Commission of the European Communities

environment and quality of life

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Harpenden, U.K.

November 1983

Earthworms are important in the later stages of soil formation and in maintaining soil structure and fertility (Darwin, 1881; Edwards and Lofty, 1977). They contribute in many ways, such as by incorporating decaying organic matter into soil, and turning over and mixing it with other soil fractions, and help in improving soil aeration, drainage and also its moisture-holding capacity. Different species are common in a wide range of soils, both in temperate countries and in the tropics.

Earthworms have been reported to move as much as 250 metric tonnes of soil and organic matter per ha annually (Evans, 1948), and even this is probably a considerable underestimate. An active worm population produces a well-broken down and thoroughly mixed soil down to a depth of about 15 to 20 cm (Evans and McGuild, 1947). During their activities, worms form soil aggregates which resist wetting, erosion and compaction and contribute considerably to fertility and to a good soil structure. (Guild, 1955).

As worms burrow through soil they increase its macroporosity and aeration (Evans, 1948; Teotia, 1950). These pores and the earthworm burrows improve drainage but at the same time, capillary water around the finer soil particles tends to be retained better and the field capacity for moisture of most soils is improved (Stockdill and Cossens, 1966). Certain species, particularly *Lumbricus terrestris*, pull organic matter down into the soil, fragment it and mix it with mineral particles (Bailey, 1961). It has been calculated that the entire leaf fall in temperate forests (2.5 to 3.5 tonnes/ha/year can be consumed by the average population of earthworms in the soil under these forests (Bray and Gorham, 1964) and similar conclusions were recorded for orchards.

The importance of earthworms in organic debris consumption is clear when soils with few earthworms (mor soils)are studied. These soils tend to accumulate a mat of undecomposed organic matter at the soil surface and have a strongly stratified structure. There is very good evidence that it is important to maintain a healthy earthworm population in soils and to avoid them becoming polluted with chemicals which kill earthworms.

Earthworms are eaten by many vertebrates including birds, poultry and pigs. Ecologically they are near the bottom of the terrestrial trophic food chains and have a tendency to concentrate compounds such as organochlorine insecticides (Edwards and Thompson, 1973) heavy metals (Gish and Christensen, 1973; Ireland, 1975) and P.C.B.'s into their tissues. These chemicals seldom harm the worms directly but can either kill vertebrates that eat worms or be taken up into their tissues and this indirectly even affects other animals higher in terrestrial food chains.

Earthworms have a number of characteristics which identify them as one of the most suitable soil animals to be used as a key bioindicator organism for testing for pollution by soil chemicals. In addition to their importance and key role in soil fertility, they are common in the great majority of soils and also in organic matter, they are large in size and easy to handle, they can be collected readily and identified and are known to be affected by and take up into their tissues a number of organic and inorganic chemicals. Earthworms are easily bred quite rapidly and in large numbers in the laboratory for toxicity testing and their longevity makes it unlikely that few worms would die during the period of a toxicity test in untreated media. Several species are available commercially from fish bait breeders.

Because of these characteristics, and since the earthworm is such a typical and important member of the soil fauna, it has been selected as a key indicator organism for the ecotoxicological testing of the toxicity of industrial chemicals not only by the European Economic Community (E.E.C.) but also by the Organization for Economic Co-operation and Development (O.E.C.D.) The Food and Agriculture Organization of the United Nations (F.A.O.) and by many national pesticide registration authorities and environmental pollution committees.

REVIEW OF EXISTING METHODS OF TESTING TOXICITY OF CHEMICALS TO EARTHWORMS

The effects of chemicals on earthworms and methods of investigating these effects have been reviewed by Satchell (1955) Davey (1963) and Edwards and Thompson (1973), Edwards and Lofty (1977) and Edwards (1980).

Most reports in the scientific literature refer to the field testing of the effects of chemicals, particularly pesticides, on earthworm populations. These are too numerous to list (Edwards and Thompson, 1973) but range from the effects of lead arsenate (Polivka, 1951; Escritt, 1955) copper sulphate (Raw and Lofty, 1960) organochlorines (Edwards and Dennis, 1960; Davey, 1963, Edwards *et al*, 1967;) carbamate compounds (Thompson, 1970; organophosphorus compounds, (Edwards *et al*, 1968; Thompson, 1970; Way and Scopes, 1968) and herbicides (Edwards 1970). When all these results are reviewed they are so variable that it is extremely difficult to assess reliably the relative toxicity of pesticides because test sites, soils, formulations, doses and methods of application differ so greatly. Field tests are adequate to identify chemicals which are extremely toxic to earthworms but do not identify accurately moderately toxic compounds, and cannot assess slight toxicity at all.

Some workers have tested the effects of pesticides on earthworms kept in soils in boxes or other containers in the laboratory (Edwards and Lofty, 1973; Hoy, 1955) or in pots (Heungens, 1969; Caseley and Eno, 1966; Altavinyte, 1975, Agarwal, 1978; Stringer and Wright, 1973; Kale and Krishnamoorthy, 1979; Loft - Holmin, 1980). Such tests tend to produce more consistent and reproducible results than field tests because standard numbers of test earthworms of a single species are used and the worms are kept in intimate contact with the chemical. However, a wide range of soil types have been used of greatly differing adsorptive properties have been used in these laboratory tests, and the L.C.50 for any particular chemical can differ greatly, depending on the test soil.

There are reports in the literature on testing the toxicity of chemicals to earthworms by immersing them in dilute solutions of chemicals for set periods of time then transferring them to clean soil to assess mortality (Goffart, 1949; Martin and Wiggans, 1959; Edwards and Lofty, 1973; Stringer and Wright; 1973, Stenersen, 1979). Such tests give very reproducible results and a relatively exact L.C.50 can be calculated for any species of earthworm but this can refer only to the concentration of a chemical in the test solution and does not really duplicate the normal routes of exposure. However, Lord, *et al* (1980) showed that there was a good correlation between the toxicity to earthworms of many chemicals in water and their toxicity in soil, but there were many exceptions. Hence, it would be impossible to predict reliably whether a chemical which was very toxic to earthworms in an immersion test would be equally toxic or hazardous if it reached soil as a pollutant. This is particularly true because chemicals are adsorbed differentially on to the clay mineral and organic matter fractions in soils.

There have been some attempts to assess the toxicity of chemicals to earthworms by injecting them through the mouth (Stringer and Wright, 1976) or into the body cavity (Stenersen *et al*, 1979). Unfortunately, it is impossible to relate such toxicity data to field toxicity on the basis of dose because this is a very artificial type of exposure to chemicals bearing little relationship to natural exposure in the field.

One group of workers (Stringer and Wright, 1973) investigated the possibilities of feeding *Lumbricus terrestris* with leaf tissue that had been treated with chemicals. The main drawback to such methods is the difficulty in controlling the dose of chemical applied to the earthworms, since different species and ages of worms consume very different quantities of leaves.

One group of workers attempted to overcome the problems outlined so far by using a carefully characterized and readily available soil type for running tests of chemicals against *Lumbricus terrestris* (Karnak and Hamelink, 1982). A similar method of using a well-defined internationally common Loess soil (25% clay, 50% silt and 25% sand) has been adopted by the U.K. Pesticides Safety Precautions Scheme (P.S.P.S.) for pesticide registration purposes.

Clearly, all of these methods suffered from various drawbacks and did not fulfil the requirements of a reliable ecotoxicity testing method with a high degree of reproducibility and relevance to the natural methods of exposure of test organisms to a chemical in the field.

DEVELOPMENT OF TEST METHODOLOGY

There are a number of criteria that must be fulfilled in the development of any suitable ecotoxicological test methodology. Firstly, the species of test organism chosen must have a thorough databank relating to its biology, ecology and environmental requirements. It should be possible to breed it in the laboratory easily, quickly and with a minimum of labour and expertise and cultures must be readily available. Its susceptibility to chemicals should be reasonably typical and representative. A test organism that is extremely sensitive to chemicals would have limited usefulness in testing programmes. Secondly, the test method developed should be as simple as possible using readily available materials; it should be quick to run, and able to be performed by operators with little specialist knowledge, should produce reproducible results and it should be possible to relate the results to the exposure of test organisms to chemicals under natural field conditions.

The development of the test methodology described in this report was sponsored by the Council of the European Communities. In the Sixth Amendment of their Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous chemical substances, (15th October 1979), certain ecotoxicological tests were made mandatory from 18th September 1981. In Annex VIII of these requirements at Level 1, any substance placed on the market in quantities greater than 10 tonnes per year or a total of 50 tonnes should be required to submit to the Commission ecotoxicological data on an algal test, a prolonged toxicity to *Daphnia magna*, a higher plant test, a prolonged toxicity to a fish test, a test for species accumulations, a prolonged biodegradation study and a test on an earthworm.

In November 1980, Dr. C.A. Edwards was asked to design suitable testing methodology, draw up a formal draft protocol and organize a discussion meeting of specialists to consider this protocol, who would criticize it and suggest modifications. As a result of this meeting he was charged with developing a final draft protocol by organizing a 'ring' test involving more than twenty collaborating laboratories whereby three unknown chemicals and a standard reference substance would be supplied to each laboratory which would test them according to the draft protocol. The results of this ring test would be analyzed statistically and presented in the form of a preliminary report to representatives of collaborating laboratories who would have the opportunity of attending a meeting at which problems would be discussed and suggested modifications proposed so that a further draft protocol could be prepared with the proviso that a further ring test would be made if necessary.

All of the methods described in the introduction were tested and rejected for reasons given earlier or because they gave poor results and a number of possible methods involving exposure both by contact and in the liquid phase considered and tested. After considerable preliminary work, two methods were developed by Dr. Edwards and a third by Dr. Bouche in France and as these were so completely different it was decided to test these methods in a ring test with laboratories that had agreed to collaborate.

Assessing the effects on chemicals to earthworms involves some difficulties not encountered in some of the other EEC tests. Earthworms can be exposed to toxic chemicals which are (i) dissolved or in suspension in the aquatic phase as, for instance, when chemicals dissolved in the capillary water surrounding soil particles pass into earthworms or (ii) by direct contact with chemicals adsorbed on to the surface of soil particles. This is complicated further, because chemicals may become adsorbed on to soil colloids that make up the clay fraction or on to soil organic matter. When adsorbed in this way they may be held loosely or so completely adsorbed that they are not toxic to earthworms or other soil-inhabiting organisms at any dose.

The first test developed involves the simple contact of earthworms with a chemical applied to a moist substrate and there is no adsorption involved. The second test uses an artificial soil which was specially developed for the test with an adsorptive capacity resembling that of a typical loam soil, the chemical being mixed in to this soil. The third French test involved exposing earthworms to chemicals applied to a matrix of very fine silica supported on a skeleton of glass balls. This test is intermediate, with some degree of adsorption but not as much as that of a true soil. Tests with the first method constitute a simple screening method which only selects out chemicals potentially hazardous to earthworms and other soil-inhabiting organisms. The artificial soil test exposes earthworms to chemicals in a manner much more relevant to a natural field exposure and selects out those chemicals not only toxic but also not strongly adsorbed and likely to be hazardous in field conditions. Both tests can provide useful information, and to some extent are complementary. The silica test is intermediate with some characteristics of both tests. It is the task of collaborating laboratories and specialist Committees of the Commission to decide which of these tests provides the most appropriate information for assessing the environmental hazards of chemicals or to recommend that a preliminary screening test which would select more hazardous chemicals for further testing using the artificial soil method which would account for adsorption.

The simple contact test is by treatment of defined standard filter papers either by dipping them in solutions of known concentrations, spraying the chemicals with a chromatograph or other fine spray, or applying drops to the surface with a fine pipette. The main problem is to obtain an even and adequate deposit of the test chemical on the surface of the filter paper. This can be achieved either by dissolving the test compound in water, or in organic solvents (such as acetone, hexane or chloroform) if relatively insoluble in water, and then pipetting a known concentration on to the filter paper and evaporating the solvent. If the chemical is poorly soluble, even in organic solvents, repeated pipetting may be necessary to achieve the required range of deposits.

In the second test a carefully defined artificial loam soil was developed, with the advice of pedologists to overcome the variability between different soil types for a soil toxicity test. This consists of:

10% sphagnum peat

20% kaolinite clay

69% industrial quartz sand

about 1% calcium carbonate (to bring pH to approximately 6.0.)

This artificial soil has an adsorptive capacity of about 25 m.e.q., approximately that of a typical loam soil. The toxicity of chemicals in the soil is tested at a moisture content of 25 - 42% (This is to some extent dependent upon the source of peat. The key criterion is that the soil must be wetted to a point where there is no standing water.)

The test is made using batches of ten worms exposed to a range of doses of the test chemical applied as a fine chromatographic spray in water or an organic solvent or, for insoluble compounds, mixed with a small sample of the artificial soil and subsequently mixed thoroughly with into the whole.

The 'Artisol' test was developed by Dr. Marcel Bouche of Dijon, France. The principle is to apply the chemicals to a matrix of very fine particle amorphous hydrated silica. This material has a tendency to pack down and not give the test earthworms sufficient air so the matrix is supported on glass balls (1.5 - 2.0 cm diameter) to keep it open. The chemicals are applied in a similar manner as to the artificial soil using 10 test earthworms in about 1.5 Kg of the test substrate and mortality is assessed after 14 days. This is an independent test which was tested by only four laboratories in the first 'ring' test.

A difficult problem was the selection of the best test species of earthworm. There are three species that would seem to be most suitable for earthworm toxicity testing:

(i) *Lumbricus terrestris*

This species is a large true soil-inhabiting species that is the species most exposed to chemical residues in the field because of its habit of moving over the soil surface at night so that it comes into contact with residues of chemicals on the soil surface and also because it feeds on surface organic matter that may be contaminated with chemicals. The main drawback to this species is that it is a slow-growing worm that takes about six months to reach maturity and cannot be bred easily under laboratory conditions.

(ii) *Allolobophora caliginosa*

This is a small, but extremely common species which moves extensively through the upper layers of soil. It breeds faster than *L. terrestris* but is still difficult to produce in large numbers in culture althoughit can be collected readily in large numbers from the field.

(iii) *Eisenia foetida*

E. foetida which resembles *A. caliginosa* in size, is not found in large numbers in soil although it can live in soils with considerable organic matter. It is common in sewage beds, particularly in trickling filters, where it is often exposed to industrial chemicals. It is a species with a short life cycle, reaching maturity in seven to eight weeks at 15 - 20°C. It is very prolific; a single worm produces 2 - 5 cocoons per week each of which will give several worms. It can be bred readily in a wide range of animal or vegetable organic wastes. This means that laboratories could easily breed their own stock if supplied with cocoons from a central source, and a standard strain could be used. Much more is known about the biology and ecology and environmental requirements of this species of earthworm than any other. Although Stenersen *et al* (1979) reported differential susceptibility of species of worms to carbamate insecticides, there is good recent evidence that the relative susceptibility of different species of earthworms to a range of chemicals does not differ significantly, both in my own laboratory and elsewhere (Heimbach, *in litt*; Edwards, P.J. *in litt*). A species may be rather more sensitive to one type of chemical but slightly more tolerant of another. For these reasons, *E. foetida* was selected as the test species. A paper comparing the relative susceptibility of this species and several other species to a range of chemicals is being prepared.

TIMETABLE FOR THE INTERCALIBRATION TEST

The draft protocol was developed and tested according to the following programme:

1. November 1980 - Dr. C.A. Edwards was charged by the Commission of the European Communities with developing a suitable earthworm toxicity test method and organizing an intercalibration or ring test.
2. December 1980 - Preliminary protocol for the test prepared and sent out.
3. April 1981 - Final draft protocol sent out, and test chemicals sent out.
4. October 1981 - Deadline for receipt of ring test results.
5. November 1981 - Preliminary report prepared
6. January 1982 - Meeting in Brussels with the Commission and collaborating laboratories to discuss ring test results and draw up new protocols based on ring test results. (Appendices I, II, III & IV)
7. March 1982 - Meeting of the Sub-Group Ecotoxicology of the EEC Directorate for Environment and Consumer Protection to decide future action. (Appendices V & VI)
8. February, 1982- Final detailed report of first ring test submitted to EEC.

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9. June 1982 - A decision was made to run a second confirmatory intercalibration ring test to be limited to about 20 laboratories all of which had experience in the test and preferably belonged to member countries of the EEC. New

protocols and three new test chemicals and standard would be sent out to laboratories that had indicated their willingness to participate and submit results of their tests by April 1983. (Appendix VII)

10. January 1983 - June 1983. Reminders sent out to collaborating laboratories. (Appendices VIII & IX)

ORGANIZATION AND RESULTS OF THE INTERCALIBRATION TEST

At the meeting in March 1981, there was considerable discussion over which unknown test chemicals should be distributed. It was decided that they should include a freely water-soluble chemical, a relatively insoluble compound, a viscous material, a chemical readily adsorbed on to soil and a known vermicidal pesticide. The final selection was as follows:

A standard reference substance - Copper sulphate

Compound A - Pentachlorophenol

Compound B - Carbaryl (1-naphthyl methylcarbamate)

Compound C - Trichloroacetic acid

They were as diversified as possible and representative of the sort of compounds that would be tested in practice and all have dose/toxicity curves with quite different slopes and different toxicities to earthworms. They were also chosen as being relatively difficult to apply. None had a high mammalian toxicity. A list of laboratories that would be willing to collaborate was prepared. These were obtained by wide canvassing and included government, university and industrial laboratories, the majority of which had no previous experience of earthworm toxicity testing.

By December 1981, results were received from 37 of the 60 that had agreed to do the test. They came from the following countries:

Austria	1 laboratory
Belgium	2 laboratories
Denmark	1 laboratory
England	5 laboratories
Federal Republic of West Germany	6 laboratories
France	1 laboratory
Ireland	3 laboratories
Japan	1 laboratory
Netherlands	4 laboratories
Norway	1 laboratory
Philippines	1 laboratory
Portugal	1 laboratory
Spain	1 laboratory
Switzerland	2 laboratories
U.S.A.	7 laboratories

In view of the short time allowed for laboratories to complete both parts of the ring test and the difficulties many workers had in obtaining test organisms and test media from local sources, the response was excellent.

Results on the contact test were received from 35 laboratories on the artificial soil test from 23 laboratories; and on the 'artisol' test from 4 laboratories. This was quite adequate for an analysis of the validity of the testing methods. Unfortunately, some laboratories did not give confidence limits for their $L.C._{50}$ estimates.

The presentation of the data from the participating laboratories was in very varied forms, ranging from carefully printed reports to merely lists of L.C.$_{50}$'s that differed between laboratories ranging from almost 'guesstimates' to sophisticated probit computer programmes. Fortunately, most laboratories kept reasonably carefully to the proposed methodology and environmental conditions. The greatest reasons for variations were the different sources of test organisms and media and, in the case of the artificial soil test, inadequate time to complete other than the range-finding test.

ORGANIZATION AND RESULTS OF THE SECOND INTERCALIBRATION TEST

The decision at the meeting in January 1982 to run a second intercalibration test using different chemicals was based on the offer of all the laboratories who had done the first test to participate. This was originally 21 laboratories distributed as follows:

Country	No. of laboratories
Belgium	2
England	5
Federal Republic of West Germany	5
France	3
Ireland	3
The Netherlands	2
Spain	1

Later several other laboratories volunteered to participate:

Greece	3
Italy	1
Switzerland	1
U.S.A.	2

This gave a total of 29 laboratories. Three unknown test chemicals and a new standard reference substance chloracetamide and the fully revized protocol was sent out to all collaborating laboratories in July 1982.

The unknown test chemicals were selected to include water-soluble and water-insoluble compounds, a viscous material, a chemical readily adsorbed on to soil and a known vermicidal pesticide in much the same way as in the first 'ring' test. Pentachlorophenol was included in both tests to assess within laboratory reproducibility.

The chemicals chosen were:

Compound A - Potassium bromide

Compound B - Pentachlorophenol

Compound C - technical Chlordane

Some results came in late in 1982 or early in 1983 but a few were not available until late 1983 and several laboratories opted out of their agreement to collaborate altogether.

Results were actually obtained from the following countries:

No. of laboratories providing test results

Country	Filter paper	Artificial Soil	Artisol
Belgium	1	1	-
England	5	4	1
Federal Republic of West Germany	4	5	3
France	3	3	3
Italy	1	1	-
The Netherlands	3	3	1
Switzerland	1	1	-
U.S.A.			-
Total	20	20	8

The results were reported in a much more precise fashion than in the first intercalibration test. It was unfortunate that all of the 3 Irish laboratories and the Spanish laboratory were unable to complete the test and also that because of a comparatively late start, the 3 Greek laboratories had not sent in results to date.

The results were analyzed in detail for between laboratory variability. There was also an opportunity to assess within laboratory variability because one of the chemicals (pentachlorophenol) was included in both the first and second intercalibration tests as an unknown.

In the analysis of the ring test results the following data are included:

- number of participating laboratories
- mean L.C.$_{50}$
- median L.C.$_{50}$
- reproducibility standard deviation (representing variability of results by different operators in different laboratories
- number of laboratories whose results were inadequate
- for pentachlorophenol reproducibility standard deviation (representing within laboratory differences).
- 0.95 confidence interval for the general mean

Some modifications had to be made to results received, to account for such factors as:

- only a range of results being given
- only raw data being given
- mortality in controls not being accounted for
- results being expressed in the wrong units

The results are summarized in Tables 1-10 and Figs. 1-10.

INTERPRETATION OF RESULTS

In interpreting the results, it must be remembered that there were a number of factors which increased the variability of the results:

- Worms were obtained locally so that they were of different strains and bred on different materials. This was because there had been considerable mortality of worms sent out in the first test. When the test is adopted it will be recommended that worms of a standard strain bred from cocoons sent out from a central source be used.
- Ingredients for the artificial soil were obtained locally so there was some lack of uniformity and deviation from the precise

materials recommended, particularly with reference to the peat.

- The variability in size and age of worms was sometimes greater than that recommended in the protocol.
- The methods used for drawing the toxicity/dose probit lines still varied between laboratories although standard methods e.g. LITCHFIELD and WILCOXON, FINNEY'S OR BLISS' methods were recommended.
- The only assessment of within-laboratory reproducibility was for pentachlorophenol.
- One of the reasons for choosing the test substances was their differential solubilities in water and other solvents in order to ensure that the collaborating laboratory had to determine the best solvent. Both chloracetamide and potassium bromide are water-soluble and presented little difficulty but technical chlordane and pentachlorophenol are sparingly soluble in water. The best solvent for these chemicals was acetone. Some laboratories used solvents that were not ideal, to apply the chemicals.

Figure 1. Typical mortality/concentration curve in the contact test

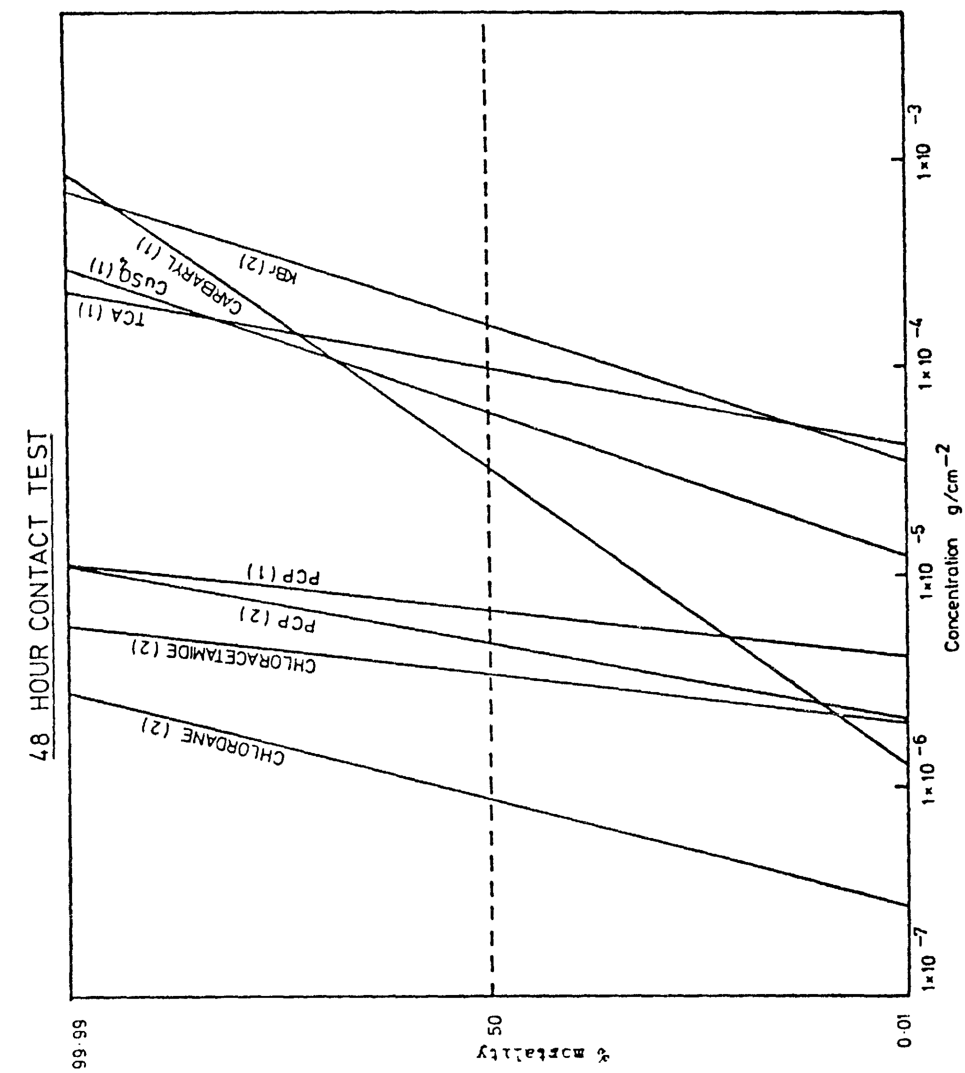


Figure 1. Typical L.C.$_{50}$ curves for the compounds tested by contact

Figure 2. Typical mortality/concentration curve in the artificial soil test

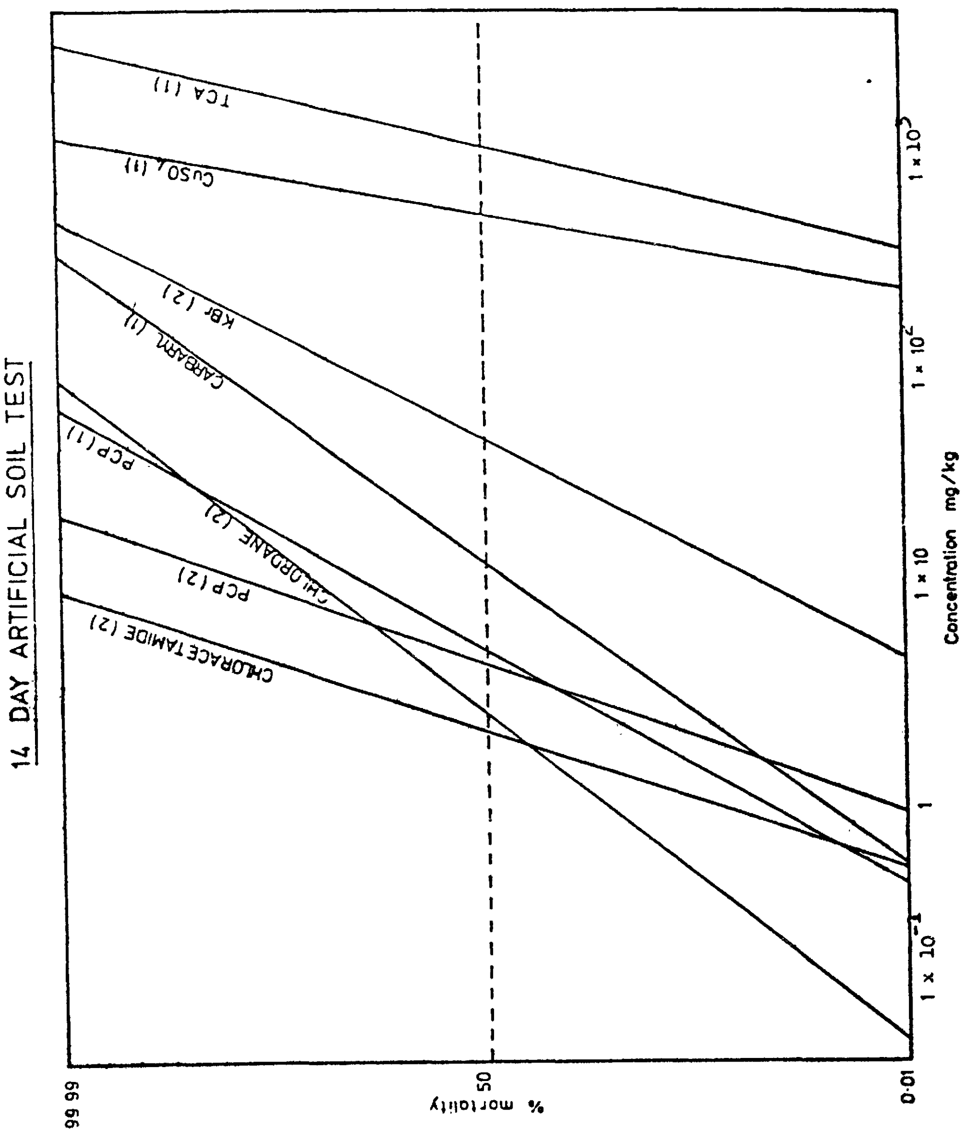


Figure 2. Typical L.C.$_{50}$ curves for the compounds tested in artificial soil

Table 1

Contact test toxicity of

A. Potassium bromide

1.	Number of laboratories	-	20
2.	Number of laboratories with data not used	-	0
3.	Mean $L.C._{50}$	-	0.453 mg.cm⁻
4.	Median $L.C._{50}$	-	0.460 $mg.cm^{-2}$
5.	Reproducibility standard deviation	-	0.301 $mg.cm^{-2}$
6.	0.95 confidence interval for the general mean	-	0.209 $mg.cm^{-2}$

Figure 3 Contact test toxicity of substance A - Potassium bromide

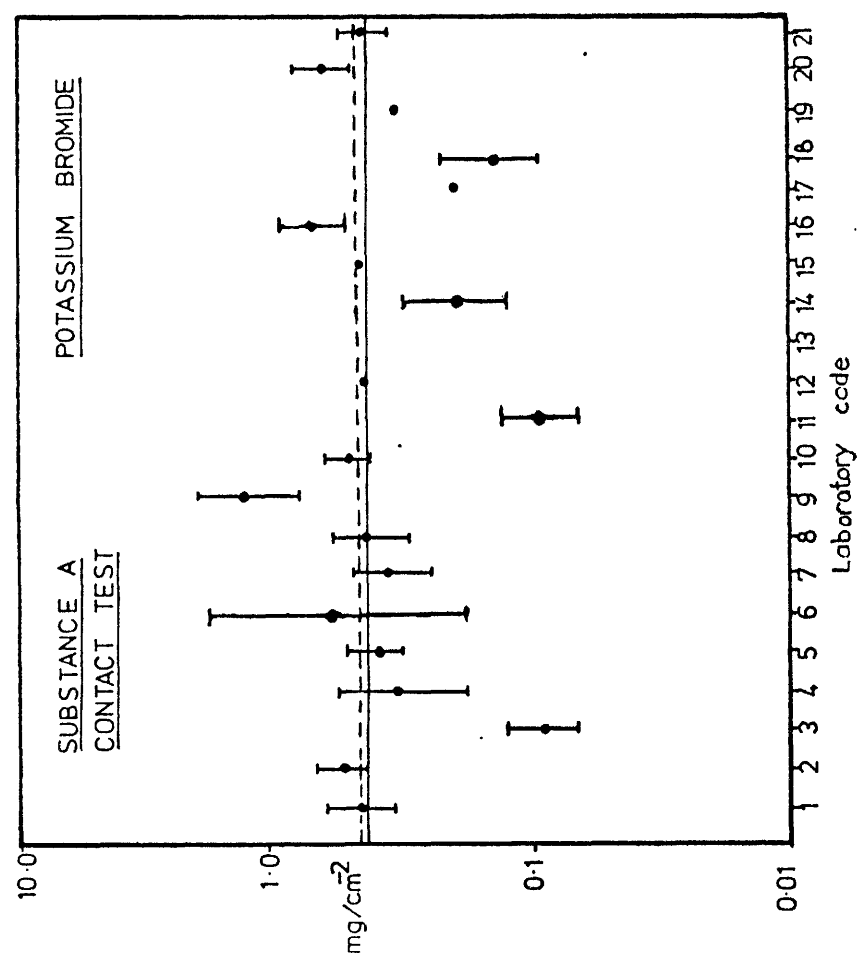


Table 2

Contact test toxicity of

B. Pentachlorophenol

1. Number of laboratories	-	20
2. Number of laboratories with data not used	-	0
3. Mean $L.C._{50}$	-	0.0041 $mg.cm^{-2}$
4. Median $L.C._{50}$	-	0.0037 $mg.cm^{-2}$
5. Reproducibility standard deviation	-	0.0016 mg/cm^{-2}
6. 0.95 Confidence interval for the general mean	-	0.00074 mg/cm^{-2}

Figure 4. Contact test toxicity of substance B - Pentachlorophenol

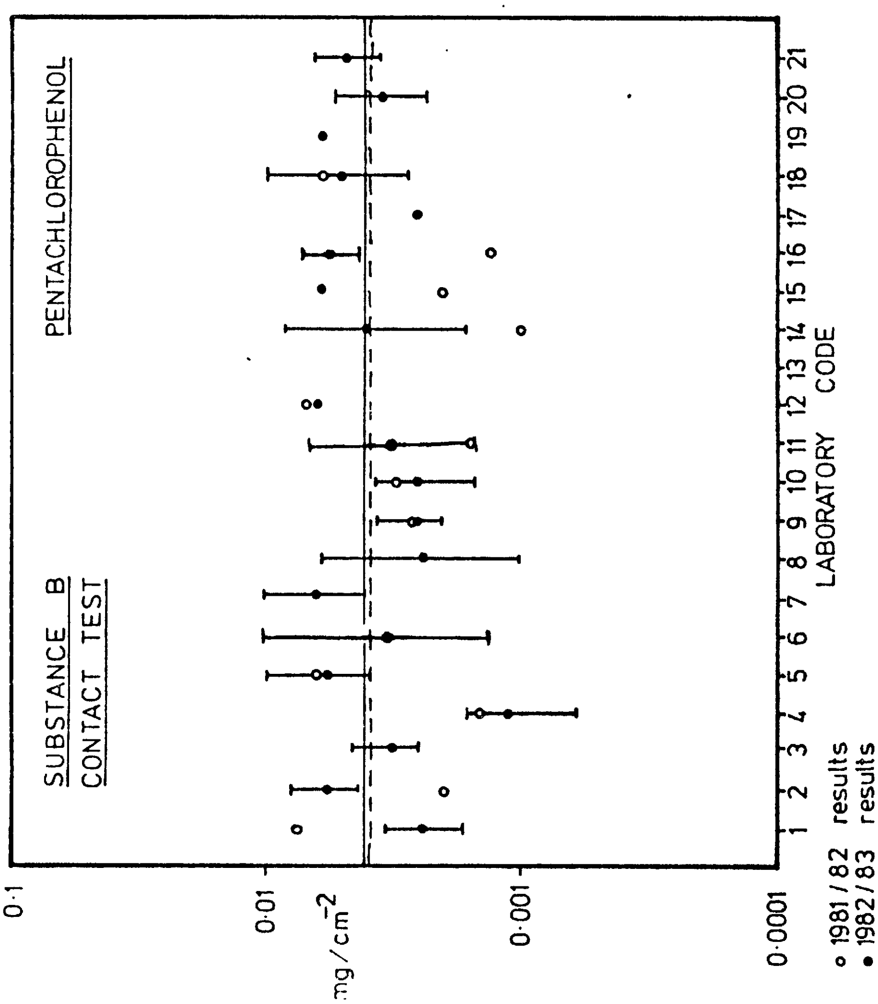


Table 3

Contact test toxicity of:

C. Technical Chlordane

1.	Number of laboratories	-	20
2.	Number of laboratories with data not used	-	0
3.	Mean $L.C._{50}$	-	0.0042 $mg.cm^{-2}$
4.	$L.C._{50}$	-	0.0018 $mg.cm^{-2}$
5.	Reproducibility standard deviation	-	0.0088 $mg.cm^{-2}$
6.	0.95 Confidence interval for the general mean	-	0.0032 $mg.cm^{-2}$

Figure 5. Contact test toxicity of substance C - Technical Chlordane

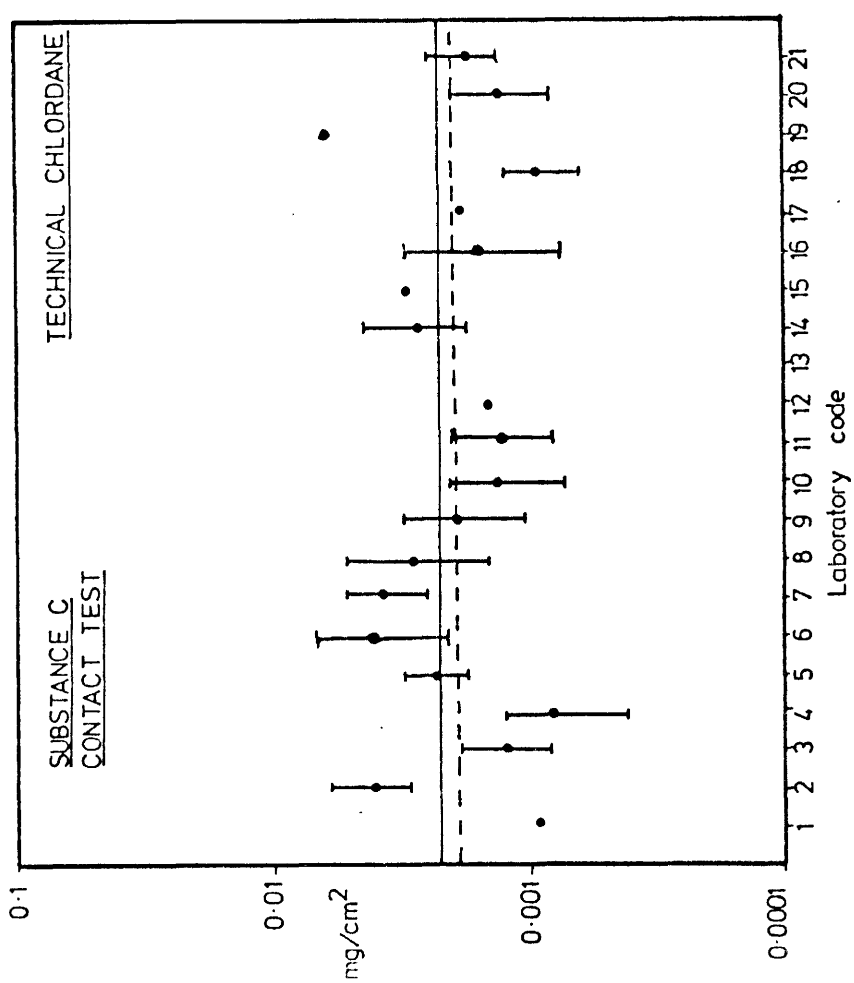


Table 4

Contact test toxicity of:

S. Chloracetamide

1. Number of laboratories	-	20
2. Number of laboratories with data not used	-	0
3. Mean $L.C._{50}$	-	0.0027 $mg.cm^{-2}$
4. Median $L.C._{50}$	-	0.0024 $mg.cm^{-2}$
5. Reproducibility standard deviation	-	0.0015 $mg.cm^{-2}$
6. 0.95 Confidence interval for the general mean	-	0.00066 $mg.cm^{-2}$

Figure 6. Contact test toxicity of reference substance -Chloroacetamide

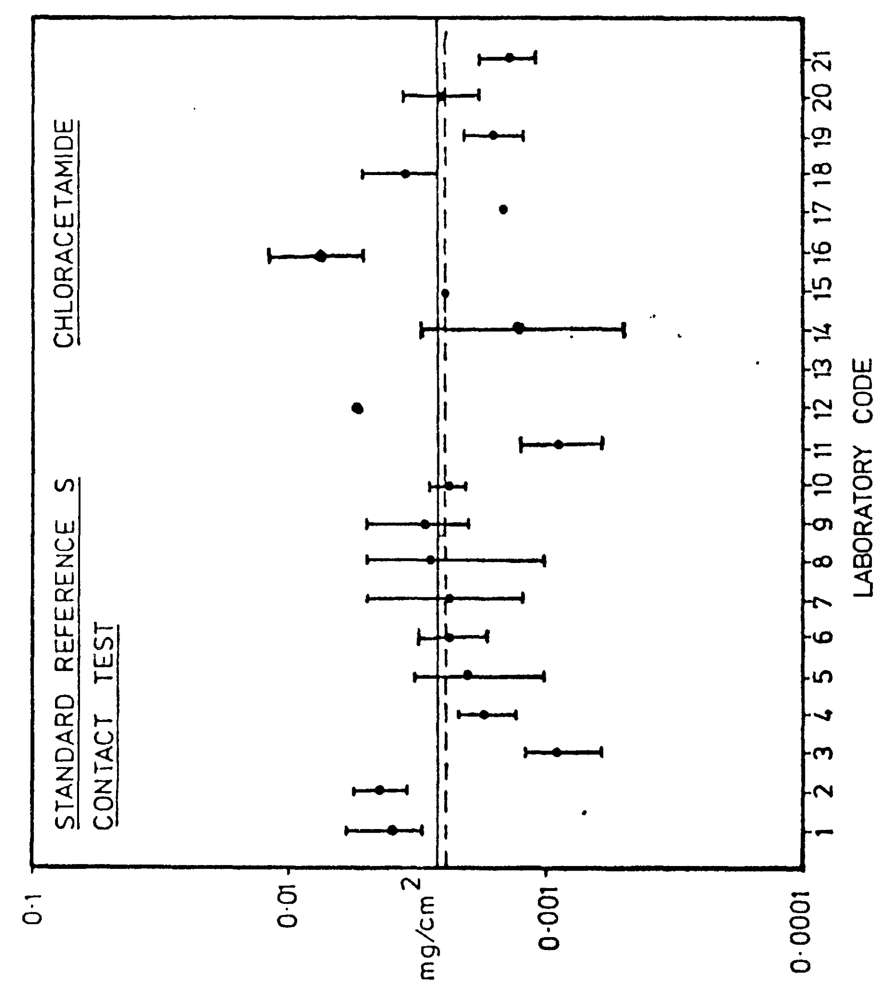


Table 5

Artificial soil toxicity of:

A. Potassium bromide

1. Number of laboratories	-	17
2. Number of laboratories with data not used	-	2
3. Mean $L.C._{50}$	-	298.0 $mg.kg^{-1}$
4. Median $L.C._{50}$	-	162.0 $mg.kg^{-1}$
5. Reproducibility standard deviation	-	346.5 $mg.kg^{-1}$
6. 0.95 Confidence interval for the general mean	-	178.1 $mg.kg^{-1}$

Figure 7. Artificial soil toxicity of substance A -Potassium bromide

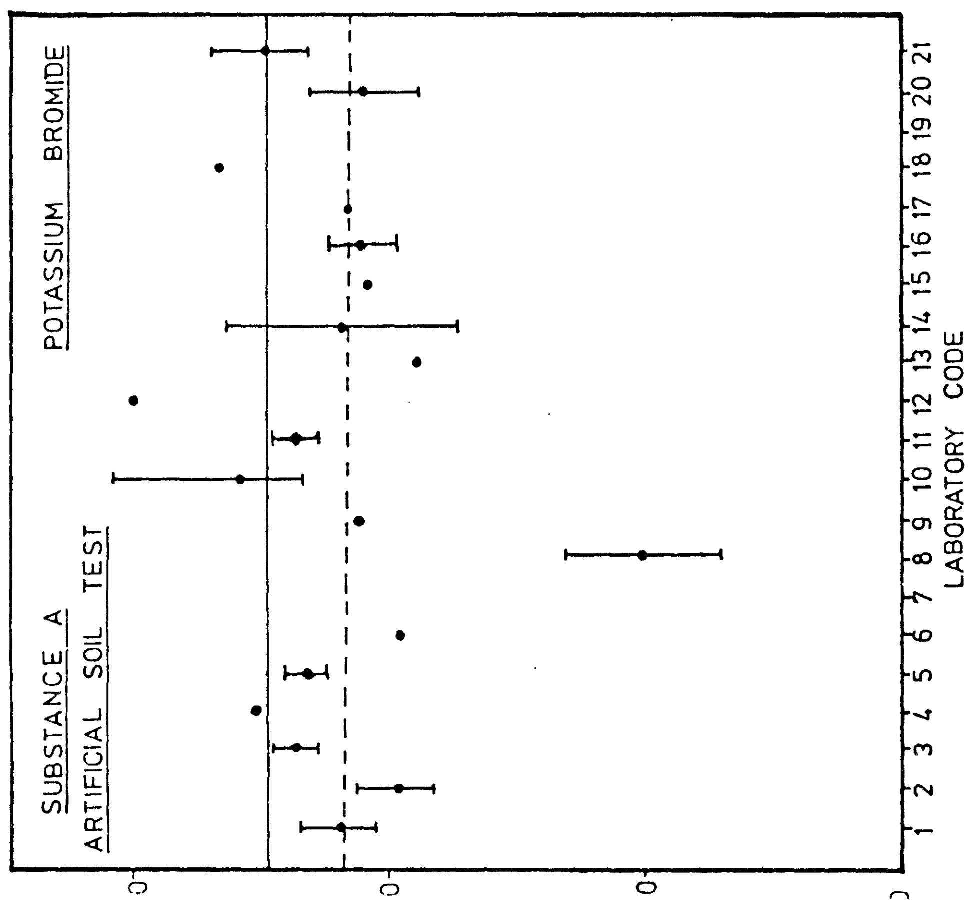


Table 6

Artificial soil toxicity of:

B. Pentachlorophenol

1.	Number of laboratories	-	18
2.	Number of laboratories with data not used	-	1
3.	Mean $L.C._{50}$	-	75.1 $mg.kg^{-1}$
4.	Median $L.C._{50}$	-	73.0 $mg.kg^{-1}$
5.	Reproducibility standard deviation	-	40.6 $mg.kg^{-1}$
6.	0.95 Confidence interval for the general mean	-	20.26 $mg.kg^{-1}$

Figure 8. Artificial soil toxicity of substance B - Pentachlorophenol

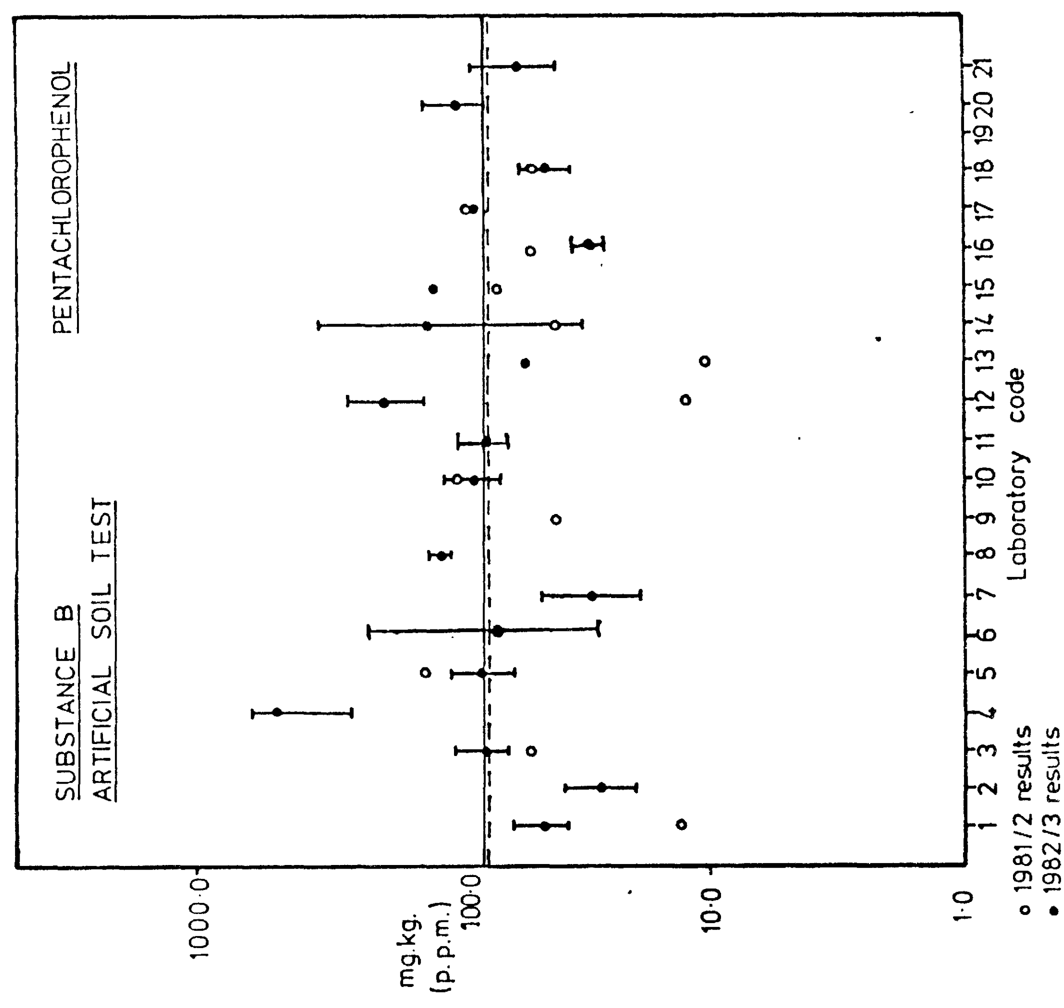


Table 7

Artificial soil toxicity of:

C. Technical Chlordane

1.	Number of laboratories	-	18
2.	Number of laboratories with data not used	-	1
3.	Mean $L.C._{50}$	-	75.3 $mg.kg^{-1}$
4.	Median $L.C._{50}$	-	47.0 $mg.kg^{-1}$
5.	Reproducibility standard deviation	-	96.6 $mg.kg^{-1}$
6.	0.95 Confidence interval for the general mean	-	48.1 $mg.kg^{-1}$

Figure 9. Artificial soil toxicity of substance C - Technical Chlordane

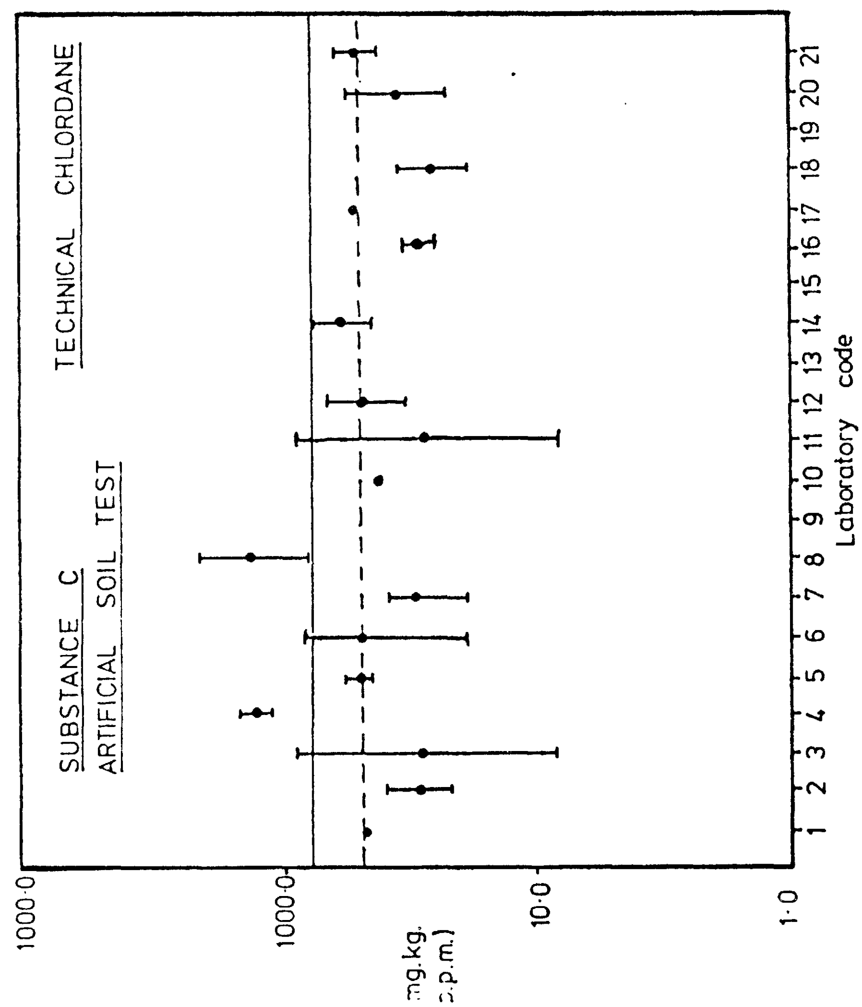


Table 8

Artificial soil toxicity of:

S. Chloracetamide

1.	Number of laboratories	-	18
2.	Number of laboratories with data not used	-	1
3.	Mean $L.C._{50}$	-	38.5 $mg.kg^{-1}$
4.	Median $L.C._{50}$	-	38.0 $mg.kg^{-1}$
5.	Reproducibility standard deviation	-	40.3 $mg.kg^{-1}$
6.	0.95 Confidence interval	-	20.05 $mg.kg^{-1}$

Figure 10. Artificial soil toxicity of reference substance - Chloroacetamide

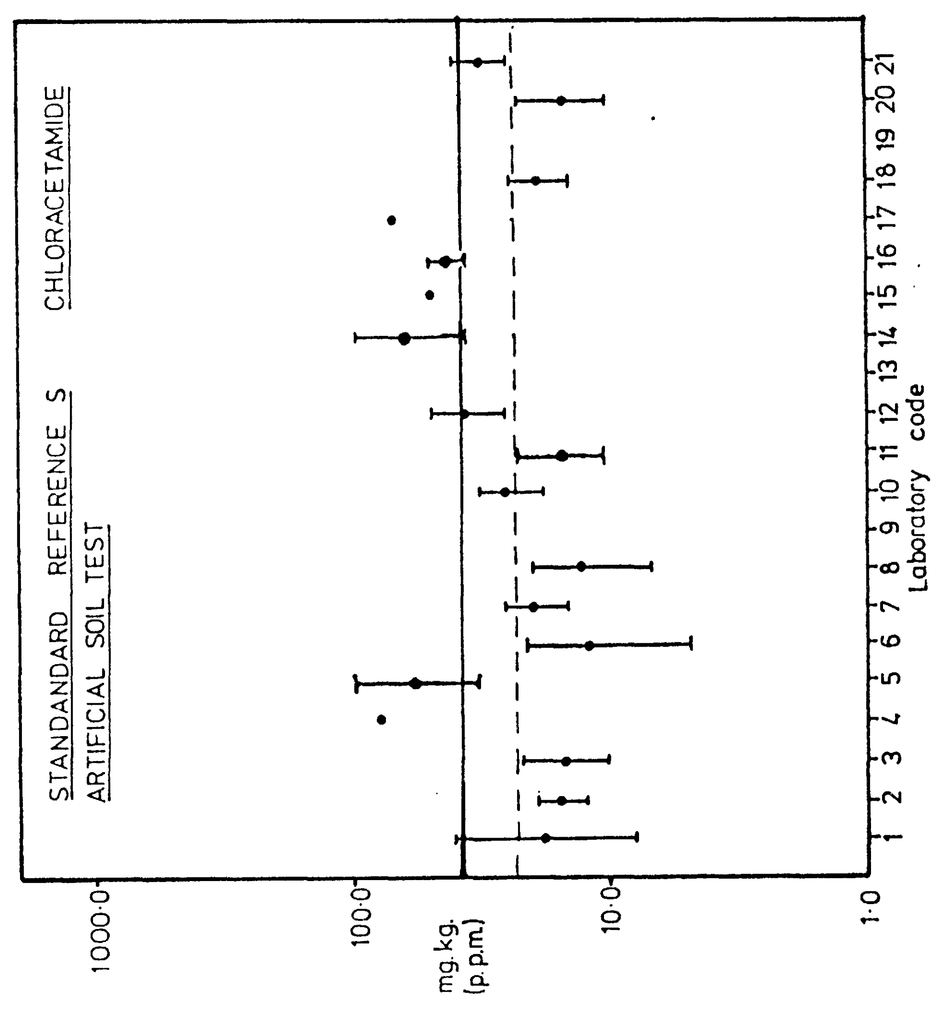
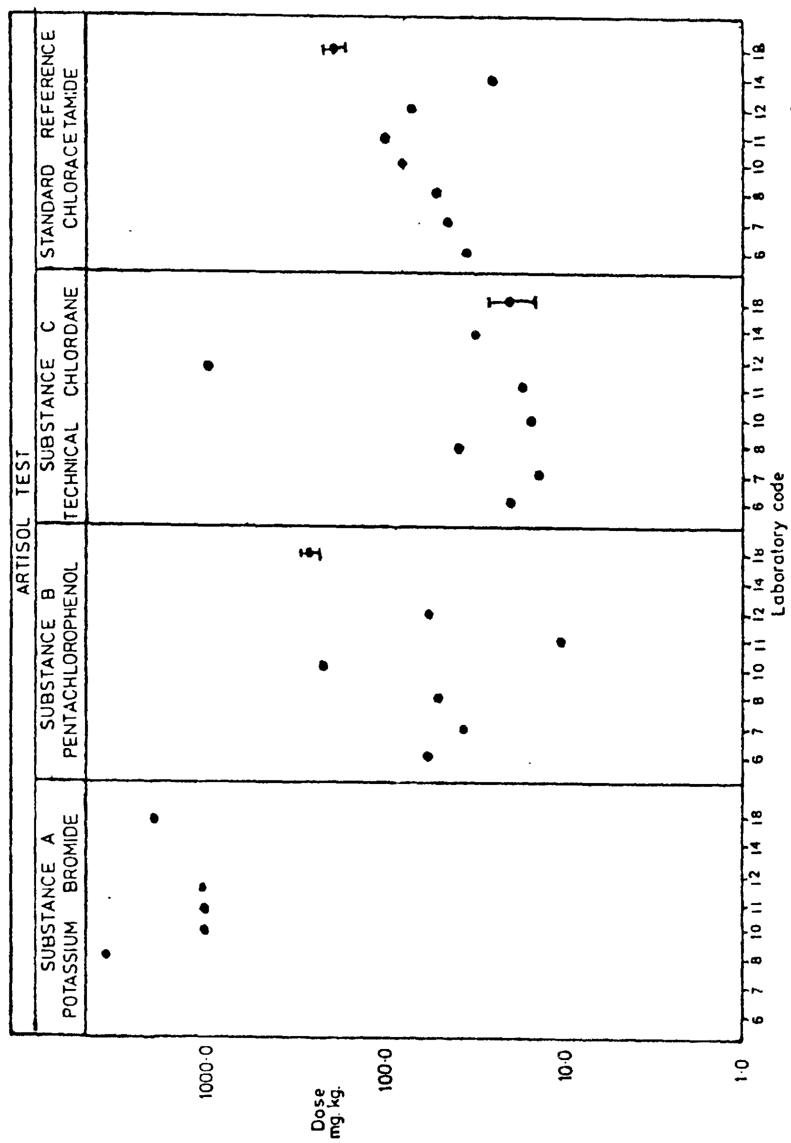


Table 9

'Artisol' test toxicity of the four test chemicals

	A	B	C	S
1. Number of laboratories	5	7	8	8
2. Number of laboratories with data not used	1	0	0	0
3. Mean $L.C._{50}$	1123.0	98.5	144.5	74.4 $mg.kg^{-1}$
4. Median $L.C._{50}$	1000.0	56.0	26.0	65.5 $mg.kg^{-1}$
5. Reproducibility standard deviation	1104.5	97.1	345.8	52.3 $mg.kg^{-1}$
6. 0.95 Confidence interval for the general mean	491.8	70.4	288.1	66.4 $mg.kg^{-1}$

Figure 11. 'Artisol' test toxicity of the four test chemicals



DISCUSSION

The results of the second intercalibration were quite satisfactory, confirming and emphasizing conclusions reached after the first test. In general, a much greater degree of precision was attained and most laboratories were able to present results on at least two methods, do both range-finding and precise tests and give their results with much greater detail and comment.

1. Contact Test

The conclusion that there were no major problems in using the contact test was confirmed. It again gave very reproducible results. Most laboratories seemed to find the contact test easy to perform, simple, with no problems in availability of materials, ease of operation, reproducibility or cheapness. As previously the only adverse comments were to question the relevance of this test to the soil situation and possible difficulties in interpretation of results, in terms of environmental hazard.

Most results fell within one order of magnitude for all chemicals which would seem to be an adequate degree of precision for assessment of environmental hazard.

2. Artificial Soil Test

The results tended to be slightly more variable than in the contact test as in the first ring test. However, with the exception of one laboratory which recorded exceptionally low toxicities for all chemicals in the artificial soil test, most of the results fell within the limitations of one order of magnitude.

The problems of obtaining the ingredients for the artificial soil found with the ring test seemed to have been minimized by relaxing the specifications of the relevant materials. There were relatively few adverse comments on the time taken to do the test.

Nearly all the results of L.C.$_{50}$ estimates after 14 and 28 days did not differ significantly and it would seem justified to conclude that a 14 day test would be adequate.

3. 'Artisol' Test

Although the materials were made readily available to participants on request from Dr. Cabridenc, only eight laboratories completed the test. The data are presented but from such a small number the variability was too great to enable valid conclusions to be made. The method did not seem to be popular with participants.

4. General comments

The relative attributes of the different tests were discussed extensively after the first ring test. Although the contact test is simple, inexpensive and gives highly reproducible results it does not take into account metabolism of chemicals in soil, the influence of ingestion of soil by worms and adsorption of chemicals on clay and organic matter, all of which affect the toxicity of chemicals.

The 'artisol test' is intermediate, because there is some degree of adsorption of chemicals but since it is a relatively sterile medium, little metabolism of chemicals. The artificial soil resembles a natural clay loam soil in its adsorptive capacity and the peat is a rich source of microorganisms which can degrade chemicals. It tests toxicity in a way closely related to field exposure.

In the first ring test, the ranking of toxicity was pentachlorophenol > carbaryl > copper sulphate > trichloroacetic acid and the same ranking occurred with both the filter paper and artificial soil tests. However, in the artificial soil the relative toxicities of copper sulphate and trichloroacetic acid were decreased considerably, presumably by adsorption on to soil fractions.

In the second ring test the results of the two methods were much closer. They both ranked the chemicals in the order chloracetamide > technical chlordane ⩾ pentachlorophenol > potassium bromide and the relative toxicities were very similar indicating that there had been little adsorption of any of these chemicals (Table 10).

Table 10 Mean toxicity levels from all laboratories

	A (potassium bromide)	B (pentachloro-phenol)	C (chlordane)	D (chloracetamide)
Contact ($mg.cm^{-2}$)	0.45	0.0041	0.0042	0.0027
Artificial soil ($mg.kg^{-1}$)	298.0	75.1	75.3	38.5
'Artisol'($mg.kg^{-1}$)	963.7	91.1	301.2	98.8

There is little doubt that if there had been such good agreement in the first ring test the contact test might have been adopted as a suitable screening method and representative of field toxicity.

As in 1982, some chemicals gave more variable results than others. Potassium bromide was more variable than any of the other chemicals and chloracetamide was the least variable. The degree of variability seems to be correlated with toxicity, the less toxic a chemical was the more the results varied. This is a reassuring result because it means that the more toxic chemicals would yield more precise toxicity data.

Chloracetamide, which was used as a standard reference chemical in 1982/3 was very much more satisfactory than the 1981/2 standard, copper sulphate. It was the most toxic of the chemicals tested, gave results which varied little between laboratories and because its relative toxicity was greatest in all tests there was no evidence of adsorption occurring.

One chemical, pentachlorophenol, was included as an unknown in both 1981/2 and 1982/3 in order to assess within laboraotry variability and also whether the mean toxicity estimate differed appreciably in the two separate ring tests. The results (fig. 4 and 8) show that results were extremely close. The overall $L.C._{50}$ estimate for its toxicity in 1981/2 was 0.0054 $mg.cm^{-2}$ for the contact test and 0.0042 $mg.cm^{-2}$ in 1982/3. For the artificial soil test, the estimates were 68.9 $mg.kg^{-1}$ in 1981/2 and 75.1 $mg.kg^{-1}$ in 1982/3.

The results of this second intercalibration test must be considered in making the decision as to which test is the more suitable for the Commission or whether a two-stage test should be used. This must be resolved by discussion at a forthcoming meeting. It is unfortunate that the data available on the 'Artisol' testing method is still inadequate for a considered decision to be made. This will have to be discussed further. The results obtained indicated that results were more variable than with the other two tests.

CONCLUSIONS

1. *E. foetida* has proved to be a suitable, readily obtainable and easily bred test species with a representative susceptibility to chemicals.

2. Both the contact and artificial soil tests provided reproducible toxicity assessments for chemicals with an accuracy within an order of magnitude for most chemicals. The 'artisol' test seemed to give more variable results, but was not tested in sufficient laboratories to give conclusive results.

3. Both the contact and artificial soil test could be used, the former providing an inexpensive screening procedure, followed by the latter which may be used only for compounds that are recorded as relatively toxic in the contact test. If only one test is used then the artificial soil test would seem the best because it provides data more readily interpretable in terms of doses and environmental hazard. The contact test, although giving very reproducible results is difficult to interpret in environmental terms and might record a chemical that degrades to a more toxic chemical in soil as harmless.

4. The artificial soil method is suitable not only for water-soluble substances, but also viscous liquids and powders and substances soluble or dispersible only in organic solvents, either by spraying or physically mixing the chemical into the test substrate.

5. There is probably no need for range-finding tests and the use of a wider range of doses in the first test could obviate this. The maximum dose tested in the artificial soil test should be 1000 $mg.kg^{-1}$. However, if there is any detectable mortality at this dose higher doses should be tested.

6. The main need for the use of a standard reference substance, is for a check on the laboratory procedure at least once a year or once in every five tests and to demonstrate that under the laboratory test conditions the responses of tested species have not changed significantly.

7. If the mortality in the controls exceeds 10% the test should be repeated.

8. Chloracetamide is recommended as a suitable reference standard.

SUMMARY

The inter-laboratory intercalibration ring test assessing the validity and reproducibility of proposed contact filter paper, artificial soil and an 'artisol' toxicity test for the earthworm *E. foetida* in 1981/2. This involved the assessment of the toxicity of pentachlorophenol, carbaryl, trichloracetic acid, labelled by code as unknown test chemicals, and a reference standard chemical, copper sulphate, by 60 laboratories in 18 countries. The order of toxicity were pentachlorophenol > carbaryl > copper sulphate > trichloroacetic acid. Results were received from 38 of these laboratories for the contact test, 24 laboratories for the artificial soil test and 4 laboratories for the 'artisol' test. The contact test gave the most reproducible results, but was difficult to interpret and the data gave only potential toxicity hazards rather than real ones. the artificial soil test although slightly less precise and rather more time-consuming gave data much more readily interpreted.

The 1982/3 intercalibration test assessed the toxicities of a new reference standard chloracetamide, and also pentachlorophenol, potassium bromide and technical chlordane. This test was done by 21

laboratories, 17 of which were from member countries of EEC. The order of toxicities reported was chloracetamide > technical chlordane > pentachlorophenol > potassium bromide. Nearly all results varied by less than an order of magnitude. Insufficient results (8) were received on the 'artisol' test to assess its potential but it seemed more variable than the other tests. The selection of the most suitable method(s) should now be relatively easy based on these results.

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APPENDIX I.

E.E.C. GUIDELINES FOR THE TESTING OF CHEMICALS

MINUTES OF THE MEETING ON METHODS FOR TESTING THE TOXICITY OF CHEMICALS TO EARTHWORMS

BRUSSELS, 21 and 22 January 1982

The chairman Dr Amavis welcomed the participants and thanked them for having attended at the invitation of the Commission.

He outlined the background to the Directive and the purpose of the meeting which was to review the results of the interlaboratory ring test summarized in the report presented by Dr Edwards and to suggest modifications to the method(s) to be incorporated in the final protocol.

The delegates (listed in ANNEX) introduced themselves and their interests. There were 24 participants representing mainly laboratories within the Community although results had been received from other countries (Switzerland, Spain, Sweden, Norway, Japan, U.S.S. and the Philippines).

Dr Edwards reviewed the testing procedure and the response. He had received 32 results for the contact test and 20 for the artificial soil test at the time of the meeting. Four results from Dr Bouche's 'Artisol' test were given to Dr Edwards at the meeting but were not in time for the report. Dr Edwards pointed out that a two stage testing system was unusual and one of the main tasks of the meeting was to decide whether this was necessary or whether a single test was preferable. Although the original aim was to see which test was preferable there was some evidence that the tests were complementary.

The paper contact test was cheap, quick and easy to do and tended to give less variable results. However, it was difficult to interpret the results in terms of soil contamination with chemicals and because there is adsorption of many chemicals on to soil the soil the simple contact test may indicate a greater toxicity than would occur in soil.

The artificial soil test is not quite so simple and requires more laboratory space and time. However, the results obtained are much easier to interpret and relevant to environmental hazards.

The discussions produced a number of important points:

1. General A few laboratories had trouble with supplies of worms and ingredients for the artificial soil. Dr Edwards suggested that there sould be no problems with worms when the test was adopted because cocoons for breeding stock could be distributed from a central source. The ingredients for the soil were rather too precisely defined and in the final protocol this would be avoided.

3. Some laboratories considered that a range-finding test was unnecessary and it was decided that it should be an optional recommendation done only if the laboratory considers it necessary.

4. Discussion on the need for tests with a standard reference substance concluded that such a test should be made only occasionally to maintain standardization. Chloracetamide was recommended as the best chemical.

5. The need for food for the worms in the artificial soil test

was discussed but it was concluded that it was unnecessary.

6. There was an extensive discussion on the length of the artificial soil test, some laboratories favouring a 7 day test and others a 28 day test. Agreement was reached on a compromise of 14 days with an additional assessment at 7 days.

7. The method of application of test chemicals to artificial soil was discussed and it was concluded that for some chemicals a physical mixing with a subsample was prefereable to a spray. Both methods were suitable.

8. The point was made that worms should void their guts and be preconditioned in a similar medium for a short period prior to a test.

9. Some laboratories preferred an intermittent light regime because they already used this for other tests. However, some testing laboratories might not have such facilities.

10. It was decided that a test temperature of $20^{o}C > 2^{o}C$ should be recommended.

11. It was agreed that the artificial soil should be brought to a pH of 6.0 to avoid adding too much calcium carbonate.

12. There was considerable discussion on preference for either or both of the tests. Only one participant preferred only the contact test. Eleven participants thought that the contact test should be retained as a trigger for the artificial soil test or as an optional extra test. Eleven participants thought the artificial test would be adequate on its own. It would seem that the contact test should be retained, at least in an optional form, for the time being although the overall opinion seems to be that the artificial soil test should be the definitive one.

APPENDIX II

E.E.C. INTERCOMPARISON EXERCISE

TOXICITY FOR EARTHWORMS

CONTACT FILTER PAPER TEST

LEVEL 1 EARTHWORMS: FILTER PAPER (Rev 4)

1. METHOD

1.1. INTRODUCTION

The laboratory test described using filter paper as test medium determines the direct effect of a substance on earthworms and provides an efficient method of screening substances for toxicity.

1.2. DEFINITION AND UNITS

LC_{50}: The concentration of a substance killing 50% of the worms during the test period.

1.3. REFERENCE SUBSTANCE

A reference substance will be used periodically as a means of demonstration that under the laboratory test conditions the response of tested species have not changed significantly.

Chloroacetamide is recommended as the reference substance.**

1.4. PRINCIPLE OF THE TEST

Single adult earthworms, of the species Eisenia foetida (see note in Annex) are kept in glass vials the sides of which are lined with strips of standard filter paper treated with different concentrations of the test substance. After 48 hours, the number of dead earthworms at each concentration is counted.

** for the confirmation exercise

1.5. QUALITY CRITERIA

The test is designed to be as reproducible as possible with a carefully standardized test medium and organisms.

Results will be compared with those from a reference substance in periodic assays.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. Materials

1.6.1.1. Test substrate

As test medium a standard quality filter paper such as Whatman's 80-85 g/m^2 about 0.2 mm. thick medium grade is used. Suitable strips of this filter paper are used to line the sides of glass vials. The test substrate consists of the filter paper, the test substance and deionized water and if necessary organic solvents.

Care should be given in obtaining a sufficient deposit of the test chemical on the surface of the filter paper. The way of applying the test substance to the substrate must be reported.

Control substrate

The control substrate consists of standard filter paper and water. If an additive agent is used an additional control should contain the same quantity of the additive agent.

1.6.1.2. Test containers

Glass vials, 8 +/- 1 cm long x 3 +/- 0.5 cm diamter are recommended. As solutions of the test substance have to be evaporated to dryness the length of the vials should be dried evenly.

1.6.2. Test conditions

The vials should be kept lying on their sides in a climatic chamber at a temperature of 20 +/- 2 °C in continuous darkness.

The test period is 48 hours.

1.6.3. Test procedure

Test concentrations

Range-finding test

The concentrations causing mortalities of zero to 100 percent should be determined in a range-finding test providing information about the range of concentration to be used in the definitive test.

The concentrations are calculated in terms of mg of test substance per cm^2 of filter paper. The range of concentrations suggested for this preliminary test are as follows: 1.0; 0.1; 0.01; 0.001; 0.0001 mg substance per cm^2.

Definitive test

Depending on the results of the range-finding test, at least 5 concentration steps are determined in a geometric series so that the LC_{50} value may be found as exactly as possible.

For each concentration, the control as well as at least ten replicates each with one worm per vial are necessary. It is not recommended to use more than one worm per vial because of possible side effects resulting from the death of one of them.

Application of the test substance

The test substance should be applied to the standard filter paper, whenever possible without any additional agents.

Immediately before starting the test, 1 ml of the solution, emulsion or dispersion of the test substance in deionized water or other solvent is pipetted into each vial and evaporated to dryness.

To achieve sufficient distribution of the test substance on the paper surface, appropriate solvents may be useful. Care should be taken that only agents which volatilise readily are used.

If additives are used, an additional control should contain the same quantity of the additive agent.

After drying in a suitable way (compressed air, rotating the vial horizontally may be useful) one ml of deionized water is added to rewet the filter paper.

In addition one earthworm per vial, kept previously for a minimum of 3 hours on clean moist filter paper, is placed on the filter paper inside the vial. The vials are covered with a suitable plastic film or perforated plastic cap. 48 hours after starting the test, the numbers of dead earthworms are determined. Earthworms are considered dead if they do not respond to a gentle mechanical stimulus to the front end.

1.6.4. Test organisms

Test organisms should be adult Eisenia foetida (see note in Annex) (at least 2 months old with clitellum) wet weight 300 - 600 mg. (for breeding method: see Annex).

2. DATA

2.1. TREATMENT AND EVALUATION OF RESULTS

The concentrations of the test substance tested are reported with reference to the corresponding percentages of dead earthworms.

When the data are adequate the LC_{50} value and the confidence limits ($p = 0.95$) should be determined using standard methods. The LC_{50} should be given in mg test substance per cm^2 filter paper (Litchfield and Wilcoxon, 1949 or equivalent method).

In those cases where the slope of the concentration curve is too steep to permit calculation of the LC_{50}, a graphical estimate of this value is sufficient.

When two consecutive concentrations at a ratio of 1.8 give only zero and 100% mortality, the two values are sufficient to indicate the range within which the LC_{50} falls.

3. REPORTING

3.1. TEST REPORT

The test report should include the following information:

- statement that the test has been carried out in accordance with the above-mentioned quality criteria

- tests carried out (range-finding test and/or definitive test)
- exact description of the test conditions or statement that the test has been carried out in accordance with the method; any deviations have to be reported
- exact description how the test substance has been applied to the filter paper
- information about test organisms (species, age, mean and range in weight, keeping and breeding conditions, supplier)
- method used for determination of LC_{50}
- test results including all data used
-- description of observed symptoms or changes in behaviour of test organisms
-- mortality in control animals
-- LC_{50} or highest tested concentration without mortality and lowest tested concentration with a mortality of 100%, 48 hours after setting up the test
-- plotting of the concentration curve
- results obtained with the reference substance if used
- date and signature

3.2. INTERPRETATION OF THE RESULTS

LEVEL 1 EARTHWORMS: FILTER PAPER (Rev 4)

4. REFERENCES

1) EDWARDS, C.A. and LOFTY, 1977 Biology of Earthworms. London: Chapman and Hall, 331 pp.

2) BOUCHÉ, M.B., 1972. Lombriciens de France. Ecologie et Systematique. Published Institut National de la Recherche Agronomique, 671 pp.

3) LITCHFIELD, J.T. and F. WILCOXON, 1949. A simplified method of evaluating dose-effect experiments. Journal of Pharmacology and Experimental Therapeutics, 96, 99-113

ANNEX

Breeding and keeping of the worms before testing

For breeding the animals, 30 to 50 adult worms are put in a breeding box with fresh substrate and removed after 14 days. These animals may be used for further breeding batches. The earthworms hatched from the cocoons are used for testing when they are mature (under the prescribed conditions after 2 to 3 months).

Keeping and breeding conditions:

Climatic chamber : 20 +/- 2°C temperature preferably with continuous light (intensity 400-800 lux).

Breeding boxes : suitable shallow containers of 10 - 20 l volume

Substrate : *Eisenia foetida* may be bred in various animal excrements. It is recommended to use as breeding medium a mixture of 50% by volume peat and 50% cow or horse dung. The medium should have a pH value of about 6 to 7 (regulated with calcium carbonate) and a low ionic conductivity (less than 6 mmhos or 0.5% salt concentration).

The substrate should be moist but not too wet.

Other successful procedures may be used besides the method given above.

APPENDIX III

E.E.C. INTERCOMPARISON EXERCISE

TOXICITY FOR EARTHWORMS

ARTIFICIAL SOIL TEST

LEVEL 1 EARTHWORMS - ARTIFICIAL SOIL (Rev4)

1. METHOD

1.1. INTRODUCTION

The laboratory test described uses artificial soil to determine the effect of a substance on earthworms.

1.2. DEFINITION AND UNIT

LC_{50}: The concentration of a substance which killed 50% of the test animals during the test period.

1.3. REFERENCE SUBSTANCE

A reference substance will be used periodically as a means of demonstration that under the laboratory test conditions the response of the tested species have not changed significantly. Chloroacetamide is recommended as the reference substance.**

1.4. PRINCIPLE OF THE TEST

Soil is a variable medium so for this test a carefully defined artificial loam soil is used. Adult earthworms of the species *Eisenia foetida* (see note in Annex) are kept in a defined artificial soil treated with different concentrations of the substance. The content of the containers is spread on a tray, 14 days (and optionally 7 days) after the beginning of the test, and the earthworms surviving at each concentration counted.

** For the confirmation exercise.

LEVEL 1 EARTHWORMS - ARTIFICIAL SOIL (Rev4)

1.5. QUALITY CRITERIA

The test is designed to be as reproducible as possible with a carefully standardized test substrate and organism. Mortality in the controls must not exceed 10% at the end of the test or the test in invalid.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. Materials

1.6.1.1. Test substrate

A defined artificial soil is used as a basic test substrate.

(a) Basic substrate (percentages are in terms of dry weight).

- 10% sphagnum peat (as close of pH 5.5 - 6.0 as possible with no visible plant remains and finely ground).
- 20% kaolinite clay with preferably more than 50% kaolinite.
- About 69% industrial quartz sand (dominant fine sand with more than 50% of particle size 0.05 - 0.2 mm. If the substance is not sufficiently dispersible in water, 10 g per test container should be kept available for mixing with the test substance later on.
- About 1% calcium carbonate ($CaCO_3$), pulverised, chemically pure added to bring the pH to 6.0 +/- 0.5.

(b) Test substrate

The test substrate contains the basic substrate, the test substance and deionized water. Water content is 25%-42% of the dry weight of the basic substrate. The key criterion is that the artificial soil must be wetted to a point where there is no standing water. Care should be taken in mixing to obtain an even distribution of the test substance and the substrate. The way of introducing the test substance to the substrate has to be reported.

LEVEL 1 EARTHWORMS - ARTIFICIAL SOIL (Ref4)

Control substrate

The control substrate contains the basic substrate and water. If an additive agent is used, an additional control should contain the same quantity of the additive agent.

1.6.1.2. Test containers

Glass containers of about one litre capacity (adequately covered with plastic lids, dishes or plastic film with ventilation holes) filled with an amount of wet test or control substrate equivalent to 500 g dry weight of substrate.*

1.6.2. Test conditions

Containers should be kept in climatic chambers at a temperature of 20° +/- 2°C with continuous light. Light intensity should be 400 to 800 lux.

The test period is 14 days, but mortality can be assessed optionally 7 days after starting the test.

1.6.3. Test procedure

Test concentrations:

Range-finding test

The concentrations causing mortalities of zero to 100 percent may be determined in a range-finding test to provide information on the range of concentrations to be used in the definitive test.

The substance should be tested at the following concentrations: 1000; 100; 10; 1; 0.1 mg substance kg^{-1} test substrate (dry weight).

* A temperature of 104°C should be used for oven drying to constant weight.

If a full definitive test is done, one test batch per concentration and one for the untreated control, each with ten worms, could be sufficient for the range-finding test.

Definitive test

Depending on the results of the range-finding test, at least 5 concentration steps are determined in a geometric series so that the LC_{50} value may be found as exactly as possible.

In the definitive test, at least 4 untreated controls and 4 test batches, each with ten worms per concentration, are necessary. The results of these replicate batches are given as a mean and standard deviation.

When two consecutive concentrations, at a ratio of 1.8 give only 0% and 100% mortality, these two values are sufficient to indicate the range within which the LC_{50} falls.

Mixture of the basic test substrate and the test substance

The test substrate should, whenever possible, be made up without any additional agents other than water. Immediately before the start of the test, an emulsion or dispersion of the test substance in deionized water or other solvent is mixed with the basic test substrate, or sprayed evenly over it with a fine chromatographic or similar spray.

If insoluble in water, the test substance can be dissolved in as small a volume as possible of suitable organic solvent (e.g. hexane, acetone or chloroform).

If the test substance is not soluble, dispersible or emulsifiable in organic solvents, 10 g of a mixture of fine ground quartz sand and a quantity of test substance necessary to treat 500 g dry weight of artificial soil are mixed with 490 g of dry weight of test substrate.

LEVEL 1 EARTHWORMS - ARTIFICIAL SOIL (Rev 4)

Only agents which volatilise readily may be used to solubilise, disperse or emulisify the test substance. The test substrate must be ventilated, before use. The amount of water evaporated must be replaced. The control should contain the same quantity of any additive agent.

For each test batch, an amount of wet test substrate equivalent to 500 g dry weight is placed into each glass container and 10 earthworms, which have been conditioned for 24 hours in a similar wet basic substrate, and then washed quickly and surplus water absorbed on filter paper before use, are placed on the test substrate surface.

The containers are covered with perforated plastic lids, dishes or film to prevent the substrate drying and they are kept under the test conditions for 14 days.

The assessments should be made 14 days (and optionally 7 days) after setting up the test. The substrate is spread on a plate made of glass or stainless steel. The earthworms are examined and the numbers of surviving earthworms determined. Earthworms are considered dead if they do not respond to a gentle mechanical stimulus to the front end.

The substrate is refilled into the container and the surviving earthworms are replaced on the surface of the same test substrate.

LEVEL 1 EARTHWORMS - ARTIFICIAL SOIL (Rev 4)

1.6.4. Test organisms

Test organisms should be adult *Eisenia foetida* (see note in Annex) (at least 2 months old with clitellum) wet weight 300 - 600 mg. (For breeding method see Annex.)

2. DATA

2.1. TREATMENT AND EVALUATION OF RESULTS

The concentrations of the substance tested are reported with reference to the corresponding percentages of dead earthworms. When the data are adequate, the LC_{50} value and the confidence limits ($p = 0.95$) should be determined using standard methods (Litchfield and Wilcoxon, 1949 or equivalent method). The LC_{50} should be given as mg of test substance per kg of the test substrate (dry weight).

In those cases where the slope of the concentration curve is too steep to permit calculation of the LC_{50}, a graphical estimate of this value is sufficient.

When two consecutive concentrations at a ratio of 1.8 give only 0% and 100% mortality the two values are sufficient to indicate the range within which the LC_{50} falls.

3. REPORTING

3.1. TEST REPORT

The test report should include the following information:

- statement that the test has been carried out in accordance with the above-mentioned quality criteria.
- test carried out (range-finding test and/or definitive test)

- exact description of the test conditions or statement that the test has been carried out in accordance with the method; any deviations have to be reported.
- exact description how the test substance has been mixed into the basic test substrate.
- information about test organisms (species age, mean and range in weight, keeping and breeding conditions, supplier)
- method used for determination of LC_{50}
- test results including all data used
- description of observed symptoms or changes in behaviour of test conditions
- mortality in control animals
- LC_{50} or highest test concentration without mortality and lowest tested concentration with a mortality of 100%, 14 days (and optionally 7 days) after setting up the test
- plotting of the concentration-response curve
- results obtained with the reference substance if used
- date and signature

3.2. INTERPRETATION OF RESULTS

4. REFERENCES

EDWARDS, C.A. and LOFTY, 1977. Biology of Earthworms. London: Chapman and Hall, 331 pp.

BOUCHE, M.B. 1972. Lombriciens de France, Ecologie at Systematique. Published Institut National de la Recherche Agronomique, 671 pp.

LITCHFIELD, J.T. and WILCOXON, J. 1949. A simplified method of evaluating dose-effect experiments. Journal of Pharmacology and Experimental Therapeutics 96, 99-113.

LEVEL 1 EARTHWORMS - ARTIFICIAL SOIL (Rev4)

ANNEX

Breeding and keeping of the worms before testing

For breeding the animals, 30 to 50 adult worms, are put in a breeding box with fresh substrate and removed after 14 days. These animals may be used for further breeding batches. The earthworms hatched from the cocoons are used for testing when they are mature (under the prescribed conditions after 2 to 3 months).

Keeping and breeding conditions:

Climatic chamber	:	20° +/- 2°C temperature, preferably with continuous light (intensity 400-800 lux).
Breeding boxes	:	suitable shallow containers of 10-20 l volume
Substrate	:	*Eisenia foetida* may be bred in various animal excrements. It is recommended to use as breeding medium a mixture of 50% by volume peat and 50% cow or horse dung. The medium should have a pH value of about 6 to 7 (regulated with calcium carbonate) and a low ionic conductivity (less than 6 mmhos or 0.5% salt concentration). The substrate should be moist but not too wet. Other successful procedures may be used besides the method given above.

LEVEL 1 EARTHWORMS - ARTIFICIAL SOIL (Rev 4)

NOTE

Eisenia foetida exists in two races which some taxonomists have separated into species (Bouche, 1972). These are morphologically similar but one E. foetida foetida has typically transverse striping or banding on the segments and the other E. foetida andrei lacks this and has a variegated reddish colour. Where possible E. foetida andrei should be used. Other species may be used if the necessary methodology is available.

APPENDIX IV

E.E.C. INTERCOMPARISON EXERCISE

TOXICITY FOR EARTHWORMS

ARTISOL TEST

1.1. INTRODUCTION

The laboratory test described determines the effect of a substance on earthworms, in a basic artificial substrate.

1.2. DEFINITION AND UNITS

LC_{50}:The concentration of a substance which killed 50% of the test animals during the test period.

1.3. REFERENCE SUBSTANCE

A reference substance will be used periodically as a means of demonstration that under the laboratory test conditions the response of tested species have not changed significantly.

Chloroacetamide is recommended as the reference substance.**

1.4. PRINCIPLE OF THE TEST

Adult earthworms of the species Eisenia foetida (see note in Annex) are kept in a basic artificial substrate called "artisol", which is treated with different concentrations of the test substance. After 14 days the surviving earthworms at each concentration are counted.

** For the confirmation exercise

1.5. QUALITY CRITERIA

The test is designed to be as reproducible as possible with a carefully standardized test medium and organisms.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. Materials

1.6.1.1. Test substrate

The earthworms are put into cutaneous and intestinal contact in a basic artificial substrate "artisol". The artisol is composed of two elements:

- a skeleton of glass balls:diameter 1.5 - 2.0 cm: 1425 g of glass balls (+/- one glass ball) per container
- a matrix composed of two elements:
- 90 g amorphous hydrated silica (trade mark "Levilite") per test container
- deionized water: 215 ml per test containe.

The test substrate contains the basic substrate, the test substance and deionized water. Care should be taken in mixing to obtain an even distribution of the test substance and the substrate. The way of introducing the test substance to the substrate should be reported.

1.6.1.2. Test containers

Glass containers about 1.5 - 2.0 l covered with perforated plastic film

1.6.2. Test conditions

Containers should be kept in a climatic chamber at a temperature of 20 +/- 2°C in continuous dark.

The test period is 14 days, at the end of which mortality is assessed.

1.6.3. Test procedure

Test concentrations:

The concentration is expressed as the ratio between the test substance and dry weight silica. The support glass balls are not taken into account in the calculation. The dry weight of silica is calculated on the basis of samples dried at 105 °C.

Range-finding test

The concentrations causing mortalities of zero to 100 percent may be determined in a range-finding test to provide information about the range of concentrations to be used in the definitive test.

The substance should be tested at the following concentrations: 1000, 100, 10, 1; 0.1 mg test substance per kg dry weight silica.

If a full definitive test is done, one test batch with 10 earthworms per concentration and one for the untreated control could be sufficient for the range-finding test.

Definitive test

Depending on the results of the range-finding test, at least 5 concentration steps are determined in a geometric series so that the LC_{50} value may be found as exactly as possible.

In the definitive test, at least 4 untreated controls and 4 test batches, with 10 earthworms per concentration, are necessary. The results of these replicate batches are given as a mean and standard deviation.

Mixture of the basic test substrate and the test substance

The test substrate should, whenever possible, be made up without any additional agents other than water. Immediately before starting the test, 215 ml of an emulsion or dispersion of the test substance in deionized water or other solvent is mixed with 90 g silica to obtain a homogeneous matrix.

If the test substance is not soluble, dispersible or emulsifiable, 10 g of a mixture of silica and a quantity of test substance necessary to treat 90 g dry weight of silica are mixed with 80 g silica and 215 ml deionized water.

If insoluble in water, the test substance can be dissolved in as small a volume as possible of a suitable solvent (e.g. hexane, acetone or chloroform).

Only agents which volatilise readily should be used to solubilise, disperse or emulsify the test substance. The test substrate must be ventilated before use. The amount of water evaporated must be replaced. The control should contain the same quantity of any additive agent.

Mix this matrix of silica, deionized water and test substance with 1425 g of glass ball (+/- 1 glass ball) and knead it. Place this test substrate in a test container and 10 earthworms, which have been washed and surplus water absorbed on filter paper before use, are placed on to the surface of the substrate.

After 14 days, the earthworms can be separated rapidly from the test substrate by washing through a 1 mm sieve. The earthworms are examined and the numbers of surviving earthworms determined. Earthworms are considered dead if they do not respond to a gentle mechanical stimulus to the front end.

1.6.4. Test organisms

Test organisms should be adult *Eisenia foetida* (see note in Annex) (at least 2 months old with clitellum), wet weight 300 - 600 mg.
(For breeding method see Annex).

2. DATA

2.1. TREATMENT AND EVALUATION OF RESULTS

The concentrations of substance tested are reported with reference to the corresponding percentages of dead earthworms.

When the data are adequate, the LC_{50} value and the confidence limits ($p = 0.95$) should be determined using standard methods. (Litchfield and Wilcoxon, 1949 or equivalent method.) The LC_{50} should be given as mg test substance/kg dry weight of silica.

In those cases where the slope of the concentration curve is too steep to permit calculation of the LC_{50}, a graphical estimate of this value is sufficient.

When two consecutive concentrations at a ratio of 1.8 give only zero and 100% mortality, the two values are sufficient to indicate the range within which the LC_{50} falls.

3. REPORTING

3.1. TEST REPORT

The test report should include the following information:

- statement that the test has been carried out in accordance with the prescriptions of the above mentioned quality criteria
- tests carried out (range-finding test and definitive test)
- exact description of the test conditions or statement that the test has been carried out in accordance with the method; any deviations have to be reported
- exact description how the test substance has been mixed into the basic test substrate
- information about test organisms (species, age, mean and range in weight, keeping and breeding conditions, supplier)
- method used for determination of LC_{50}
- test results for including all data used
-- description of observed symptoms or changes in behaviour of test organisms
-- mortalities in control animals
-- LC_{50} or highest tested concentration without mortality and lowest tested concentration with a mortality of 100%, 14 days after setting up the test
-- plotting of the concentration-response curve
- result obtained with the reference substance if used
- date and signature

3.2. INTERPRETATION OF THE RESULTS

4. REFERENCES

1) EDWARDS,C.A. and LOFTY, 1977. Biology of Earthworms. London: Chapman and Hall, 331 pp.

2) BOUCHÉ,M.B., 1972. Lombriciens de France, Ecologie et Systematique. Published Institut National de la Recherche Agronomique, 671 pp.

3) LITCHFIELD,J.T. and F. WILCOXON, 1949. A simplified method of evaluating dose-effect experiments. Journal of Pharamcology and Experimental Therapeutics, 96, 99-113.

ANNEX

Breeding and keeping of the worms before testing

For breeding the animals, 30 to 50 adult worms are put in a breeding box with fresh substrate and removed after 14 days. These animals may be used for further breeding batches. The earthworms hatched from the cocoons are used for testing when are mature (under the prescribed conditions after 2 to 3 months).

Keeping and breeding conditions:

Climatic chamber : 20 +/- 2°C temperature preferably with continuous light (intensity 400-800 lux).

Breeding boxes : suitable shallow containers of 10 - 20 l volume

Substrate : *Eisenia foetida* may be bred in various animal excrements. It is recommended to use as breeding medium a mixture of 50% by volume peat and 50% cow or horse dung. The medium should have a pH value of about 6 to 7 (regulated with calcium carbonate) and a low ionic conductivity (less than 6 mmhos or 0.5% salt concentration). The substrate should be moist but not too wet.

Other successful procedures may be used besides the method given above.

NOTE

Eisenia foetida exists in two races which some taxonomists have separated into species (Bouché, 1972). These are morphologically similar but one *E. foetida foetida* has typically transverse striping or banding on the segments and the other *E. foetida andrei* lacks this and has a variegated reddish colour. Where possible *E. foetida andrei* should be used. Other species may be used if the necessary methodology is available.

APPENDIX V

E.E.C. GUIDELINES FOR THE TESTING OF CHEMICALS

Report of the 4th meeting of the Sub-Group Ecotoxicology of the E.E.C. Directorate for Environment and Consumer Protection. (Section - Toxicity to Earthworms)

9 - 10 March 1982

The meeting was attended by 17 delegates from Belgium, Denmark, West Germany (F.D.R.), France, Greece, Ireland, Italy, the Netherlands and the United Kingdom and 5 members of the Commission.

The results of the ring test were summarised by Dr. Edwards. All the participants agreed on the value of this first test. Several delegations commented on the good organization of this test and recommended that similar ring tests should be organised for other methods concerning level 1 of Annex VIII.

A number of delegates commented that the filter paper was a test of maximal contact between a chemical substance and the test organism. Opinions differed as to whether such a test would be suitable for the testing programme without support data, although several delegates liked its simplicity and reliability. However, it was considered generally, that the artificial soil test was more representative of natural environmental conditions. It was generally agreed to present the two methods (filter paper and artificial soil) to the Coordination Committee.

Dr Edwards stated that 20 laboratories had agreed to participate in a 'confirmation' ring test in 1982 using the three methods: filter paper test, artificial soil test and 'Artisol' test (Bouche). The Sub Group agreed that such a test was desirable before any implementation of the test protocol.

The Chairman, Dr. Amavis, emphasized that the work on toxicity to earthworms was being coordinated with work carried out by OECD.

C A EDWARDS

APPENDIX VI

E.E.C. GUIDLINE FOR TESTING OF CHEMICALS - EARTHWORMS

Draft minutes of the 4th Meeting of the Sub-Group "Ecotoxicology" of the group "testing methods for dangerous substances"

Bruxelles - 9-10 March 1982

After having welcomed the participants (list in Annex) the Chairman Mr. R. Amavis gave a general view of the objectives of this meeting: Preparation of testing methods related to information requested in Annex VIII of Directive 79/831/EEC. In addition he informed the participants of decisions taken during the last meeting in Brussels of the National Competent Authorities (19-20 January 1982): a certain flexibility should be obtained for the choice of information requested in Annex VIII but protocols must be prepared in a similar way than those concerning Annex VII. Nevertheless this question will be also discussed during the next meeting of the Coordination Committee (6 and 7 May 1982) in Brussels.

The draft agenda was approved.

1. Toxicity on earthworms (doc. XI/127/82, XI128/82 and XI/129/82) Dr EDWARDS, in charge of the ring test on the determination of toxicity on earthworms, gave a global view of the results obtained. All the participants agreed on the interest of this first exercise. Several delegations asked that similar ring tests should be organised for other methods concerning level 1 of Annex VIII.

Some participants emphasized that the filter paper test is a test of maximal contact between a chemical substance and the tested organisms. Artificial soil is more representative of natural environmental conditions.

A general agreement was reached to present these 2 methods (filter paper and artificial soil) to the Coordination Committee.

Dr EDWARDS recalled that 20 laboratories agreed to participate in a "confirmation" exercise with the following methods: filter paper, artificial soil and 'Artisol' (Dr. Bouche's medium). This confirmation exercise must be agreed upon by the Coordination Committee.

Any written comments in this field should be sent to the Secretariat before 8 April 1982.

The Chairman underlined that this work on toxicity on earthworms is coordinated with work carried out in OECD in this matter.

2. Algal growth inhibition test (doc. XI/723/81)

It seems that this test is now studied by different international bodies (OECD, I.S.O.).

Some national delegations (Belgium, Federal Republic of Germany and France) asked to use the results of the I.S.O. ring test (available in June 1982 during a meeting in Stockholm) to prepare a draft protocol for Annex V.

The OECD Guidelines in this matter seem to have many errors (for example: culture medium, algal species, counting of results, recipient capacity, duplications of tests, problems raised by volatile solutions). The Group recommended to present these points to OECD up-dating as CEE proposals.

3. <u>Prolonged toxicity for daphnia</u> (doc. XI/78/82)

Following the 79/831/EEC Directive, this study should also include determination of the "no effect level" for reproduction and the "no effect level" for lethality. The Group agreed that a simultaneous measurement of EC_{50} (lethality) and no effect level is not possible with one test. Priority must be given to the determination of no toxic effect level for the reproduction and no effect level for parent lethality. An agreement of the Coordination Committee will be requested on this point.

After examination of the proposed document, delegates underlined the need to validate this method by a practical exercise. Dr CABRIDENC (F) agreed to coordinate the preparation of testing protocols and a mini ring test between a small number (5) of experienced laboratories. Dr. BROWN (UK), Dr. HAMBURGER (FRG), Dr. DE HENAU (B) a, d, Dr KONEMANN (NL) agreed to help Dr. CABRIDENC in this work.

4. <u>Prolonged toxicity for fish</u> (doc. XI/70/82)

For this test, a general discussion took place on the need to test larvae, young or adult fish. According to some delegates difficulties arise by mentioning of reproduction studies only in level 2.

In conclusion delegations agreed to have some national consultations with experts and to send comments and proposals to the Commission before 15 April 1982. These will be presented to the coordination Committee.

5. <u>Toxicity test on higher plants</u> (doc. XI/130/82).

This test is based on a very preliminary draft of the OECD. The Group agreed to wait for results of discussions in the Ecotoxicology ad hoc Group in the OECD.

6. Any other business

The Chairman circulated an OECD document on glossary of terms. He asked for reactions of the Ecotoxicology Sub-Group on special topics, to try to have an international agreement on this matter.

The meeting was closed by the Chairman, Mr. AMAVIS, at 4.30 p.m.

APPENDIX V.II

E.E.C. GUIDLINES FOR THE TESTING OF CHEMICALS

June 1982

I apologise for the delay in sending you details of the latest developments in the E.E.C. earthworm toxicity testing methodology in which you have cooperated until now. There was a meeting of 24 delegates from 8 countries in January 1982 (Annex 1) a small meeting with Dr. Becker (F.D.R.), Dr. Cabridenc (France) and Dr. Amavis (E.E.C.) in February 1982, and a meeting of the Ecotoxicology Sub-Group on the E.E.C. programme in March 1982 (Annex 2), all in Brussels. I have waited until these meetings were over and I had received more results before writing to inform you of the future programme. I should like to thank you very much for all your cooperation and for sending me your results so promptly.

All of the data received have been combined into a final report which will be published by E.E.C. I hope this will be available shortly when I will send you a copy.

The conclusion of the various meetings are summarised in Annexes 1 and 2. The general opinion was divided equally between (a) the use of a two-stage test i.e. filter paper test (or 'artisol'test) followed by an artificial soil test to be used only when hazard was indicated by the first test or (b) only an artificial soil test. This is going to be resolved by a 'confirmatory' ring test which will involve about 20 laboratories that volunteered to do the test

in 1982. It is hoped that the results of these tests will be available by early 1983 to enable a final decision on which test is to be adopted by the E.E.C. to be made. Samples of three unknown test chemicals will be sent out to collaborating laboratories in June 1982. It is hoped that worms for the test can be obtained by collaborating laboratorties locally, but where this is impossible they will be supplied by me. The specications of materials for the artificial soil have been made broader and there should now be no difficulty in obtainiong these locally. The materials for the 'artisol' test will be supplied by Dr. Bouche.
It would help in organising the test if you could write to me immediately, if you anticipate any difficulties in obtaining worms, materials for the artificial soil or any other items. If there is likely to be any difficulty in completing the test could you let me know by return so that I can find an alternative collaborator. Please confirm your intended collaboration to avoid sending you unnecessary chemicals.
I am most grateful for your continued interest and help and look forward to continued fruitful collaboration.
Best wishes
Yours sincerely

APPENDIX VIII

E.E.C. GUIDELINES FOR THE TESTING OF CHEMICALS - EARTHWORMS

PROTOCOL FOR MEASURING THE TOXICITY OF CHEMICAL SUBSTANCES TO EARTHWORMS

28 January, 1983

Dear

I hope all goes well with your test of the three unknown chemicals and the standard (chloracetamide) that I sent to you last year and that you have had no problems in obtaining substrate, materials or worms for the test.

I enclose a slightly revized draft of the three protocols. these have been redrafted to bring them into exact line with the official E.E.C. format and terminology, and it is hoped that little further revision will be needed for whichever of them is finally adopted. There are no substantial changes which would in any way affect the way in which you have done the test; or the results.

The full support of the first ring test is now in press and I hope to be able to send you a copy of this in the very near future. As you probably know the unknown chemicals for this first test were:

A. Pentachlorophenol

B. Carbaryl

C. Trichloracetic acid

S. Copper sulphate

The time-table for the present ring test is that I require the results in April to prepare a report in May which will be considered at a meeting in Brussels in late May or early June. This meeting will decide on which method(s) should be recommended for adoption. All collaborating laboratories will be invited to this meeting to discuss their experience and problems with the test and some funds will be available for E.E.C. participants.

A similar protocol was accepted by the OECD in September 1982 and is now in process of being revized and finalized. This may be updated later in light of the results of the present ring test.

I hope you have had no problems in completing the ring test because we have limited it to about 20 volunteer laboratories and it is essential that I have 20 sets of results to validate the decisions on adoption of a suitable test.

In case you have had problems in obtaining glass balls for the 'Artisol test' you should write to:

Dr. R. Cabridenc
IRCHA
Centre de Recherche B.P. No 1
91710 Vert-le-Petit
Paris

APPENDIX IX

E.E.C. GUIDELINES FOR THE TESTING OF CHEMICALS - EARTHWORMS

PROTOCOL FOR MEASURING THE TOXICITY OF CHEMICAL SUBSTANCES TO EARTHWORMS

The purpose of this letter is to enquire as to progress with the above ring test. I hope that you have been able to complete at least part of the tests beause this year's test is a relatively limited one and I am heavily dependent upon receiving results from most of those laboratories that agreed to participate. I now have over half the results in but I need all possible results to make the test a valid one.

I would be most grateful if you could report on progress and give me some idea when I might expect your results. This will enable me to schedule a firm date for preparing a report and organizing a meeting to review the results and finalize the eventual protocol for implementation in the EEC testing procedures.

Many thanks for all your co-operation and I hope to hear from you very soon. I would like results by end of June if possible and if we can keep to deadlines the meeting would be in September or October.

Yours sincerely

C A Edwards

FRANCE

He will send you a suitable quantity.

Please contact me if you have had any problems or think you may not be able to complete the test. It is essential that I know this as soon as possible.

Once again, many thanks for your help and wiling collaboration.

All best wishes for 1983.

Yours sincerely

C A Edwards