>2</sub> changes linked to photosynthesis. Phytoplankton is very poor in the Bay of Calvi. Besides seagrass beds do not move. However, they are covered with epiphytes and epifauna and form an habitat hosting many animals. We therefore measure an overall response including chemical events (production of carbonate shells, chemical precipitation or dissolution of carbonates ; degazing of water colder than air ; bacterial and chemical processes in the sediments). It is hoped that a quantitative estimate of all sources and sinks will be possible since an intensive study of the seagrass bed is going on since many years by interdisciplinary teams from belgian Universities or from other countries (Netherlands, Denmark, U.S.A., etc).

The primary production has namely been studied in a continuous way and during several years by D. BAY (Thesis 1978) and in 1979 an international team has studied the processes going on in the "matte" and the sediments under the leadership of Dr. BLACKBURN (Aarhus University, Denmark). The results are still being processed but it will be possible to correlate them with our data and those of Prof. WOLLAST (University of Brussels) and MACKENZIE (Northwestern University, U.S.A.) in the water column. Informations on physical air-sea interactions are also available but have not yet been completely analyzed under the leadership of Prof. NIHOUL (University of Liège) and BERGER (University of Louvain). This year further work will be carried out in the same direction. Besides the study of secondary production and of the biological processes involved in the dissolution of shell debris have been undertaken under the direction of Prof. JEUNIAUX (University of Liège) and Prof. GODEAUX and co-workers (University of Liège) follow the population dynamics of phyto- and zooplankton. We thus hope to obtain rather important informations complementary to our own work on the CO<sub>2</sub> system.

#### Preliminary interpretation of the in situ results of 1980

Fig.1 shows that the pH fluctuation observed in July at the level of the seagrass bed propagates through the whole water column with little modifications. Fig.2 shows a small gradient changing sign above the plants after sunrise and sunset, fig.3 the correlation with light intensity (total incoming radiation). The water column is well mixed.

The situation in May is given in fig.4 and 5. An important gradient is visible above the seagrass bed where water is in laminar flow about 40 time less turbulent than in the upper layer (ratio of slopes in the morning) The whole signal is still transmitted over the whole water column as shown by profiles taken every 3 hours, together with water samples for O<sub>2</sub> and total alkalinity determinations. Advective processes do not seem to affect the CO<sub>2</sub> distribution (the seagrass covers about 1100 hectares, half of the bay).

The total alkalinity (A<sub>T</sub>) is given by :

$$A_T = (\text{HCO}_3^-)_T + 2(\text{CO}_3^{2-})_T + (\text{B}(\text{OH})_4^-) + (\text{OH}^-) - (\text{H}^+)$$

It is not modified by addition of CO<sub>2</sub>, no ions being added to the rightside sum. Speciation is carried out using the method described by MILLERO (1979)

the set of apparent constants given by MEHRBACH et al (1973). The pH scale is calibrated on the NBS scale using a conventional pH meter and standard buffers to further calibrate the slope of the seagoing probe of the type designed by DISTECHE (1959). This probe has a special junction to minimize junction potential problems. It is pressure compensated.  $A_T$  is measured by Gran titration and corrected for boric acid, as well known, to give  $A_C$  the carbonate alkalinity.

Table I shows the speciations obtained in May and July (in moles/kg). The theoretical partial equilibrium pressure of  $CO_2$  in the air (atm) (see WEISS, 1974) is indicated ( $PCO_2$ ) and illustrates the  $CO_2$  change between sunrise and sunset. This  $CO_2$  absorption in the whole water column is followed by reemission, which crudely can be attributed to global oxidative processes. It is too early to make precise calculations taking into account the actual use of  $CO_2$  corrected for simultaneous emission even during day time taken equal or not to the increase in the night, but first estimates give data within the range of the observations of BAY who showed that the plant respiration amounted to 10%. However the region is found to emit  $CO_2$  at all times and the release is only modulated by photosynthesis. The source might correspond to animal life, bacterial activity, plant respiration, degazing of cold water fed from local upwellings from the nearby deep canyon. The continental plateau even when covered with seagrass and probably the whole Mediterranean with its poor phytoplanktonic activity is apparently therefore a  $CO_2$  source to the atmosphere. During fall the plants become senescent the leaves fall and partially decompose. No pH oscillations can be detected and  $CO_2$  emission increases strongly. One is thus faced with the distribution of an internal source whether it be the water column during day time or during the night the water layer about 1m high where the seagrass lives, animals dwell, bacteria are active and shells are formed or degraded with precipitations of Mg-Ca  $CO_3$  on exposed sites in 300-400% supersaturated water.

With further experiments, namely the detection of uptake or emission in the air column using an I.R.  $CO_2$  analyzer for field work which is now being calibrated having been bought only a few months ago, we hope to quantify the total flux of  $CO_2$  to obtain eddy diffusivities and eventually relate these to vertical profiles of turbulence. A complete model of this system will then be constructed to show how  $CO_2$  is transferred through shallow depth water. We will carry out similar tests to find the transport through deeper layers in the open sea (300m is actually the maximum operational depth of cable attached pHmeter). Corrections for advective processes will however have to be taken into account from physical oceanography data we hope to obtain.

#### Laboratory measurements

pH electrode calibration : the type of electrode used is very stable but suffers from a long equilibrium time when introduced successively in different calibration solutions. This seems not to be due to the junction and reflects probably adsorption on the glass. Other glass bulbs would have to be tested. Further the slope varies with pH. The same difficulties appear in the junctionless system used in the laboratory where filtered (0.8  $\mu$ ) natural seawater is used or prepared solutions where the chlorinity is either known or kept constant inside and outside the electrode.

Speciation : Speciation requires a knowledge of all constants involved and two experimental data : pH and  $A_C$  ( $A_T$ ) or pH and  $\Sigma CO_2$  for instance

(see MILLERO, 1978) of the Na<sub>2</sub>CO<sub>3</sub> and NaHCO<sub>3</sub> systems.

Since the dissociation constants and  $A_C$  or  $A_T$  are also calculated from pH measurements a coherent set of constants and pH scale must be established. The coherence can be tested when using supplementary experimental data : paH,  $A_C$ ,  $\Sigma CO_2$ , f.ex.

It can be shown experimentally or from calculations how  $\Sigma CO_2$  varies as a function of pH when  $CO_2$  increments are added without gas phase.<sup>2</sup> Several experiments have been made by mixing samples of the same water with different  $CO_2$  contents but equal  $A_m$  (injection of small amounts of acid water under silicone oil cover). The results fit nearly the theoretical curve showing that the apparent dissociation constants do not vary too much with pH and that the pH electrode slope is well under control between pH 7.5 or even 6 and 8.2. However when  $CO_2$  added to NaCl at the same ionic strength is added, taking solubility data<sup>2</sup> found in the literature (see WEISS, 1974) rather important difficulties arise at pH < 7.5. Whether the solubility data in NaCl are not coherent with the rest of the unknowns and whether the use of acidified seawater (saturated at pH 2.2) would improve the situation is still a matter we want to study.

To emphasize the importance of the problem we have calculated the theoretical equilibrium  $PCO_2$  partial pressure in the atmosphere from the data using  $A_m$  and paH and those where  $\Sigma CO_2$  is calculated from the addition of small increments of  $CO_2$  saturated NaCl. Fig.6 shows pH as a function of  $\Sigma CO_2$ . The buffer factor  $\Delta \ln PCO_2 / \Delta \ln CO_2 = \beta$  has been calculated in both cases. Fig.7 indicates the scatter of the results and the importance of the uncertainty which we hope to remove by further experiments. Currently  $\beta$  is estimated from calculations at constant carbonate alkalinity and that is using equations which do not take the boric acid dissociation into account. At paH 8.151, 13.5 is found. The correction for borates seems essential since it reduces  $\beta$  to 10.8. To emphasize the importance of the borate correction (neglected by MACINTYRE, 1978), it should be noted that straight forward calculation show that the ratio  $\Delta(CO_3^{2-})/\Delta(HCO_3^-)$  is equal to 2 when  $A_C$  is constant, but 2.47 when  $A_m$  is constant. Borate thus adds to the buffer capacity of seawater,  $H_2CO_3^*$  reacting with  $B(OH)_4^-$  to produce more  $HCO_3^-$ . The approximation  $A_C$  constant is therefore not valid. DE LUCA REBELLO and WAGENER (1978) have recently found that  $\beta$  reduced to 7 by equilibrating seawater with a controlled  $CO_2$  gas phase. For comparison, fig.8 shows  $\log PCO_2$  calculated as a function of paH, from our experiments and the curve obtained by these authors after addition of strong acid, the  $CO_2$  being monitored in the gas phase. The discrepancy around pH 8.10 is evident and support the need for more experiments both in presence or absence of a gas phase. It should be remembered that halving  $\beta$  reduces atmospheric retention by 1/3. At  $\beta = 10$  an increase of  $CO_2$  in the atmosphere distributes as 1/11 in the ocean and 10/11 in the atmosphere.

Literature values oscillate between 6 and 14 (see DE LUCA REBELLO and WAGENER, 1978). The effects of pressure are known from previous experiments (DISTECHE, 1974).

Solubility and surface chemistry of calcite crystals submitted to high pressure : preliminary experiments show that the use of Auger spectrometry to detect surface phase changes (Mg-Ca) after prolonged high pressure (1000 atm) exposition and subsequent stepwise release of pressure is unreliable unless extreme precautions are taken to avoid contamination by dust particles.

### CONCLUSION - FUTURE PERSPECTIVES

1. The seagrass bed (*Posidonia*) and the whole local ecosystem in relation with it produces during spring and summer a daily excellent regular, almost sinusoidal signal of  $\text{CO}_2$  corresponding to absorption (photosynthesis) and release (mainly oxidative processes). As a whole the system proves to be excellent for in situ verification of laboratory experiments dealing with the buffering of  $\text{CO}_2$  added or subtracted in a water column. Further experiments are required to assess the net flux to the atmosphere (use of I.R. analyzer).  
Detailed examination as to the chemistry of the various parts of the ecosystem will permit a better understanding of their relative degree of importance but in a first stage the bulk water surrounding the seagrass bed can be taken as sink or source.
2. Difficulties of experimental nature appear with the use of various pH scales, associated constants, electrode stability and slope. They have to be resolved and in particular the solubility of  $\text{CO}_2$  in either acid seawater or NaCl at the same ionic strength as to be investigated, as well as the coherence between the dissociation or solubility constants used and the pH scale.
3. The calculations of the buffer factor will be reassessed using  $\text{CO}_2$  I.R. analysis in the water column as well as in the atmosphere and in laboratory tests.
4. The topics mentioned in the initial objectives will not be achieved within a one year contract. An extension in time is wished, since many experiments require long time intervals (effect of pressure on the solubility of carbonates, on the destruction of organic matrices by enzymes or bacteria, etc) are required. Special devices to maintain samples under high pressure are in construction.
5. The effect of the presence of a gas phase (air +  $\text{CO}_2$ ) and a solid phase (carbonates or clays or mixtures) has to be investigated.

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ANNEX : TABLE AND FIGURE.

Table I

Extreme values of pH in seawater in May and July ; corresponding speciation of CO<sub>2</sub> and Borate

<u>MAY</u> A <sub>T</sub> : 2,67 T° : 286K SZ. : 37,8				<u>JULY</u> A <sub>T</sub> : 2,67 T° : 293K SZ. : 37,8			
	<u>4h</u> pH min	<u>18h</u> pH max	Δ		<u>4h</u> pH min	<u>16h</u> pH max	Δ
(B(OH) <sub>4</sub> <sup>-</sup> )	8,011	8,080	+0,069	(B(OH) <sub>4</sub> <sup>-</sup> )	8,055	8,136	+0,081
A <sub>C</sub>	0,0686	0,0784	+0,0098	A <sub>C</sub>	0,0834	0,0969	+0,0135
(HCO <sub>3</sub> <sup>-</sup> )	2,6014	2,5916	-0,0098	(HCO <sub>3</sub> <sup>-</sup> )	2,5866	2,5731	-0,0135
(CO <sub>3</sub> <sup>=</sup> )	2,3281	2,2781	-0,0500	(CO <sub>3</sub> <sup>=</sup> )	2,2251	2,1519	-0,0732
(CO <sub>2</sub> <sup>*</sup> )	0,1367	0,1568	+0,0201	(CO <sub>2</sub> <sup>*</sup> )	0,1807	0,2106	+0,0299
(CO <sub>2</sub> <sup>*</sup> )	0,0261	0,0218	-0,0043	(CO <sub>2</sub> <sup>*</sup> )	0,0203	0,0163	-0,0040
ΣCO <sub>2</sub>	2,4909	2,4567	-0,0342	ΣCO <sub>2</sub>	2,4261	2,3787	-0,0474
PCO <sub>2</sub>	645	538	-107	PCO <sub>2</sub>	615	494	-121

Conc. in millimoles/kg seawater.  
Partial pressure in μatm.

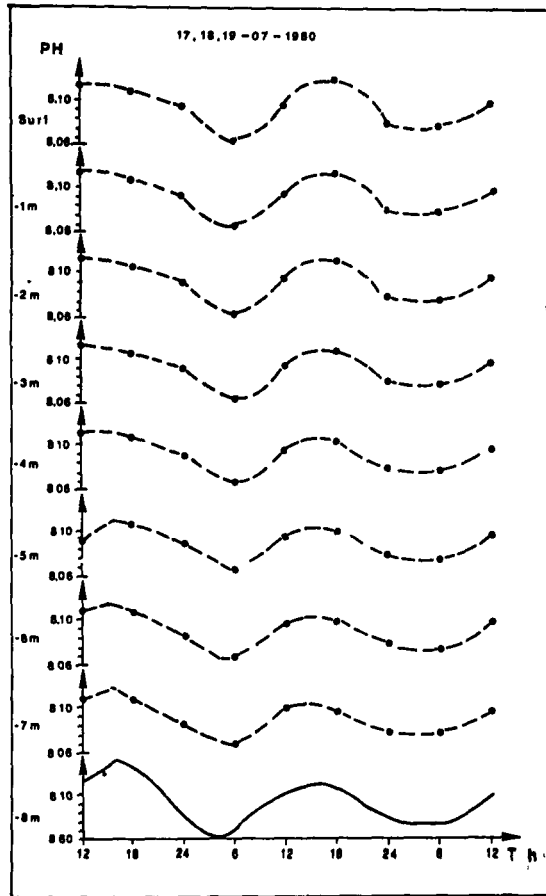


Fig.1. : pH changes in seawater during 48 hrs. at 8m depth  
above seagrass bed.  
Vertical pH profiles every 6 hrs.  
July 1980.



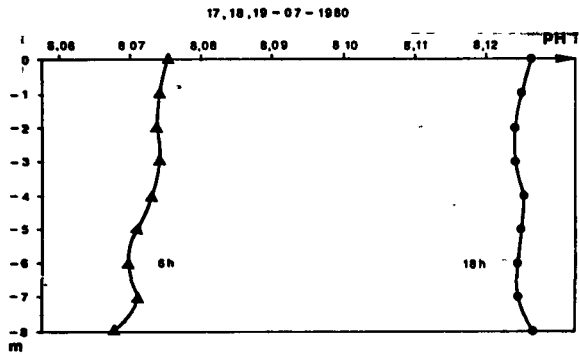


Fig.2. : Vertical pH profiles at sunrise and sunset  
July 1980.

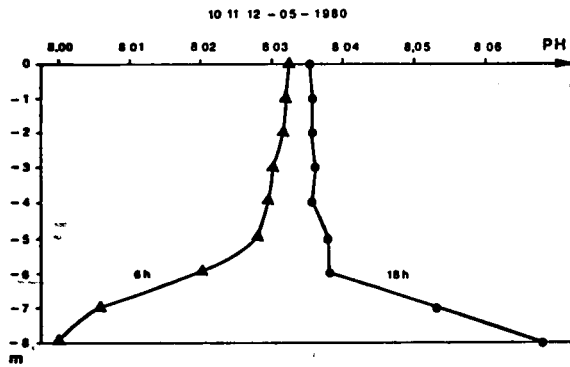


Fig.4. : Vertical pH profiles at sunrise and sunset  
May 1980.

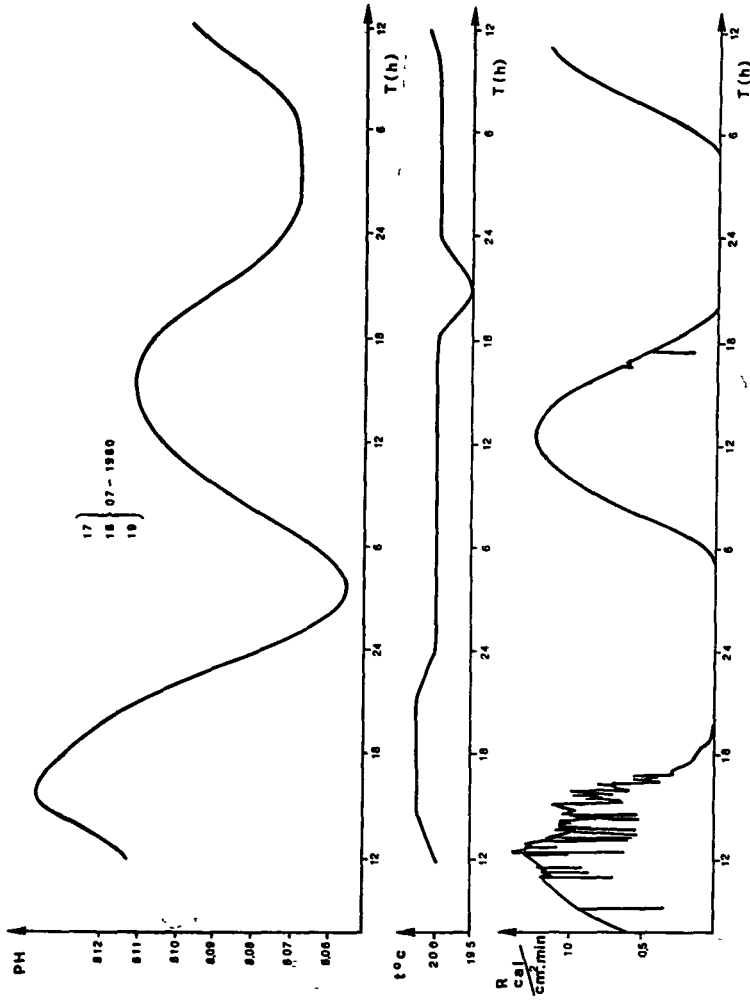


Fig. 3. : pH changes during 48 hrs at 8m depth above seagrass bed;  
correlation with total incoming radiation;  
water temperature. July 1980.

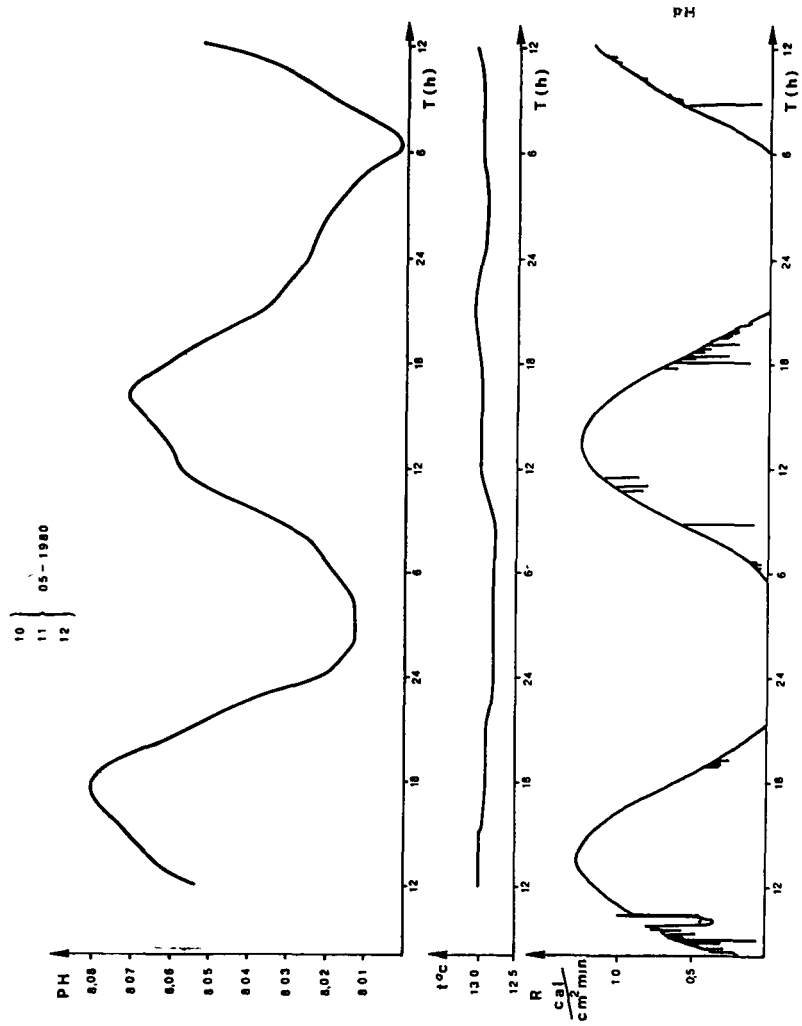


Fig. 5. : pH changes during 48 hrs. at 8m depth above seagrass bed; correlation with total incoming radiation; water temperature. May 1980.

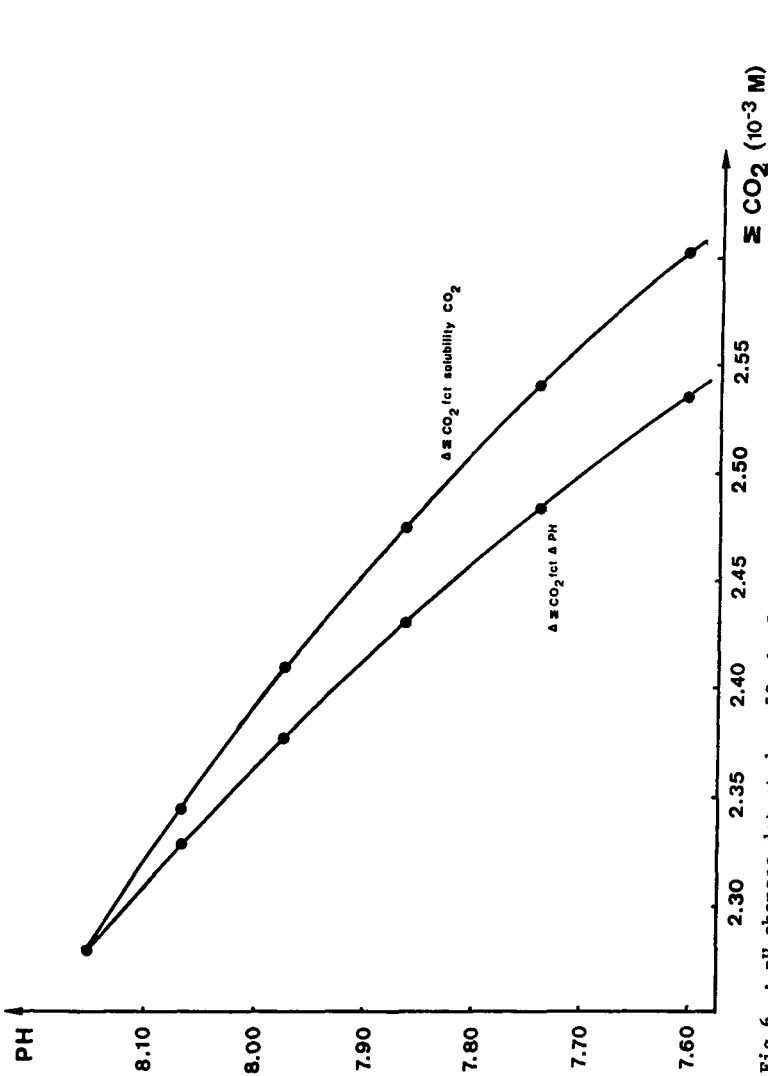


Fig. 6. : pH changes detected in 50 ml of natural seawater ( $A_T$ : 2.58 mM, S: 37.8‰, T: 294.2°K) produced by progressive addition of 0.1 ml CO<sub>2</sub> saturated NaCl.  $\Sigma CO_2$  calculated from pH changes and  $\Sigma CO_2$  calculated using CO<sub>2</sub> solubility data in NaCl (WEISS 1974)

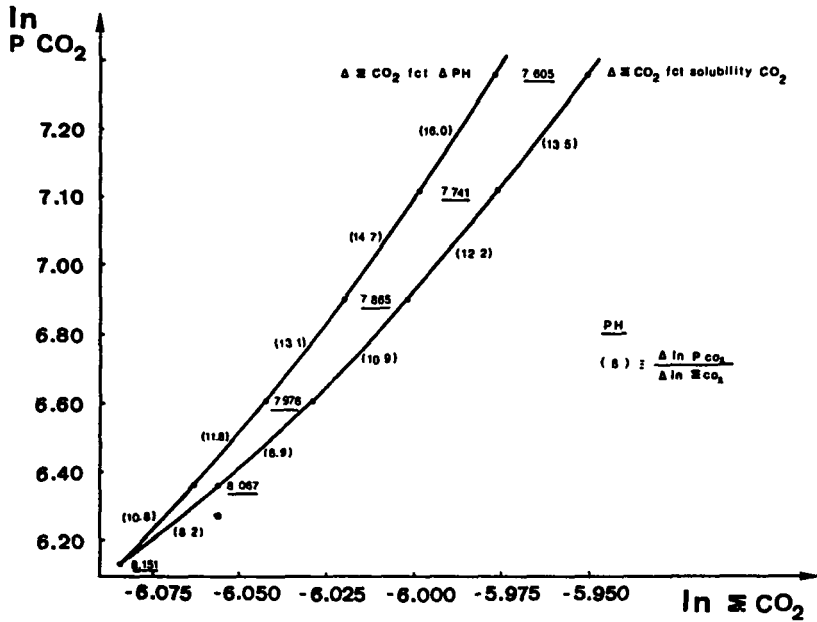


Fig.7. : Variation of theoretical  $\ln PCO_2$  in function of  $\ln E_{CO_2}$   
 (data from fig.6.)  
 $\beta = \Delta \ln PCO_2 / \Delta \ln E_{CO_2}$  and pH are indicated  
 (data from fig.6.)

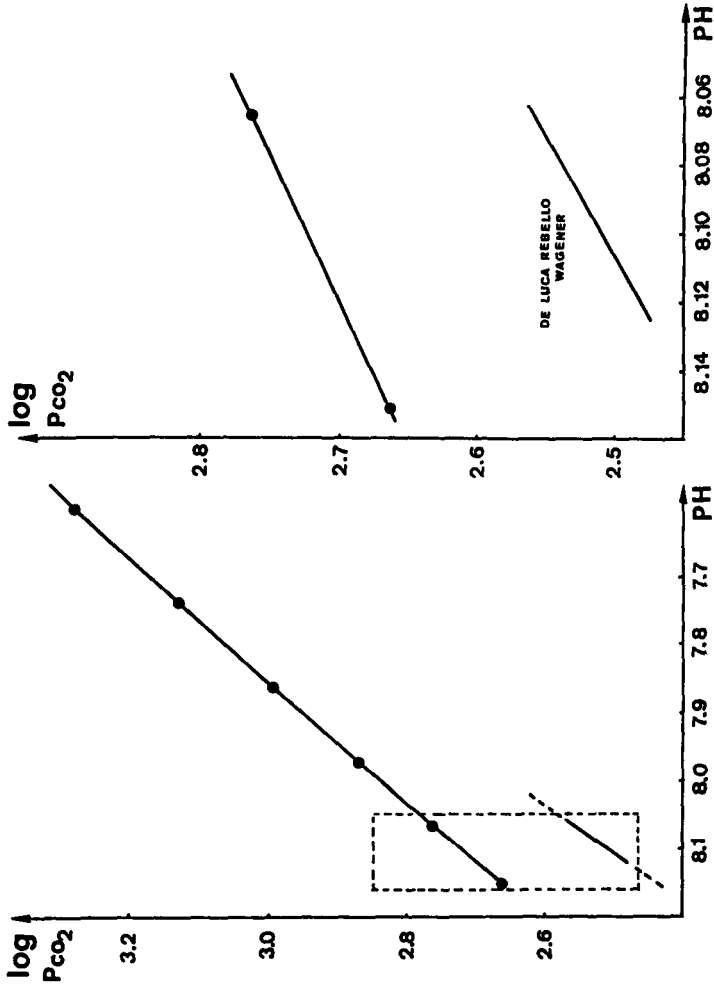


Fig.8. : Variation of  $\log P_{CO_2}$  in function of pH (data from fig.6.)  
comparison with the results of De Luca Rebello and Wagener (1979).



TOPIC 42 : RECLAMATION OF DERELICT LAND



Contractor : Umweltbundesamt, Fachbereich Umweltplanung, Ökologie  
Bismarckplatz 1, 1000 Berlin 33

Contract n° : ENV-422 D

Project leader : Dr. D. ROSENKRANZ

Title of project : A feasibility study for the reclamation of heavily  
polluted areas

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The progress report includes a draft of the structure of the investigation procedure, a list of questions which are to be answered in the course of the different working cycles as well as already obtained results.

In a first step, the relevant scientific literature that is internationally available has been evaluated with respect to the elaboration of remedial measures for contaminated land.

Furthermore have been collected numerous proposals for reducing the contamination of land by heavy metals. Thereby, it became clear that the proposals for solving the problem generally referred to single aspects and that only a small number of "integrated strategies for solving the problem" has been submitted.

The proposed measures refer to human medicine, redevelopment areas (also with respect to soil), redevelopment of town districts, agriculture, safety at work, reduction and control of emissions and air quality.

Lacking consummation of measures in the area of Oker can not exclusively be ascribed to insufficient proposals for solution. It is also the consequence of obviously insoluble conflicts between different groups of interests (Federal State, community, industry and citizen).

Therefore, such remedial measures can only be realized if the above conflicts are to be reduced. It is planned to directly contact the persons/institutions involved and to talk with them.

The progress report includes a list of the persons involved and an evaluation of the possibilities of conflict between the persons concerned. According to this report, a phase of ideation shall follow the phase of information. In this phase of ideation the proposals shall be extended and contrined. Subsequently shall be elaborated the structural conditions for the realization of the proposals for solving the problem. And finaley an estimation shall disclose how successful these proposals may be.

A first draft of this conception of remedial measures shall be discussed and enodified with in a group of experts and of the persons concerned.

Contractor : G.E.R.S.A.R. - 8, rue Jean Goujon - 75008 PARIS (France)

Contract n° : 202 77 1 ENV F

Project leader : PORTIER Jean

Title of project : Research on recovery of red muds by planting

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## I - Objective of the research

Red muds are the industrial waste left after alumina has been extracted from bauxite. In Provence (France) these muds take the form of a coagulated flow covering about 15 hectares. They are composed of sterile and inert mineral matter, of massive structure clotted together and contain 42 %  $Fe_2O_3$  and 6 %  $Na_2O$ .

From 1978-1979 a preliminary study was carried out, which enabled us to define the best soil conditions for the development of ray grass. In this case the various substances necessary to fertilize the red muds were provided by wastes produced massively in the area surrounding the dumping site for red muds.

This study was carried out in vegetation tubs (of 50 litres) and concerned only one species.

The second part of the study (1979 - 1980 - 1981) has been carried out in lysimetric tanks (6 cubic metres) and simultaneously on an experimental plot (2 000 m<sup>2</sup>) using several species of vegetation. The purpose of this experiment is to observe the growth of the vegetation which has been planted, taking into account the evolution of the substrate.

## II - Materials and methods

1) Lysimetric tanks (located at Le Tholonet, SCP). Two tanks each of 6 m<sup>3</sup> (2 x 2 x 1,50 m) were filled with the following materials :

- pebbles at the bottom, to a depth of 50 cms, to facilitate drainage,

- on this foundation the following mixture :

- 1/3 of the volume red muds

- 1/3 of the volume sewage sludge

- 1/3 of the volume composted household refuse and sawdust.

• in October 1979 this was planted with 4 species of vegetation

- Atriplex halimus
- Tamarix africana
- Festuca eliator
- Medicago lupulina.

For a year (October 1979 to October 1980) one of the tanks was intensively irrigated in an effort to reduce the sodium content. The other tank was submitted to the natural rainfall.

2) Implementation of the experimental plot at Vitrolles (Bouches du Rhône - France) located at the foot of the dumping site. On this plot which covers 2 000 m<sup>2</sup>, the following operations were carried out - the necessary materials having been deposited in the vicinity of the working area.

- 300 m<sup>3</sup> = 15 cms of sewage, dumped in a heap on the site, was levelled,
- Sawdust and composted household refuse was spread (100 m<sup>3</sup> + 200 m<sup>3</sup> = 15 cms).
- A bull dozer criss-crossed the surface and dug up the red muds to a depth of 30 to 35 cms to mix in the added materials.
- A crosskill plough then broke up the sods of red muds, thus finishing the mixing process.

8 species of vegetation were planted at a distance of 2 m x 2 m - cyprus, broom, atriplex, Provence's cane, aleppo pine, olive trees (Bohème), mulberry and tamaris.

These operations can only be carried out in summer. During the winter the dump is not accessible to mechanical equipment as the ground is very slippery.

III - Results

1) In lysimetric tanks

From October 1979 to October 1980 the irrigated tank received 2 250 mm (25 mm per week in winter, 50 mm per week in summer). The non-irrigated tank, 805 mm which corresponded to the rainfall.

The effects of the use of the different amounts of water were visible on the development of vegetation and on the structural evolution of the soil. In the case of atriplex and tamaris (both of which can stand a certain amount of sodium) irrigation has not changed the growth much in comparison to the same type planted in the non-irrigated tank. On the other hand fescue and lucern grew considerably better in the irrigated tank.

The physico-chemical and microbiological aspects of the evolution of the soil in the lysimetric tanks is controlled on samples taken every three months.

The following table shows that the substrate has not been greatly affected by irrigation.

Soils	pH	C %	N %	C/N	P <sub>2</sub> O <sub>5</sub> ass. p.p.m.	K éq meq %	T meq %	Na/T %	Usable water %
<u>Irrigated tank</u>									
October 79	9,8	7,3	6,5	11,2	51	0,86	21,1	91,4	37
July 80	8,8	6,35	5,4	11,8	20	0,75	32,3	52,7	17
<u>Non irrigated tank</u>									
October 79	9,35	8,1	7,0	11,6	47	0,90	26,6	93,5	32
July 80	8,75	6,25	4,85	12,9	18	0,77	29,3	39,1	17

In spite of having received an abundant amount of water (nearly three times the amount of the natural rainfall) the "soil" of the irrigated tanks shows, after 9 months, an amount of exchangeable sodium assimilated to the absorbing complex of up to 52 % -whereas the non-irrigated tank reaches 59%. It can be concluded that leaching is not very effective and that the sodium content in the red muds is not very soluble. But in spite of these very high rates of exchangeable sodium the vegetation continues to grow, largely because of the high organic matter content.

As for the global microbiological activities (carbon, nitrogen and phosphorous cycles as determined by the functional groups method), they are considerable during the whole period under consideration, and vary little with regard to the depth ("soils" of the tanks 1 m deep).

However, no significant difference was brought to light, between irrigated and non-irrigated tanks.

As far as the relationship between the seasons and the micro-biological activity is concerned, it is to be noticed that the denitrification process is at its lowest in the summer, whereas the alkaline phosphorous activity follows the reverse pattern.

## 2) On the experimental plot

Planting having been carried out in November 1980 a first count was carried out in March 81. It is estimated that approximately 65 % of the plants on the plot has taken. Tamaris and Atriplex and pines have a very high survival rate, whereas among the cyprus and broom several plants have died.

This can be explained in part by the rainfall, which has been particularly low during the winter months :

November 1980 to February 1981	87,4 mm
November to February (average over 20 years)	240 mm

## IV - Conclusion

This second part of the research study entails applying the results obtained in the laboratory to the field.

After a year, trials in lysimetric tanks have proved satisfactory. The vegetation has grown well. It does, however appear to be very difficult to bring down the level of exchangeable sodium assimilated to the absorbing complex and with a level of 50 % only a limited number of species can grow.

It is as yet too soon to come to conclusions with regard to the development of vegetation on the experimental plot -especially taking into account the fact that over the past few months the area's rainfall has been considerably below average.

Contractor: UNIVERSITY OF YORK, UNITED KINGDOM  
Contract no.: 271-77-10 ENV UK  
Project leader: Dr. M.J. CHADWICK  
Title of project: Chemical characteristics of colliery spoil  
in relation to the long-term performance of  
grass and legume species under various  
fertilizer regimes

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1. INTRODUCTION

The second phase of the contract has concentrated upon the relationship between the physical and chemical relationships of colliery spoil; the nitrogen economy of colliery spoil; the potential utility of amenity grass cultivars (and non-agricultural grass species) for long-term colliery spoil reclamation.

2. PHYSICAL CHARACTERISTICS OF COLLIERY SPOIL

It is the experience from work at the University of York, and elsewhere, that chemical characteristics of colliery spoil are initially more important in relation to the establishment and maintenance of vegetation than physical features. Once chemical limitations have been ameliorated, however, then the influence of the physical conditions of the spoil become more apparent.

Due to the lack of organic matter (other than fossil organic matter) in spoil, problems arise in relation to spoil structure. There is a deficiency of water-stable aggregates which in turn reduces the macropore space in the spoil. This results in spoil moisture problems and problems of compaction. As well as affecting plant growth, these problems give rise to tip surface instability and erosion.

Spoil physical features relating to these problems are field- (or field-determined bulk-) density, compaction, moisture content and specific gravity. Field density is determined by the sand displacement method (using a 150 mm pouring cylinder), compaction using a standard rammer and a Proctor mould (at a range of moisture levels), moisture content is determined gravimetrically and specific gravity by the displacement method. Table 1 gives some values of physical parameters for soiled, reclaimed and undisturbed (natural) colliery spoil sites.

The results do not differentiate clearly between soiled, reclaimed and 'natural' spoil sites although the unsoiled sites do show the highest field density, highest (or equally high) standard compaction, lowest moisture content at standard compaction and lowest specific gravity. None of the sites has field density values as high as the standard compaction values but the ratio of these two (expressed as a percentage) is highest on the unsoiled sites. It must be emphasized that the values in Table 1 only give

TABLE 1. Density and related physical measures for soiled, reclaimed and undisturbed (natural) colliery spoil sites

	A Field density (g cm <sup>-3</sup> )	B Standard compaction (g cm <sup>-3</sup> )	C Moisture content at maximum compaction (%)	D A x 100 B (%)	E Specific gravity (g cm <sup>-3</sup> )
<u>Soiled sites</u>					
Wilmington	1.36	1.63	16	83	2.61
Blackcroft	1.23	1.56	20	78	2.45
Edymoor	1.35	1.59	17	85	2.48
Wharfedale	1.11	1.33	25	83	2.39
Leeds	1.43	1.65	12	86	2.42
Wetherby	1.58	1.69	16	94	2.60
Mean	1.34	1.57	17.6	85	2.49
Standard deviation	0.17	0.13	4.4	5.3	0.09
<u>Reclaimed spoil sites</u>					
Wilmington	1.45	1.69	15	85	2.48
Wetherby	1.60	1.80	13	89	2.36
Edymoor	1.62	1.70	15	95	2.46
Wharfedale	1.30	1.34	21	96	2.06
Wetherby Race	1.55	1.68	12	92	2.05
Wetherby	1.55	1.66	15	93	2.54
Mean	1.51	1.64	14.9	92	2.29
Standard deviation	0.13	0.16	3.5	4.1	0.22
<u>'Natural' spoil sites</u>					
Wetherby Tip	1.29	1.61	16	80	2.47
Wetherby North	1.01	1.27	24	79	1.92
Wetherby	1.60	1.74	12	92	2.34
Wetherby	1.38	1.57	17	88	2.34
Wetherby	1.74	1.76	12	99	2.24
Wetherby	1.20	1.40	18	86	2.14
Mean	1.37	1.56	16.3	87	2.28
Standard deviation	0.27	0.16	4.4	7.5	0.19

indications of the denser, more compact nature of spoil than soil, with a consequent reduction in moisture content; the claim is not made that the results are significantly different in a statistical sense. The other trend to be noted is the relatively close approximation of values for all measures (except specific gravity) between the soiled and undisturbed ('natural'), spoil sites.



TABLE 2. Moisture characteristics of soiled, reclaimed and undisturbed ('natural') colliery spoil sites

	Total Porosity (%)	Moisture content saturation (%)	Field capacity (%)	Available water (%)
<u>Soiled sites</u>				
Ashington	48	3	29	22
Hullcroft	50	42	34	26
Toddy Moor	54	40	33	25
Libshelf	46	37	33	24
Hattsville	41	32	26	18
Whitwood	40	38	31	23
mean	46.3	37.0	31.0	23.0
standard deviation	5.9	3.9	3.0	3.0
<u>Reclaimed spoil sites</u>				
Ashington	42	33	28	20
Bryn Lliw	32	40	35	26
Toddy Moor	34	29	25	18
Libshelf	37	43	36	28
Upper Race	25	21	17	10
Wombwell	39	35	27	19
mean	34.9	33.5	28.0	20.0
standard deviation	6.6	7.9	7.0	7.0
<u>'Natural' spoil sites</u>				
Avenue Tip	48	45	36	28
Beaufort North	47	42	36	29
Kilnhurst	32	26	21	14
Moorgreen	41	30	26	18
Tir Pentwys	23	23	18	10
Whitwood	44	44	37	28
mean	40.1	35.0	29.0	21.3
standard deviation	9.0	9.8	8.4	6.3

The effect that physical features of spoil have on moisture characteristics can be gauged from the results given in Table 2. Unsoiled sites have lower porosity, lower moisture contents at saturation and field capacity and a lower percentage available water than the soiled sites. Table 3 expresses the relationships in terms of correlation coefficients.

Factor analysis reveals the relationship between spoil physical parameters and chemical characteristics. The negative relationships between field density and standard compaction on

TABLE 3. Physical measurement correlation matrix

Field density	1.						
Standard compaction	2.	0.896**					
Specific gravity	3.	0.307**	0.539				
Porosity	4.	-0.753**	-0.491	0.313			
Field capacity	5.	-0.702**	-0.648*	0.007	0.713**		
Saturation index	6.	-0.713**	-0.638*	0.014	0.713**	0.977**	

\* p < 0.01; \*\* p < 0.001; n = 16

the one hand and porosity, field capacity and saturation index on the other, also exposes a relationship with spoil zinc and manganese levels. There is a negative relationship between the concentration of these elements and porosity, field capacity and saturation index, that in view of potential losses of these highly mobile ions is not surprising.

### 3. NITROGEN ECONOMY OF COLLIERY SPOIL

There is ample evidence from the work carried out at the University of York over five years that the nitrogen supply to plants growing on unamended colliery spoil is severely limited (Table 4). Even when nitrogen fertilizer is supplied (in a variety of forms), the annual recovery rate has only barely exceeded 20 per cent. Naturally, therefore, there is considerable interest in the use of legumes on colliery spoil and the rate of nitrogen fixation of which they are capable.

TABLE 4. The nitrogen components ( $\mu\text{g N. g}^{-1}$ ) of spoils from six sites in the U.K.

	Total	Fossil organic	Fixed ammonium	Organic	Available
	N	N	N	N	N
Abertysswg (Mid-Glamorgan)	4 600	3 200	1 350	50	20
Ashington (Northumberland)	3 350	2 860	330	160	30
Bullcroft (South Yorkshire)	4 140	3 420	590	130	20
Cefn Pennar (Mid-Glamorgan)	5 900	4 010	1 480	410	30
Thorne (South Yorkshire)	2 790	2 090	630	70	20
Tudhoe (Durham)	4 740	3 970	620	150	20

All values expressed in  $\mu\text{g N. g}^{-1}$

If legumes are sown along with grass species and the supply of fertilizer nitrogen is sufficient to allow rapid establishment of the grass, the legumes are often suppressed. Subsequent additions of nitrogen fertilizer can lead to the

virtual disappearance of the legumes from the sward. A greenhouse experiment where levels of nitrogen fertilizer applied were varied in combination with sowing Trifolium repens and Lolium perenne together, or delaying the former, showed that the best clover establishment and yield was obtained when the clover was sown with the grass, or into an established grass sward, where spoil nitrogen levels were low. This delayed sowing approach needs testing in the field.

Field trials have shown that although legume establishment may be slow on colliery spoil, once this has occurred levels of spoil mineral nitrogen rise sharply in comparison with routinely N-fertilized spoil plots. This has led to a detailed field study (using the acetylene reduction technique) of nitrogen fixation by Trifolium repens (cv. S100) on colliery spoil. The result of weekly determinations is given in Figure 1. The fixation that is evident, emphasizes that although the establishment of a legume-based sward on colliery spoil may be more difficult than a fertilizer-based sward, the advantages of the former are considerable. To this end, when grass and clover are sown together, a maximum dressing of 25 kg N. ha<sup>-1</sup> and 200 - 300 kg P<sub>2</sub>O<sub>5</sub>. ha<sup>-1</sup> are recommended. Once established, the sward should be managed to maintain the legume component by ensuring relatively frequent defoliation.

#### 4. GRASS SPECIES AND CULTIVAR EVALUATION

Experiments and trials in controlled environment rooms, the greenhouse and in the field have attempted to evaluate grass species and cultivars for colliery spoil reclamation over the last six years. It has been shown that species differences were larger than any inter-cultivar ones. In one experiment the following order of dry-weight yield was obtained: Lolium perenne and Poa compressa > Festuca rubra > Poa pratensis > Agrostis tenuis; in another Holcus lanatus > Dactylis glomerata > Agrostis tenuis > Festuca rubra > Festuca pseudovina. Poa trivialis appears to show promise on acid colliery spoil. Table 5 summarizes some results. Cultivar yields in relation to spoil fertility are given in Table 6.

TABLE 5. Species screened in three spoil experiments  
(mean N and P spoil level)

Species	mean yield g m <sup>-2</sup>
<u>Holcus lanatus</u>	3.88
<u>Poa trivialis</u>	3.17
<u>Lolium perenne</u>	3.04
<u>Poa compressa</u>	2.93
<u>Dactylis glomerata</u>	2.73
<u>Poa pratensis</u>	2.33
<u>Cynosurus cristatus</u>	2.25
<u>Festuca rubra</u>	2.06
<u>Agrostis tenuis</u>	2.02
<u>Festuca ovina</u>	2.00
<u>Festuca pseudovina</u>	0.70

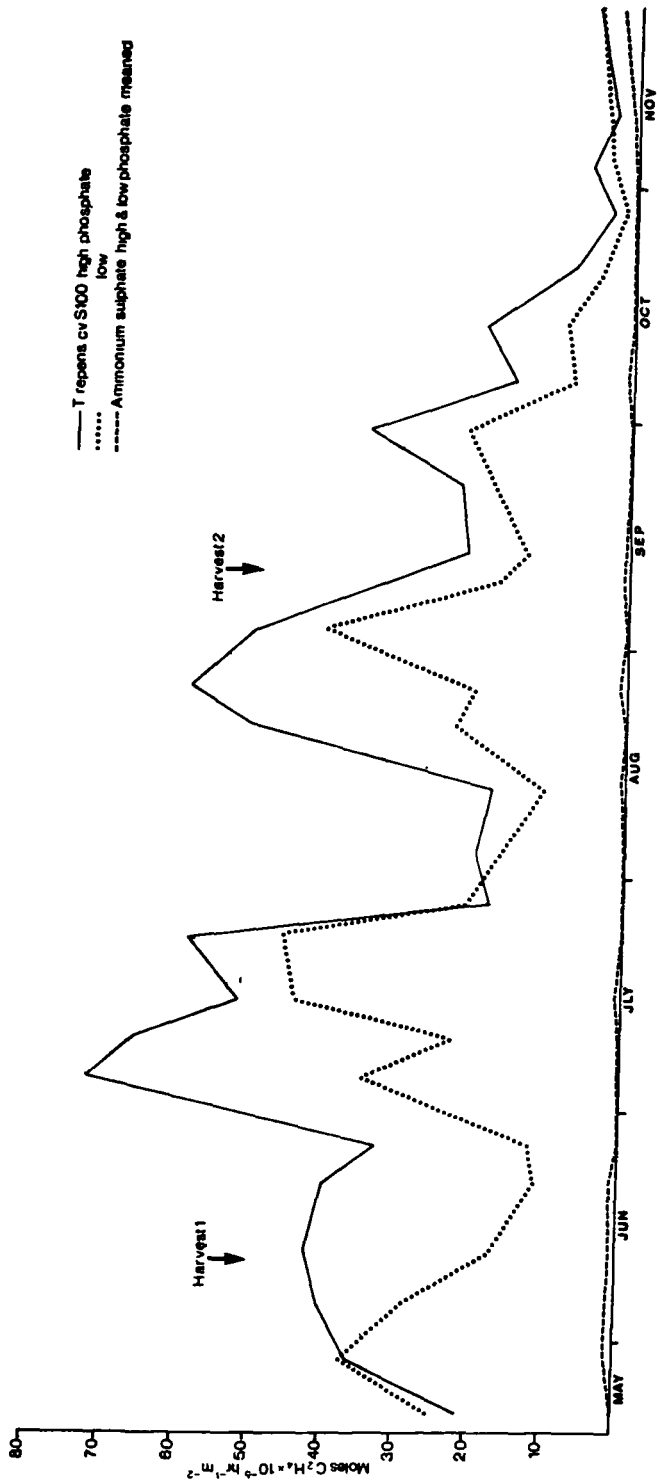


Figure 1. Nitrogen fixation by *Trifolium repens* (cv S100) at two levels of P-fertilizer additions to colliery spoil

TABLE 6. 1980 yields (kg. ha<sup>-1</sup>) of the 20 species-cultivars in a field trial, at low and high fertilizer levels

	Cultivar	Low fertility	High fertility
<u>Lolium perenne</u>	S23	65	1169
	Sprinter	74	1043
	Manhattan	66	1124
	Majestic	85	1011
<u>Festuca rubra</u>	S59	55	852
	Merlin	21	939
<u>Festuca ovina</u>		45	583
<u>Festuca tenuifolia</u>	Novina	52	548
<u>Festuca longifolia</u>	Biljart	21	591
<u>Agrostis tenuis</u>	Highland	66	797
	Tracenta	66	607
	Parys	47	596
	Goginan	119	587
<u>Agrostis stolonifera</u>	Seaside	43	660
<u>Poa pratensis</u>	Monopoly	90	532
	Nugget	30	453
<u>Poa compressa</u>	Reubens	86	1022
<u>Poa trivialis</u>	Sabre	101	664
<u>Holcus lanatus</u>		169	1056
<u>Cynosurus cristatus</u>		86	503
1sd (p < 0.05)		63	271

Generally, agricultural species and cultivars of grass do well on colliery spoil sites where fertility levels are maintained; where these cannot be maintained material bred for amenity purposes may prove useful. Holcus lanatus, Poa compressa, Poa trivialis and Poa pratensis all have potential as sward components on colliery spoil.

Contractor : UNIVERSITY OF NEWCASTLE UPON TYNE  
Contract No. : 328-79-1 ENV UK  
Project Leader : Professor P. W. Arnold  
Title of Project : Management of soils forming from colliery spoils,  
Phase II

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- Objectives of the research

To determine the effect of the amount and type of soil applied over deep-mined colliery spoil on the herbage yield and on soil forming processes in both soil and shale. The effect of incorporating soil with shale has also been examined.

- Methods

A dual approach was adopted:

1. Experimental work, in the form of two field trials and a smaller test using contained soil columns.
2. Interpreting data collected in a survey of soil-covered reclamation schemes.

- Field Trials

1. Soil Depth Trial

This was carried out on a 200 m<sup>2</sup> site at East Cramlington in N.E. England. In May 1979 three wedges of clay-loam subsoil were positioned on the shale to give soil depths ranging from 0-25 cm in a horizontal distance of 10.8 m. Each was divided into 6 plots, corresponding with mean soil depths of 0, 5, 10, 15, 20 and 25 cm. The plots were sown with a ryegrass/timothy/clover seeds mixture on 31/7/79 and harvested twice during the 1980 growing season (on 5/6/80 and 7/8/80). Field and laboratory measurements of CO<sub>2</sub> evolution from soil (a measure of biological activity) were made.

2. Soil Depth and Incorporation Trial

This two part field trial was sited at Mainsforth reclamation scheme in County Durham (N.E. England). The shale was initially covered by a clay loam subsoil to a mean depth of 10 cm, and strips of 6 plots (2 m<sup>2</sup>) were laid out. The lower three plots of each strip were then adjusted randomly to 0, 10 and 20 cm depth of soil. For the soil-shale mixing trial, the mean depth was found by augering and three of the strips were then mixed to 25 cm with deep ripping tines in 3 passes. The remainder were cultivated

with the same tines to 5 cm. The site was then sown with a ryegrass/timothy/clover mixture on 1/8/79. Harvests were taken in Oct. 1979 and in 1980 (9/6/80 and 21/7/80).

Cores of soil to a depth of 20-30 cm were examined for root distribution and chemical properties down the profile. Measurement of soil moisture equivalent (Bouyoucos, 1935) were also made.

### 3. Contained Soil Columns

The trial, carried out at Newcastle University field station at Close House, Northumberland, investigated the effect of applying soil over shale and mixing soil with the shale on herbage yield and root distribution. 30 cm of shale were packed into 25 cm diam. PVC pipes, which were buried to their rims. The various soil depth treatments were then applied. The experiment was a factorial combination of two contrasted soil types (clayey and sandy), three shales (pH 3.1, 3.8, 4.6) four soil depths (0, 5, 10, 20 cm) and each treatment was replicated three times. Columns containing 20 cm of each soil and shale alone were also included.

The mixing experiment used one soil (sandy) and one shale (pH 3.8). The soil was mixed with the shale as three equal layers (5, 10, 15 cm) and the degree of mixing was set by the thickness of shale between the layers of soil (1.66, 3.33 and 5 cm). There was an additional treatment, using 4 blocks of soil (5 cm x 6.5 cm diam.) surrounded by a band of shale 4.5 cm wide. The treatments were sown with a ryegrass/timothy/clover seed mixture in Summer 1979, and five harvests were taken between Sept. 1979 and Sept. 1980. In late Sept. 1980 the columns were dismantled and sampled.

### - Survey Methods

Thirteen soil-covered reclamation schemes in Northumberland and Durham were selected from those described by Lloyd (1978), and on advice from the County Councils. Criteria used in the site selection were:

1. that the deep-mined colliery spoil had been reclaimed using soil/subsoil covers at depths not exceeding 50 cm.
2. that the current use of such sites was for grazing or amenity grassland.

Sampling points within a reclamation scheme were chosen so as to represent as large a range of soil depth as possible and a range of chemical and physical parameters was determined as listed below:

Extractable P (Olsen et al., 1954)

Extractable Ca, K, Mg (M.A.F.F. Tech. Bull. 27, 1973)

Non-pyritic Fe, total pyrite, partially oxidisable pyrite  
(Colbourn & Stanton, 1979; Dacey & Colbourn, 1980),  
Mineralisable N, pH, Bulk density, Total porosity, Organic Matter,  
Saturated hydraulic conductivity, Water holding capacity,  
Particle size distribution (B.S. 3406 Part 2, 1963)

Biological activity was estimated as the amount of CO<sub>2</sub> produced by a given weight of moist soil when incubated for a set period, under standard conditions (Jenkinson & Powelson, 1976). The statistical packages CLUSTAN (Wishart, 1975), ORDIFLEX (Gauch, 1977) and SPSS (Nie et al. 1975) were used to analyse the data. By measuring biological activity and a large number of related parameters, we hope to evolve a theory of what influences biological activity in soils used in the reclamation of colliery spoil.

## - Results

### 1. Soil Depth Trial

At the first harvest, yield increased with each increment of depth of soil applied over shale. This trend was repeated at the second harvest, but yields were generally higher. The eight week period preceding the first cut had lower air temperatures and less rainfall than the corresponding period preceding the second cut and this may explain the difference in mean yields. The yield response to increased soil depth was greater at the first harvest than at the second harvest. Most of this effect, however, was due to the poor performance of the herbage on bare shale during the spring drought and its subsequent recovery during the wetter months of June and July. Mean total yields for the season were satisfactory ( $\approx 8$  tonnes ha<sup>-1</sup>). This compares favourably with yields of 8.5 tonnes ha<sup>-1</sup> obtained in cutting trials on grassland to which a comparable amount of N fertilizer had been applied (Morrison et al., 1980). Biological activity was higher in soil than in shale but there was no significant correlation between biological activity and yield.

### 2. Soil Depth and Incorporation Trial

At the first harvest in 1980, yield increased with soil depth on the uncultivated plots only. At the second harvest, there was no increase in yield with depth, irrespective of cultivation treatment. This difference in yield response arose mainly from the difference in performance on the bare shale plots of each cultivation treatment. Dry weight of herbage from cultivated shale was much higher than that of uncultivated shale.



Mean total yield on the experimental plots was again satisfactory (7.7 tonnes  $\text{ha}^{-1}$ ). Root distribution and moisture retention measurements showed that more water was available from the soil-treated and cultivated bare shale plots.

During the first season, there were no significant differences in herbage yield on the 10 cm soil plots irrespective of mixing treatment. Total root weights (0-15 cm) were also similar.

### 3. Contained Soil Columns.

(a) Soil Depth Trial. At each harvest there was a significant increase in yield for each increment of shale pH. However, the total yield response to increased soil depth was dependent on the pH of the underlying shale. Response to increased soil depth was least where shale pH was greatest. This suggests that soil pH is strongly influenced by shale pH and work to substantiate this is in progress. No significant differences in yield on the clayey and sandy soils were found, for any depth over shale.

(b) Mixing Trial. There were no significant treatment effects on herbage yield, however, root distribution was affected by the degree of mixing. Roots penetrated the bands of shale, and proliferated in the soil layers, thus in shales of low pH, incorporating the soil with the shale may encourage more root development at depth.

### - Survey results

For colliery spoils of this region, pH was higher than expected, most falling within the range of pH 5-8. A similar pattern was found for the pH of the overlying soils. The relatively high pH is perhaps an indication that pyrite oxidation does not proceed rapidly where shale is covered by soil. Most of the sampling points had levels of  $\text{NaHCO}_3$  extractable P less than the target of  $10\mu\text{g g}^{-1}$  for grassland soils (Cooke, 1972). As expected the amount of available P in the underlying shales was very low. However, K extracted from shale was generally higher than the amount extracted from the soils. Most soils had  $<100\text{ mg l}^{-1}$  extractable K, indicating that a response to K fertilizer might be expected (Cooke, 1972). Levels of Ca and Mg extracted from soils were generally quite high, with the exception of very acidic soils (pH  $<4.5$ ). The physical measurements showed a wide variety of soil types and structures. Similarly, there was a wide variation of

biological activity of such soils, ranging from almost zero in an acid sand, to greater than 1000 mg C kg<sup>-1</sup> day<sup>-1</sup> in a clay loam. At the time of writing, full laboratory analysis has been completed on over half the sample sites. The remainder require only shale pyrite and particle size analysis data. Statistical analysis has begun. Only the results of the SPSS regression analysis are presented as they are less affected by incomplete data.

A complete regression of biological activity with all variables (79 data sets) explained 51% of the variation. When the same regression was performed on the 36 complete data sets, R<sup>2</sup> was improved by nearly 20% due to the inclusion of extra variables. We would, therefore, expect to explain approximately 60% of the variation in biological activity with complete data. Those variables from the first regression which appeared to have most influence on activity were selected. These were soil organic matter, water holding capacity, and soil Ca, Mg. These accounted for 35% of the variation in biological activity. Those variables which can be influenced by management were also selected and a regression with biological activity was carried out. Of these, soil pH, water holding capacity and permeability accounted for 39% of the variation. Although the levels of available nutrients were low enough to limit plant growth, it is likely that the supply is adequate for the functioning of micro-organisms.

#### - Conclusions

The field trials and soil column experiment showed that herbage yield increased with increasing soil depth up to 25 cm. This increase may arise from

1. The greater capacity of the soil to retain moisture
2. pH of the soil used is generally higher than that of the shale. The overlying soil might also decrease the rate of pyrite oxidation.
3. Plant roots penetrate more easily through soil than shale.

Good moisture retention characteristics appear to be essential for soils used as covers in spoil reclamation. The optimum depth of soil could not be deduced from either field trial, as yield increased with each increment in soil depth. The contained soil column test indicated that the optimum depth of soil was dependent on the pH of the underlying shale. However, this optimum may not necessarily apply in the field, mainly because reconstituting both soil and shale in made up columns of this type would tend to make them more suitable for plant growth.

Incorporating soil with shale had little effect on herbage yield, however the cultivation used to mix soil and shale increased the yield of herbage on bare shale. It is likely that improved moisture retention is responsible for this increase. The effect is similar to that reported by Rimmer & Colbourn (1978).

In the soil column experiment, roots proliferated in the soil layers where soil was mixed with the shale. This may be due to the more favourable pH, nutrient availability or physical conditions. Work is proceeding to determine which of these factors had the greatest effect. It is interesting that when soil was mixed with a shale of high pH (pH 7) as in the 'incorporation' field trial, there was no effect of mixing on root distribution.

The survey has shown that the quality of the soil overlying shale has a far greater influence than quantity on biological activity. The physical properties of the soil, particularly water retention, have the greatest influence on biological activity of soils overlying colliery shale. Although biological activity is greatly reduced by very low pH, in the present work, with few exceptions the shales and soils were not in the very low pH category. Where biological activity is low, nutrient cycling and soil forming processes such as organic matter transformations proceed slowly, and this is associated with deteriorating physical conditions. Such processes have been linked with deterioration of swards established directly on colliery spoil (Rimmer & Colbourn, 1978). By gaining a greater understanding of factors controlling biological activity in soil overlying colliery spoil, we hope to be able to recommend changes in reclamation practice to increase the 'self-sufficiency' of the established sward.

#### Publications and oral communications on contract research

Relations between laboratory and field data on pyrite oxidation and acid production. Oral, presented by Dr. Colbourn at Symposium on Acidity in Coal Mine Spoils, held in York University, November 1980.

In preparation: Factors affecting the biological activity of soils overlying deep mined colliery spoils. For presentation by P. W. Arnold and A. Gildon at Conference on The Productivity of Restored Land sponsored by the Land Decade Educational Council, London, May 1981.

Dacey, P and Colbourn, P. (1979) An assessment of methods for the determination of pyrite in colliery spoils. Reclamation Review 2, 113-121.

**TOPIC 43 : APPLICATION OF REMOTE SENSING TECHNIQUES  
TO STUDY ENVIRONMENTAL DISTURBANCES**

Contractor: Groupement pour le développement de la télédétection aérospatiale (Aerospace Remote Sensing Development Group)

Contract N° ENV - 427 F

Project Leader: Mr. FOIN

Title of project: Medium-scale land use mapping

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#### I. Aim of the research

This study consisted of the search for a methodology which would not only improve automatic procedures for land use mapping starting with satellite imagery but would also make use of various kinds of statistical data to obtain, starting with the land use map, its division into small homogeneous regions, a commune typology and, if possible, derived thematic maps.

#### II. Equipment and methods

Use was made of

- (a) A Landsat scene covering the entire zone to be studied,
- (b) Digital files of the boundaries and chief town of each commune in the département,
- (c) Substratum Data Bank of the Bureau de Recherches Géologiques et Minières (BRGM) and
- (d) Kilometric numerical model of the surface (altimetry) of the département. The method uses a group of statistical digital procedures carried out on an IGN-F SOLAR 16 computer and described in detail below.

#### III. Results

##### 3.1. Selection of the zone studied

The study was due to be carried out for the Rhône-Alpes region but the Loire département was finally selected for the following reasons:

- (a) That département was covered by a single Landsat scene so one could avoid problems of linking one scene with another which, although feasible, would have been useless and expensive,
- (b) A fairly recent and sufficiently good quality image was available,

- (c) Other data concerning the département, particularly geological data, were available.
- 3.2. First phase: production of a land use map. This map was obtained by an unsupervised classification using an algorithm, already tested, of Large Cloud Dynamics. The innovation here, which made it possible to reduce errors, was as follows:
- 3.2.1. Firstly, using aerial photographs, general geographic knowledge and after a brief survey on the ground the département was divided up visually using a coloured composition (channels 4 - 5 - 7) of the Landsat image which had been geometrically corrected beforehand. This preliminary study made it possible to distinguish the following 11 geographically different and sufficiently homogeneous zones.
- (a) **Pilat region**  
Zone with large forests, (mainly coniferous), with natural meadows and waste land often covered with ferns. Altitude from 800 to 1400m.
  - (b) **Monts du Forez - Bois Noirs - Monts de la Madeleine**  
A long strip of territory at the western edge of the département where the forest (especially conifers) is dominant, with some natural meadows but few crops. Towards the highest zones of the Monts du Forez one encounters large stretches of heathland with heather and bilberries. Altitude 800 - 1600 m.
  - (c) **Belmont de la Loire region**  
It forms the N.E. point of the département and is situated at a slightly lower altitude than the two preceding zones (600 - 800m), but the forests still cover a very large area (often mixed forest).
  - (d) **Plaine du Forez**  
There are few forests here only deciduous trees or poplar plantations (the ground is very wet - numerous ponds). A large part of the ground is covered by meadows and crops (maize).
  - (e) **Plaine du Roannais**  
The relief here is a little more pronounced than in the Plaine du Forez, the land parcels are smaller and the conifers are rarer. Crops clearly predominate in the southern part whilst meadows are dominating in the northern part.
  - (f) **Sud Charolais region**  
Region of rich grassland. Very few woods, almost exclusively meadows (stock rearing zone).
  - (g) **Western slope of the Monts du Lyonnais**  
Low altitude mountainous zone with forests at the eastern edge of the département and on descending towards the R. Loire there are increasing amounts of meadow.
  - (h) **Eastern slope of the Forez**  
Transition region between the Monts and the Plaine du Forez. One thus finds towards the upper zones a landscape fairly close to that of the Monts du Forez with heathland and natural meadows and when

descending land parcels with increasingly dense crops. This slope is crossed by wooded valleys (mixed species).

- (i) Coteaux de Renaison  
This zone situated between the Monts de la Madeleine and the Plaine du Roannais resembles the eastern slope of the Forez but the forests are very often deciduous ones, the meadows somewhat less in number and in addition vineyards occupy a non-negligible area.
- (j) Rhone right-bank  
This region is a special one in the département because it faces the R. Rhone and is separated from the remainder of the département by the Pilat massif. Its a region of crops, e.g. vineyards, orchards in particular, with few meadows but heathland and woods as soon as one climbs up towards the Pilat.
- (k) Gier Valley  
This is a zone of dense habitat with orchards and vineyards on the slope exposed to the south, but with a meadow region larger than that in the preceding zone.

3.2.2. Each of these zones was transformed into a group of communes for transfer on to the digital image using the commune boundary file. An unsupervised classification was then carried out for each zone and was followed by accurate identification of the themes using the aerial photographs. The urban zones were studied separately because it was found that, in general, there was a considerable confusion on the image between wet ploughed ground and the towns. Consequently, a classification of the country areas was carried out without introducing any urban themes and was followed by another classification of the towns alone. The final map combining these two intermediate results was, in this case, the product of 12 partial classifications.

3.2.3. Comparison of the results so obtained with a map resulting from a single classification confirmed their advantages. A number of confusions can be avoided with this method, e.g. fields-towns, deciduous trees - meadows, and new local themes can be sought, e.g. vineyards. In addition,

- (a) not only is it possible to avoid radiometric slipping within a given scene, i.e. avoiding that a given radiometric value correctly identified in one part of the image, corresponds to something completely different in another part a sufficient distance away and in a sufficiently different context, but

(b) one can also, as the zones are already more homogeneous, search within each zone for a smaller number of land-use themes so reducing the percentage error (For example, one did not attempt to introduce "vineyard" themes in a zone like the Charolais which certainly avoided errors!). The resulting map consisted of the following 12 themes (the last one is just a reminder)

1. Water
2. Coniferous trees
3. Deciduous trees
4. Heath land
5. Meadows
6. Ploughed fields
7. Crop fields
8. Orchards - vineyards
9. Dense urban zones
10. Less dense urban zones - suburbs
11. Industrial zones
12. Bare ground - quarries

This represents a fairly complete interpretation especially if one considers that the study was based on a single image.

### 3.3. Second phase - use of the above map along with external data

3.3.1. The guiding principle in this part of the research was to complete the data obtained from the space image by other complementary data (altimetric model, geological data, human and agricultural statistical data, etc.), i.e. to take the environment into account in the best possible way in order to obtain a more complete description of the landscape and to be able to divide it into homogeneous regions with respect to a larger number of criteria. The main problem which arises is a technical one: the different data sources a priori are not compatible from the computer processing point of view and the production of interfaces is a hard and long phase but it must be carried out before any advance can be made. The second problem encountered is a practical one: obtaining the data from different organizations which are not normally associated with remote sensing operations; in our case this problem has given rise to some delays in the development of the research. One would like to use in this study:



- (a) Digital model of the surface represented by a 1 km interval grid (produced by the IGN-F),
- (b) BRGM geological data,
- (c) DRA-Cadastre agricultural statistics, not yet obtained.

3.3.2. Starting with the land use map having a 100 m x 100 m pixel, a pseudo-image with 1 km x 1 km pixels comprising 12 channels was produced, each channel representing the percentage of ground covered by the corresponding theme over a 10 x 10 grid square. A classification of that image made it possible to define the main land-use types in the département and consequently to divide it into homogeneous zones in which one sees, in broad general outline, its geographic zones such as they have been previously described. However, one now obtains more details not only of the thematic aspect but also about positions in space; in addition, other features are revealed; for example, in the Plaine du Forez, one sees the contrast between the R Loire left bank where one finds more woods and meadows, and the right bank where the crops are dominant.

As the latest division is more precise than that used in the first instance for producing the map, one might envisage an iterative procedure for improving once again the thematic description of the land use. The use of a digital model of the surface makes it possible, in addition, to differentiate between analogous land-use zones at the kilometre level, (where the relief induces effective differences of land-use, differences of parcels, yields, etc.). Thus one can distinguish the Sud Charolais from the western slope of the Monts du Lyonnais (northern region) although these two zones are both predominantly covered with meadows. The taking into account of the altimetry makes it possible to obtain a better description of the environment, by displaying differences that land-use criteria alone could not reveal.

3.3.3. Analog processing should then make it possible to amalgamate zones into small groups of communes (here the pseudo-image will no longer be the percentage of each theme in a kilometre grid square but the percentage over the commune) and thus find once again or redefine the small agricultural regions. That latter classification could

be improved by adding BRGM position data and agricultural statistics (in addition to the digital surface model) in order to supply a more complete communal typology. (The geological data and agricultural statistics will not be used with the kilometric image because they are too small in number, e.g. about one hundred for pedology, one for each commune for the agricultural data, to be used at this level).

3.3.4. Finally, starting with the gridded pseudo-image, a digital surface model and geological data, one can obtain an approximate geological map of the département. Unfortunately in this particular case, the amount of BRGM data which can actually be used (fully positioned) is too low for that latter operation to be interesting other than for methodology purposes and will have to be carried out in a zone with more interesting geological data.

#### IV. Conclusion

This research has thus revealed the practical interest of the joint use of remote sensing along with geographic, topographic, geological and economic data in order to obtain a better description of the environment.

The division beforehand of the zone to be studied makes it possible to improve very considerably the validity of the land use map. The taking into account of general data concerning the geographic environment completes the remote sensing data and in return the environmental description so obtained is more complete and more accurate so making it possible to plan an iterative procedure. Nevertheless the method is still rather unwieldy because it requires the manipulation, which is not completely automatic, of a large number of files; however, practical progress is planned in the near future.

As concerns the second research phase, operations are still being carried out but one can already consider that the method so developed will make it possible in a fairly general way to define a division into small homogeneous regions and giving each one a very complete description of its environment.

In the future, with the launching of the SPOT satellite, one might follow the environment over the years because the better space resolution will make it possible to "see" local environmental changes, often small in extent but significant in the evolution of a zone, that the current accuracy of satellite imagery does really make it possible to examine.



TOPIC 44 : ECOLOGICAL CONSEQUENCES OF LAND USE PLANNING

Contractor: Dornier-System GmbH, Friedrichshafen  
Contract no.: 243 - 77 - 10 ENVD  
Project leader: Dr. Peter Boese/Burghard Rauschelbach  
Title of project: Study on the ecological consequences of traffic development in certain regions  
(Untersuchung der ökologischen Folgen der verkehrlichen Erschließung ausgewählter Regionen)

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Contents:

1. Objective of the research
2. Materials and methods
3. Results and conclusions
4. Recommendations
5. List of references

1. Objective of the Research

The research contributes to the development of the environmental assessment methodology for regionally important planning. The ecological consequences and the environmental effects of traffic development (opening up for development by road network enlargement and road construction) in northern Schleswig-Holstein (FRG) and South-Jylland (Denmark) are being investigated to provide the empirical basis of the methodology to be introduced.

An area of about 4000 km<sup>2</sup> had to be evaluated in respect to its different suitability and conservation value. This assessment enabled a pre-choice of areas which belong to different sensitivity classes according to the impact of the regionally significant measure (in particular land use changes).

The positive and negative effects on the environment could be demonstrated with the new built motorway E 3 between the cities of Rendsburg (FRG) and Haderslev (Denmark). The mediate environmental effects and their causes also had to be taken into account.

Finally the aggregation of effects allowed a system analytical review of economic and environmental factors.

## 2. Materials and Methods

The research comprehends three main levels of investigation:

- an analysis covering the whole investigation area,
- an investigation of the effects which directly come from the road line and road construction, including the mediate consequences,
- a more detailed analysis of problematic areas where land use conflicts are to be expected.

The levels demand different procedures while the general methodological approach remains more or less the same.

The research is based on the data acquisition by a geographic inventory of the area where mainly the material which is available without further inquiry shall give the input for the further analyses. However, because of the fact that the available material had been very diverse, fragmentary, of different quality and different scales, additional survey had been necessary. This meant that the results show different reliability rates for the planning statements (e.g. about the risk of a proposed land use change).

The methodological research, as the main objective of this study, aims at the development of means for the environmental assessment of regionally significant measures. It concentrates on substantiations of aggregation procedures and on the improvement of their applicability in the planning practice. The empirical part of the project covers the acquisition of socio-economic and geographical data, and the tests of the mentioned methods for environmental planning.

The processing of the basic data is manually done by direct interpretation (topographic and thematic maps, aerial photographs, traffic frequencies, etc.), by means of an interactive digital cartographic device and a standard computer.

The methodology is particularly based on the procedures as developed for the "Handbook for Ecological Planning" (Umweltbundesamt, (Hrsg.): Abschlußbericht zum FE-Vorhaben "Handbuch zur ökologischen Planung", 1978).

The main methodological task lies in aggregating area-related informations. The most important principles can be described as:

- indicator concept: The environmental situation and processes are characterized by means of informations which stand for more complex facts. E.g. the degree of air pollution of an area can be derived from the energy consumption, the different energy sources and the topographic and climatic factors.
- assessment and aggregation: The informations to be aggregated are transferred into a uniform scale according to instructions which had been developed for different applications and potential land uses. This includes an assessment of the many basic data. E.g. the recreational suitability becomes assessed by the diversity of land uses.
- grid-transformation: The basic regional unit is a grid of a certain size. The grid allows the spatial combination of different geographical factors.
- EDP-assisted procedure: The informations are processed by means of a computer and partly a device for interactive digital cartography. This facilitates updating and changing of assessments and planning or scientific assumptions (as shown in the aggregation instructions).

### 3. Results and Conclusions

New planning possibilities for road construction result from the valuation of ecological consequences, i.e. of environmental pollution and natural resources. This fact bases on the area-covering and synoptical examination of the region, as it was made possible by applying the Instruments for Regional Planning. Not only restricted conflict cases relating to individual route sections are discovered, but one can get an idea of the marked restrictions varying in intensity of a region and in this way chooses the route course with the minimum conflicts.

The profile of the alignment hardly met with opposition among others because of the poor regional conflicts with the environmental fields in the region under consideration. The number of ecological conflict zones is comparatively low. The extensive dissection of biotopes is to be considered as the most important conflict. Nevertheless, by applying the Instruments for Regional Planning, it could be shown how a more favourable route course was to be found a few kilometers to the west with regard to the potential ecological conflicts.

The analysis of the highway A 7 showed that this highway in fact produces a traffic-shifting effect for the previous parallel road connections, but, on the other hand, that additional traffic is, at the same time, attracted or produced, respectively. Therefore,

the complete number of relieving effects (e.g. number of accidents or nuisance caused by noise) is not as high as would be expected when assuming a constant traffic volume.

The evaluation of accessible materials and documents is not sufficient for environmental compatibility assessments at the level of a more exact route planning. Nonetheless, ecological consequences can generally be evaluated already on account of the basis of available regional information in form of a rough and general estimation. This statement is of course only applicable in a certain range and for comparable ecosystems. After all, it showed that statements pertinent to plans were already possible for the superimposed standard on the basis of usually available information.

On account of the updating routines for topographical maps, their interpretation results in an ecological weighting of areas that is higher than in reality. The regeneration potential of the landscape is thus in many cases already lower than it seems to be according to the topographical map.

The results gained in a number of interviews show that the regional economic consequences in the industrial area of the BAB (Federal highway) section under consideration are not very important. In view of the highway, big industrial regions were nevertheless shown, the position of which is to be considered as "highway-dependent".

The application of the discounting calculation using formulas relating to financial mathematics to the valuation of natural resources will not do justice to their ecological importance. They assume a heavily decreasing weighting of annual benefit in the accumulation of lost benefits over a future period.

The frequently applied method to convert assessed effects into monetary units by means of the cost-benefit method via so-called coupling quantities seems dubious merely for purely methodical reasons. A cost-benefit (point) scale of a nature whatsoever is limited in upward direction, while monetary values are not limited. A linear coupling of both scales is therefore admitted only punctually. According to tendency it involves the danger of an underestimation of the effects valued according to the cost-benefit scale, i.e. of so-called intangible ecological benefits or damages.

It could be shown that a great part of so-called intangible aspects in the ecological field are accessible to a monetary valuation, despite the fact that it is still incomplete. In the present case, the ecologically orientated cost and benefit components are of the same order as cost and benefit relating to traffic economy. This fact indicates that the ecological aspects must no longer be included only verbally or in a relative valuation into the decision concerning road construction measures.



#### 4. Recommendations

- The valuation of the construction of a highway must not base on a "before/since"-comparison but on a "with/without"-comparison, whereby the "without"-case includes the status quo with adaption measures (basing on general traffic development). It has to include the relief and load effects in the traffic network and adjacent areas.
- The basic effects of a highway construction on regional development can be generalized in systematics and flow. Their forms are however largely and individually varying according to the special time and geographical conditions. Accordingly, these effects can be derived only approximately without detailed study and long-term observation.
- The monetary valuation of natural resources strained by road construction measures must be orientated to the aims of a long-term resource policy. If, for instance, the agricultural or forestry soil is assessed only by means of the productive contributions of agricultural or forestry enterprises to the national product, then the long-term importance of the resource "soil" with respect to ecology, food and social policy is neglected.

Since the majority of environmental fields is lacking of a founded system of measured quantities, indicators must frequently be gained on the basis of measured quantities, whose significance according to indicators is not yet empirically secured to a sufficient degree. For this reason, the development of an ecological indicator system with the concrete aim of planning applicability should be promoted for the different subjects. The consequence is among others that the expenditure of data collected can be optimized to the extent that the expressiveness of defined measured quantities is to bring in relationship to their expenditure of data collected.

- The subsequent estimation of a status-quo-ante is always involved with methodical and data-technical difficulties which have insecurities in the effect analysis as a consequence and unnecessarily diminish the empirical basis for questions which can be generalized. For this reason, an economic-ecological inventory should precede concrete measures having a regional effect and support them in form of a documentation on a medium term or over a defined project-dependent period (for instance in the scope of a pilot project).
- For reasons of expenditure, it has to be referred to available materials and data for rough planning at the regional level. Therefore, the empirical calibration of valuation approaches and the estimation of the indicating capability of topographical elements are of decisive significance in the judgement of regional measures. A number of contributions could be made for individual areas and sub-regions in this project. Moreover, the reasoning for valuation flows (aggregation steps and valuation functions)

set the methodical background. Before a standardized (at least in some parts) method can be introduced, additional valuation proposals should however be tested by different geographical situation. Pursuant to this, demands could be defined and the applicability of ecological mapping in the EEC could be demonstrated.

### List of References

a) Communications in particular with representatives of:

- Miljøministeriet, Copenhagen
- Fredningstryrelsen, Copenhagen
- Sozialministerium, Abt. Umwelt, Kiel
- Straßenneubaubehörde-Mitte, Neumünster
- Planlegningsafdeling, Aabenraa
- Ministerium für Ernährung, Landwirtschaft und Forsten, Kiel
- Innenministerium, Kiel
- Landesamt für Naturschutz und Landespflege, Kiel
- Kreisverwaltungen
- Ämter für Land- und Wasserwirtschaft
- Sønderjylland Amtskommune, Tønder und Aabenraa
- Landbrugsministeriet, Vejle

b) Publications or papers referring to project:

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"Instrumentarium zur Beurteilung ökologischer Folgen der verkehrlichen Erschließung"; Symposium "Umweltschutz und Straße" June 1978, published in: Sozialminister des Landes Schleswig-Holstein (Hg.): Umweltschutz und Straße, Vorträge und Ergebnisse einer Veranstaltung vom 5.-7.6.1978.
- Paper, read in Den Haag:  
"Instruments for Regional Planning - experiences with its application in environmental planning projects"; Symposium "Urban Data Management" 24.-27.4.1979, published in the symposium report.

Contractor : Centre National d'Etude et de Recherche du Paysage - C.N.E.R.P -  
64, Rue de la Fédération - 75015 PARIS - France.

Contract No : 223 - 77 - 1 ENV F

Project Leader : Rémi PERELMAN

Title of Project : Typical landscapes in Europe

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### RESEARCH PURPOSE

Within the scope of the research call for tenders launched by the C.C.E. in 1976, this C N E R P project was selected. It deals with sites and landscapes quality, constantly brought up in land development policies. Up to the recent past, intuitive practice prevailed in the rough adaptation of buildings and facilities to the natural environment. Of late, attempts have been made to rationalize this effort, especially through the utilization of environment sciences and social sciences. It is within this framework that this project is undertaken to compare separately elaborated approaches.

This work comprises an interesting international aspect since it brings together the agency in charge of the research, in this case the C N E R P, and the Institut français of Rome, the Institut néerlandais du paysage (Wageningen University) and the Manchester University (Great Britain) Département d'aménagement et d'urbanisme.

The purpose of this research has been:

- to clarify the qualitative effect of the increasing occupancy of delicate and attractive sites;
- to observe man's behavior in regard to the transformations induced by these sites developments;

Attention focused on typical sites which were both:

- Remarkable on account of their structure (hillsides, hills) or on account of their location (seaside, lake side),
- Customary on account of their general existence in Europe

### MATERIALS AND METHODS

A first work symposium was held in TRAPPES (France) on March 20 and 21, 1978; there took place the selection of the precise sectors for the case studies, the framing of documentary work and the work practical aspects:

- a) - Documentation (collection and analysis),
- b) - Case studies concerning the selected areas

I - HILLSIDE AND HILL LANDSCAPES.

France - Burgundy (main research)

Italy - Latium (additional research)

II - COASTAL LANDSCAPES

The Netherlands - Central Zeeland Development Plan,

Great Britain - Essex and Suffolk Structural Planning,

- Blackwater River Estuary Study,

- Suffolk "coastal heritage" Study.

France - Brittany Coast Planning:

. Pointe du Raz Site Restoration,

. Baie d'Audierne Natural Reserve,

. Combrit Natural Coastal Base.

The general work schedule agreed upon was:

- Presentation of the Studied Sectors Area (pictures and maps),
- Planning Purpose, Involved Institutions and Agencies, Elaboration Methods, Results.
- Specific work carried out in the selected geographical sector, studied from the various viewpoints of landscape and ecology, original motivations, concrete consequences.

RESULTS

Research was based on the two following "basic landscapes": hillsides and coastal areas, in order to determine the respective contribution to the landscape studies of, on one hand, the historical sciences and, on the other hand, of the ecological sciences.

I - THE HILLSIDE LANDSCAPE - The French section was covered by an original research of Yves LUGINBUHL, a landscaper agricultural expert, a leading C N E R P researcher. This work dealt with the Burgundy area; it consists in an in-depth historical research which permits discovering the evolution of a mostly vineyard landscape and above all of the latter's relationship with the men who created and transformed it. It shows the most constant features which persisted up to our times and those having disappeared.

The Burgundy vineyards limits never ending variations in the course of history that research managed to bring out, materialize, as a matter of fact, the alternance of phases during which economic, social and technical conditions made

an expansion possible or dictated a decline - possibilist theory - while determinism is illustrated through the vineyard minimal localization on the narrow hillside strip where, by the way, choice vintages are to be found.

These variations have left an imprint on this vineyard hillside landscape, especially in regard to its limits, on account of the outstanding permanence of vineyards in that area. It is interesting, for the purpose of enrichment of the knowledge in the development field, to study the phenomenons having led to these marginal modifications. Just as captivating is the research on the most permanent phenomenons in the hillside landscape evolution, or yet that of discontinuity phenomenons. Thus, they may become a basis for the setting of landscape development study methods. The research which was geographically restructured, resting principally on the Burgundian vineyard hillside example, attempted therefore to bring out the most permanent phenomenons shown in the landscape historical evolution process. Indeed, the Burgundian hillside proved a choice field for such a research: vineyard permanence, famous district, relatively easy historical knowledge. The hillside geographical location is also an interesting item in itself in that it represents one of the preferred topographical location of individual urbanisation: watching and being watched. The few constants which have been brought out are probably not the only ones. But they seem of primary importance to us since they permit showing that a landscape development cannot depend only on economic phenomenons, but that symbolic, emotional values linked to a certain type of landscape, for a given community, in very precise environment, are important as well.

The Italian section was briefly approached, after a field assignment under the guidance of one of the research scientific consultants, Mr. TOUBERT, a history professor at La Sorbonne and an expert on LATIUM. The teachings drawn from the rapid survey of this Italian area helped support or invalidate or rather weight the conclusions drawn from the Burgundian historical survey.

Several constants are prominent:

Vineyard permanence, the most telling phenomenon of these landscapes history; stability in close relationship with other food crops, with animal husbandry and with dwellings.

Besides, the correlative nature of the hillside landscape components (flat country, hillside, coomb, stubble-fields) outstanding on two essential planes - the economic plane and the socio-cultural plane.

II - COASTAL LANDSCAPES - This research led to a very fruitful collaboration between C N E R P, the Manchester University (Town and Country Planning, Professor ROBINSON) and the Wageningen University in the Netherlands (Institut Néerlandais du Paysage, Professeur VROOM), on the basis of three case studies previously established somewhere else. The research work consisted in describing the methods, the concepts and the findings, taking into account the national context (administrative, social, traditional or recent).

More specifically, the study dealt with the share of landscape and ecological concerns having arisen recently. This comparison rested on the examination of case studies considered on one hand, as typical of each national policy and, on the other hand, with enough similarities between themselves so that problems came up in them under not too far apart aspects.

The Netherlands: Study effected by Professor M.J. VROOM, on Central Zeeland Development Plan.

The first section of this study is an outline review of regulations applying to the rural district including the specific zones involved in the DELTA ACT. Next, it gives the report on the regional development plan and then the Walcheren Island landscape structural plan and the Vrouwen-Polder detailed plan.

The choice of Central Zeeland and the peninsula of Walcheren as object for this case-study is based on the following considerations:

- The interior of Walcheren has been largely reconstructed in the early post-war years after floods caused by war time bombing had created havoc. This reconstruction work formed a starting point for new planning proposals after the 1953 floods.
- Developments here in the sense of urbanisation, influx of tourists have been accelerated by recent improvement in accessibility (see following chapter).
- Students teams of the Department of Landscape Architecture have made surveys and planning proposals during recent years. The material collected was useful for the case study

This coastal zone, typical of the Dutch landscape appears to be very rich from the ecological standpoint; this is associated with the landscape other aspects, those of the protection against the sea onslaught and the question of coastal dunes.

France: The case study performed by Mr. Rémi PERELMAN, the C N E R P Director, deals with French "Cornouaille" and more specifically with three of its main sites with a threefold interest.

- These are zones of major touristic interest and being subjected to the bad effects from the frequent presence of people.

It deals with the urbanisation decisions in conflict with associations actions and with recently elaborated protective measures.

These zones constitute non-negligible ecological reserves whose balance is delicate.

Great Britain: The study was carried out by Professor D.G. ROBINSON and J.F. WAGER of the University of Manchester Town and Country Planning Department.

This study deals with sensitive zones of the Suffolk and Essex coast (Black-water river estuary) which are, by many aspects, comparable to the zones studied in the Netherlands and in France.

The Blackwater river estuary constitutes a particularly rich zone from the ecological stand point which is also subjected to the problems involved with the presence of people on account of its proximity to heavily populated areas. The estuary development and protection have been entrusted to the MALDON DISTRICT COUNCIL in order:

1. to maintain, and enhance where possible, the wildlife conservation and landscape quality of the area.
2. to improve and extend facilities for recreation provision within the area where this can be achieved without detriment to objective 1.
3. to resolve existing conflicts between different interests in the area, to prevent further conflicts arising and to prevent congestion of the river.
4. to support, encourage and protect from excessive disturbance the agriculture and fishery enterprises in the area.
5. to prevent excessive disturbance to those living in the area.
6. Operational objective - to achieve an effective co-ordination of planning and management within the area in pursuit of the above objectives.

The study of Suffolk heritage coast plan has been directed towards examining and preparing planning policies and a programme of action which seeks to identify and manage these pressures.

Whilst the coastline has long been under pressure, problems have increased rapidly in the postwar period. The growth of industry in coastal areas, the expansion of settlements; and recreation and holiday developments in areas previously remote, have all contributed to the deterioration of coastal landscape quality. Changing agricultural practices, dereliction and eyesores, particularly the remains of coastal defences, and the physical pressures exerted on the landscape by very substantial numbers of holiday and day-recreation visitors, also pose a serious threat to coastal amenity. As well as conflicting with conservation objectives these uses and pressures are often in competition with each other for the limited space available.

The national importance of the coastline is emphasised by the fact that over half the population look to it as their main outdoor recreation and holiday outlet.

This very attraction, however, is also one of the greatest threats to the continued existence of the open coastline. It must be emphasised that all open countryside is

a non-renewable resource and once built upon or changed by inappropriate and unsympathetic use cannot be replaced.

### CONCLUSIONS.

Thus, the study of the European basic landscapes permitted tracing their evolutions throughout the various periods up to the current one. What teachings should then be drawn from it for landscapes development? Do historical studies supply other data than those from ecology, economics, sociology...?

A large number of events have left their imprint on the landscapes; sometimes they were completely upset. The unearthing of what they have been subjected to, of what imprint is left on them should therefore permit determining what they can withstand. Space development decisions should not be taken in the absence of a precise knowledge of the major historical events, of the landscape features induced by them and of the relationships established between men and the landscape.

Landscape development, as it is currently performed through urban planning documents, rests on an exaggerated functionalizing of space: any space should have a function, any space should be production. There are no vacant spaces in any urban planning, or development planning. However, economically vacant but culturally rich with symbols landscapes are reference landscapes. Their mere existence takes on a large importance for the people psychological balance. May we, by extrapolation extend this conclusion to other landscapes being currently subjected to production-oriented developments: the moors, the brush, the garrigues which, in our opinion, are also landscapes apt to offer a place of escape of primary importance. The important thing is that they do exist, for their very existence is apt to bring about a release of emotional feelings reserved to the only ones aware of them.

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### PUBLICATIONS

#### PAYSAGES ELEMENTAIRES EUROPEENS :

- LE PAYSAGE DE COTEAU *CENTRE NATIONAL D'ETUDE ET DE RECHERCHE DU PAYSAGE*  
*Rédaction Y. LUGINBUHL, Ingénieur Agronome, paysagiste*  
*Documentation et premières investigations :*  
*P. GRONGNET, Maître es Lettres.*  
(1 volume)
- LES PAYSAGES LITTORAUX :
  - . LANDSCAPE PLANNING  
IN COASTAL AREAS  
(2 volumes) *DEPARTMENT OF LANDSCAPE ARCHITECTURE*  
*AGRICULTURAL UNIVERSITY OF WAGENINGEN - NETHERLANDS*  
*by prof. M.J. VROOM, M.Sc.M.L.A. Landscape architect*  
*J.B. STRUIK, M. Sc. Landscape architect*  
*M.W.M. VAN DEN TOORN, M.Sc.M.L.A. Landscape architect*
- . FRANCE - ETUDE DE CAS *CENTRE NATIONAL D'ETUDE ET DE RECHERCHE DU PAYSAGE*  
*par Rémi PERELMAN, Directeur du C N E R P.*





**TOPIC 45 : ECOLOGICAL CONSEQUENCES  
OF MODERN AGRICULTURAL PRACTICES**

Contractor: Bayerische Landesanstalt für Bodenkultur  
und Pflanzenbau, Abt. Pflanzenschutz,  
München

Contract n<sup>o</sup> 209-77-1-ENVD

Project Leader: Prof. Dr. Wallnöfer

Title of project: Degradation of xenobiotics and their  
metabolites by soil microorganisms

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### Objective of the research

Degradation of xenobiotics in the environment is often incomplete leaving metabolites, frequently the aromatic moiety of the parent molecule which are more recalcitrant to microbial attack. There is a need to investigate the further degradation.

The purpose of this investigation was to elucidate the metabolism of phenolic compounds which are liberated during the microbial degradation of phenolbased pesticides in soil.

Chlorinated phenols are intermediates in the microbial degradation of the widely used chlorophenoxyacetic acid herbicides (Bollag et al., 1968; Loos et al., 1967). Methyl-, methylthio-, and nitrosubstituted phenols are released in soil from a number of organophosphorus and methylcarbamate insecticide and nematicides by chemical and enzymatic hydrolysis in water, soil, plants, and animals (Fest and Schmidt, 1973).

Since the acute toxicity of those nitrosubstituted phenols which are used as herbicides is considerably high against mammals, effects on the soil microflora cannot be excluded. Therefore investigations on the degradation of such compounds by soil microorganisms were included to this study (Wallnöfer et al. 1978).

### Materials and methods

Chemicals. Most substituted phenols were purchased from EGA Chemie, Steinheim, Germany, the chemicals are commercially available compounds.

The herbicides DNOC(2-methyl-4,6-dinitrophenol) and dinoseb

(2-sec-butyl-4,6-dinitrophenol were from Dr. S. & I. Ehrens-  
torfer, Augsburg, Germany.

Microorganisms. The following microorganisms were used in  
this study:

Bacillus sphaericus ATCC 12123	Arthrobacter sp. DSM 20389
Nocardia sp. DSM 43251 (=Rhodococcus rubrus)	A. sp. DSM 20390
N. restricta DSM 43199	A. paraffineus ATCC 15590
N. corallina DSM 43278	Pseudomonas fluorescens 1542
N. erythropolis ATCC 4277	P. putida 1022
Alcaligenes sp. DSM 30128	P. putida 1065
Brevibacterium sp. ATCC 19615	P. fragi 1095
Corynebacterium petrophilum ATCC 19080	Azotobacter chroococcum

Culture conditions. Degradation studies were performed using Hegeman's mineral base (Hegeman, 1966) modified by addition of 0.02 % yeast extract in 0.1 M phosphate buffer pH 7.5. Cultures were incubated on a rotary shaker (New Brunswick, G 10) at 28 °C and 220 rpm. Liquid cultures were grown in 100 ml-Erlenmeyer flasks containing 25 ml mineral base with the addition of 0.01-0.02 % of a substituted phenol and 0.4 % of fumarate or ethanol, respectively, as carbon source. For large scale preparation of metabolites, cultivation was performed in 2 l-Erlenmeyer flasks with 1 l of medium.

Degradation of substituted phenols was assayed by extraction of residual substrate and metabolites followed by quantitative UV-analysis as described (Engelhardt et al., 1977).

#### Identification of metabolites

Melting points were determined using a Kofler hot stage (Reichert, Austria). Absorption spectra were recorded on a Zeiss spectrophotometer model DM 4 and mass spectra were obtained from an AEI mass spectrometer, type DB MS 1073 at an ionization potential of 70 eV using the direct insertion probe.

### Results

#### 1. Degradation of substituted phenols

Investigations on microbial degradation of halogen- and methylthiosubstituted phenols were continued including 2, 4, 5-trichlorophenol derived from the herbicide 2, 4, 5-T and 3-methyl-4-(methylsulfinyl)-phenol derived from the insecticide fenthion.

Isolation and identification of degradation products of 2, 4, 5-trichlorophenol (TCP). Concentrations up to 10 mg/l<sub>2</sub> of TCP in the culture medium caused no inhibition of N. restricta DSM 43199, when 0.2 % fumarate was used as carbon source. Within 6 hrs of incubation more than 98 % of the TCP.

added was metabolized by the bacteria. Shortly after the decrease of TCP-concentration in the culture medium a transient metabolite could be observed, which was obviously converted further to a not yet identified compound. The metabolite was identified as 3, 4, 6-trichlorocatechol (TCC) by means of its uv-absorption spectra, melting point, and mass spectrum.

Oxidation of 3-methyl-4-(methylsulfinyl)-phenol by *Nocardia erythropolis* ATCC 4277. Since 3-methyl-4-(methylthio)-phenol is oxidized very easily to the corresponding sulfinyl derivative this compound was tested for its possible degradation by *N. erythropolis* ATCC 4277. With 0.4 % of fumarate as additional carbon source the transient formation of 3-methyl-4-(methylsulfinyl)-catechol could be observed during active growth phase.

All species of the genus *Nocardia* investigated were not able to transform this catechol under the condition of cooxidation within three weeks, although most of the strains could utilize phenol or *p*-cresol as sole source of carbon. The reasons for the low degradability of this compound are that  $-SO-CH_3$  is a substituent with a strong electronwithdrawing character rendering the electrophilic attack of the first two enzymes, phenolhydroxylase and catechol 1,2-dioxygenase more difficult, and additional methyl substituents at ring position 3 reduce the affinity of the enzymes to the phenolic substrate.

## 2. Transformation of nitrophenols used as herbicides

*A. chroococcum* was able to transform the nitrophenol compounds in a medium from which the nitrogen source was omitted. From the initial amount of 100  $\mu$ moles/l DNOC (40  $\mu$ moles/l dinoseb) *A. chroococcum* transformed 80  $\mu$ moles/l DNOC (20  $\mu$ moles dinoseb) almost quantitatively into the corresponding acetylated 6-aminophenols.

As described previously (Wallnöfer et al. 1977) it is of interest that *A. chroococcum* converts the 6-aminophenols almost immediately into the corresponding acetylated compounds. The acetylation of aromatic amines is a well-known detoxification process in various microorganisms.

In a respiratory test it could be demonstrated that 10  $\mu$ -moles/l dinoseb inhibited the respiration of A. chroococcum to a rate of 7 %, whereas the intermediate 6-amino-sec-butyl-4-nitrophenol caused an increase of the inhibition rate to 14 %. The acetylated metabolite, however, showed a considerably lower inhibition rate (10 %). This indicates that acetylation may be an important detoxification reaction in this bacterial species too.

### Conclusion

Phenols substituted by one or two halogen-substituents are readily oxidized by several soil bacteria by 1,2-dioxygenative ring cleavage accompanied by chlorine elimination from the molecule. Additional methyl-, nitro-, or halogen-substituents at ring position 3 or 5 reduce the affinity of the enzymes to the phenolic substrate. However, in soils even phenols which are degradable are often withdrawn from microbial attack for a while due to adsorption reactions to the soil organic matter. Most commonly such phenols are incorporated into the humic core, thus forming "bound residues". The first results obtained from degradation studies of model compounds of such humus-bound phenolic residues indicate cleavage of these residues by a variety of soil microorganisms Engelhardt et al. 1980).

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**Contractor :** Bundesforschungsanstalt für Getreide- und Kartoffel  
verarbeitung

**Contract No.:** 252-77-4 ENV D

**Project Leader :** Dir. and Prof. Dr. H.-D. O C K E R

**Title of project :** Toxic heavy metals in cereals and cereal products

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The objective of the research was first to receive knowledge to which constituents of cereals and cereal products heavy metals (mercury, lead and cadmium) are bound and secondly why the heavy metals are distributed in a specific and different manner within the cereal kernels, as shown in earlier publications.

Samples of commercial wheat and rye were milled on a laboratory scale mill; the corresponding milling products were examined for their content of heavy metals. It was tried to isolate organic-bound fractions of the heavy metals by extraction with organic solvents (petrol, ether, acetone). The results of these experiments showed clearly, that the fraction of organic-bound, lipophile heavy metal compounds can only be very small, if at all. Most of the heavy metals seem to be bound to fractions of the cereal proteins. Rather surprising was the fact, that cereal and cereal products can absorb mercury from contaminated atmosphere in large quantities. After a storage period of three months the mercury content of wheat grains and wheat flour was found at a level of more than 3 ppm, while the average mercury content of untreated wheat is below 0,01 ppm. Wheat bran absorbs the volatile mercury to an even higher degree (up to 15 ppm). The absorbed mercury was found to be rather strongly bound, because it cannot be removed by aeration with a mercury-free stream of air or nitrogen. About 20% of the absorbed mercury vanishes, by heating the contaminated flour to 100° C.

It is assumed that mercury reacts irreversible with certain protein fractions. Isoelectric focussing experiments have shown an influence on the activity of some isoenzymes of esterases and other enzymes, however, these indications of biochemical changes have to be checked by future work. Further experiments with cereal products of oat, maize, rye and durum wheat (figures 1-3) have shown, that the bran of rye and wheat can absorb more than 7 ppm mercury if stored for 100 days in contaminated atmosphere (figure 2), while other cereal products (figure 3) and cereal grains (figure 1) absorb mercury to a much lower degree. A correlation between the amount of absorbed mercury and the protein content of the cereal products could not be verified.

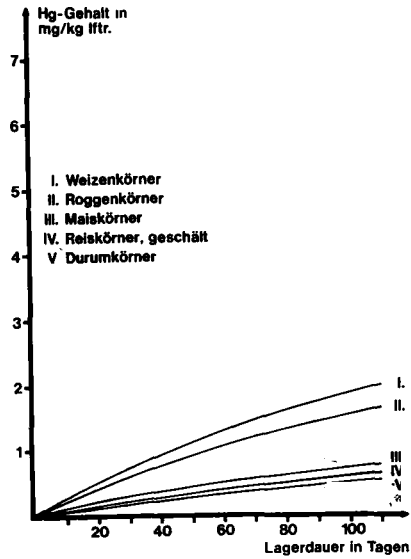


Figure 1  
Absorption of mercury in kernels of  
different cereal species

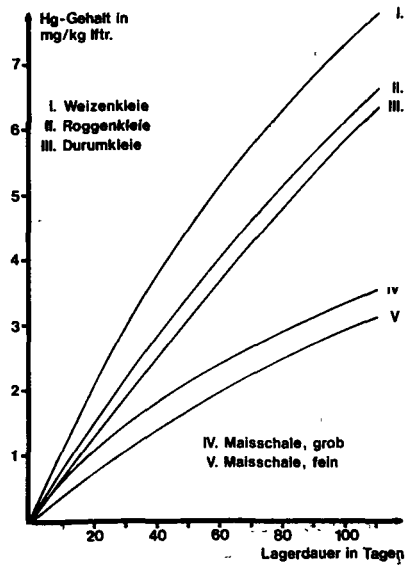


Figure 2  
Absorption of mercury in bran of  
different cereal species



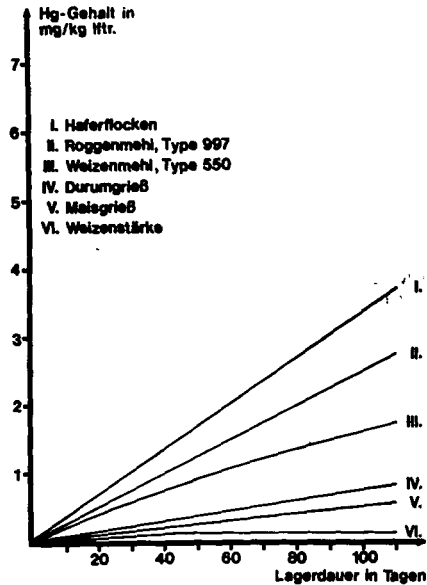


Figure 3

Absorption of mercury in different  
cereal products

Oral communications

Ocker, H.D.: Übertragung von Schwermetallen in Mühlennachprodukten  
Vortrag auf dem carry-over Symposium, Kulmbach, 19/20. Juni 1978

Ocker, H.D.: Zur Belastung des Brotgetreides durch die Schwermetalle  
Blei und Cadmium

Vortrag auf dem Deutschen Lebensmittelchemikertag, Stuttgart,  
17.-19. September 1980

Publications:

Deutsche Forschungsgemeinschaft:

Kommission zur Prüfung von Rückständen in Lebensmitteln.

Mitteilung ' II: Bewertung von Rückständen in Getreide 1980

Ocker, H.D. und W. Seibel: Rückstandssituation bei Getreide und Brot  
2. Mitt.: Schwermetallgehalte (Blei, Cadmium)

Getreide, Mehl und Brot, 34, 5 (1980), S. 118-123

**TOPIC 46 : BIRD PROTECTION**

**N.B. : Contract research on this topic is still in progress.  
No final reports are available.**



European Communities — Commission

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