Pestic. Sci. 1984, **15**, 605–611

Ref ID 0271

59245

Correlations Between Three Methods for Determining the Toxicity of Chemicals to Earthworms

Fred Heimbach

Bayer AG, Agrochemicals Division, Institute for Environmental Biology, D-5090 Leverkusen, Federal Republic of Germany

(Revised manuscript received 6 February 1984)

Three different laboratory methods for determining the toxicity of chemicals to earthworms were tested with a variety of pesticides and other chemicals. These methods, proposed by the EEC (Determination of Ecotoxicity of Chemicals), were: the Contact Filter Paper Test, in which the skin contact toxicity is tested by placing the organisms on treated filter paper; the Artificial Soil Test, in which the toxicity by skin and gut uptake is determined by adding the earthworms to an artificial soil made of sand, clay mineral and peat; and the Artisol Test, in which the toxicity by skin and gut uptake is determined by placing the worms in an artificial substrate consisting of silica, water and glass balls. (In the Artisol Test, the earthworms ingest the silica paste as they do soil.) With several chemicals, the Artificial Soil Test was conducted as described in an early guideline of the BBA. This test is similar to that proposed by the EEC. Results from the Contact Filter Paper Test were poorly correlated to those obtained by the other two methods (correlation to the Artificial Soil Test, $r=0.55$; correlation to the Artisol Test, $r=0.48$). In contrast, there was a high correlation between the Artificial Soil Test and the Artisol Test ($r=0.91$). This indicated that the mode of uptake of the chemicals (skin uptake and/or gut uptake) may be the same. As the Artisol Test seems to be readily handled and standardised, it could be useful for obtaining reproducible data on the ecotoxicity of chemicals to earthworms.

1. Introduction

Within the last few years, several methods for determining the toxicity of chemicals to earthworms have been described. A test in which the earthworms were injected with the chemical,[1–3] and a test in which they were immersed (Graff, O., 1979, unpublished), were found unreliable because the test situations could not be related to field conditions. In addition, earlier experiments with the immersion test showed that this method leads to incorrect hazard assessment when the results are used to predict the situation in the field (Heimbach, F., 1979, unpublished). Other methods, for example those using a sandy loam soil[4] or mixtures of several natural substrates,[5,6] create conditions that more closely resemble the natural environment of earthworms.

Currently, the European Economic Community (EEC) is considering three different methods for determining the ecotoxicity of chemicals to earthworms (EEC Directive 79/831, Annex V)[7]: the Contact Filter Paper Test; the Artificial Soil Test; and the Artisol Test. The first of these tests, in which the organisms are exposed to the substance on a piece of wet filter paper, can be used only to obtain information on the skin contact toxicity of the chemical. The second test is more representative of the natural environment of earthworms; an artificial soil, composed of sand, a clay mineral and peat, is mixed with the chemical and the earthworms are added to this substrate. In the third test (the Artisol Test) the substrate is completely artificial; it consists of silica mixed with glass balls and water. The earthworms ingest this material as they would on a

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natural soil. Although artificial, the substrate allows a high degree of standardisation and, if used routinely by many laboratories, may allow reproducible and reliable data to be obtained for evaluating the toxicity of chemicals to earthworms.

The aim of this paper is to compare results obtained with these three different test methods. Many people intuitively prefer the artificial soil test because it would appear to be the best simulation of a natural soil. On the other hand, if the other two methods provide the same information, that which can be best standardised and is the easiest to handle may be preferred. In this paper, results are presented that may be of help in selecting an effective method for scientific as well as legislative purposes.

2. Materials and methods

In the present studies, which were conducted in the period 1981 to 1983, the first step in determinating the LC_{50} of a substance was to perform a range finding test. With the Contact Filter Paper Test, the concentrations 1.0, 0.1, 0.01, 0.001 and 0.0001 mg cm^{-2} were tested. With the other two methods, the concentrations 1000, 100, 10, 1 and 0.1 mg kg^{-1} of artificial soil (dry wt) or silica (dry wt) were tested.

The concentrations for the definitive tests were selected to give between 0 and 100% mortality. In most experiments, a multiplication factor of 1.8 was used to derive the concentration series. Earthworms were considered dead if they did not respond to a gentle mechanical stimulus. The reproducibility of the results was satisfactory; this was demonstrated with several test chemicals including chloroacetamide. This material has meanwhile been proposed as a reference chemical for the EEC test guidelines.[7]

Table 1. LC_{50} values (as active ingredient) for the various compounds against *Eisenia foetida*, as determined by the three different methods

Compound	LC_{50} found for each method		
	Contact Filter Paper Test (μg cm^{-2})	Artificial Soil Test (mg kg^{-1} dry wt of substrate)	Artisol Test (mg kg^{-1} dry wt of silica)
1. Methidathion	0.81	3.6	NT
2. Endosulfan	5.7	6.7	3.0
3. Propoxur	21	10	NT
4. DNOC	1.6	21	NT
5. Benomyl	86	22	18
6. Chloroacetamide	2.4	24	75
7. Fentin acetate+maneb	43	27	NT
8. Chlordane	1.3[b]	42[b,c]	15[b]
9. Pentachlorophenol	4.2[b]	87[d]	316[d]
10. Mercaptodimethur[a]	190	129	147
11. Dialifos	24	133	NT
12. Carbaryl	3.0	174[d]	151[d]
13 Ethiofencarb	14	262	264
14. Methaphenamiphos	17	318	NT
15. Bupirimate	15	338	NT
16. Potassium bromate	530	393[c]	1000
17. Captafol	47	496	351
18. Captan	290	625	691
19. Copper (II) sulphate	30	NT	1930

[a] Known in the U.K. and some other countries as methiocarb.

[b] The compound was added to the substrate as a solution in acetone.

[c] These values were obtained in the Artificial Soil Test, using conditions described by the EEC (test duration 14 days, 20% kaolin, 20°C, and constant light).

[d] The compound was diluted with powdered quartz sand or silica, and this mixture was added to the substrate with stirring.

2.1. Contact Filter Paper Test

The Contact Filter Paper Test, devised by C. A. Edwards, was performed as described by the EEC proposal for an EEC test guideline 'Toxicity for Earthworms—Contact Filter Paper Test'.[7,8] The test chemicals were dissolved, emulsified or dispersed in deionised water, or, when this was not possible, they were dissolved in acetone (Table 1). One ml of solution, emulsion or dispersion was transferred to a piece of filter paper (9.5×6.7 cm, Whatman Grade 1) which was placed as a cylinder in a brown glass vial (medicine vials, 7.0×3.5 cm). Ten replicates were prepared for each concentration. The water or acetone was evaporated to dryness by ventilation with an electric fan while rolling the vials. After evaporation of the solvent, deionised water (1 ml) was pipetted on to the filter paper to rewet it. Earthworms were held on wet filter paper for about 2 h before being added to the vials. One earthworm was added to each vial. The vials were covered with plastic film ('Parafilm M') and stored at 20 (±1)°C in the dark for 48 h.

2.2. Artificial Soil Test

The Artificial Soil Test was performed as described by a proposal of the BBA (Biologische Bundesanstalt für Land- und Forstwirtschaft, D-3300 Braunschweig).[9] The test substrate consisted of 83.5% fine quartz sand (84% of which had a particle size between 0.06 and 0.2 mm), 5% bentonite (broken up, concentrated bentonite; cation exchange capacity about 45 mEq per 100 g; trade name 'Tonsil Standard'), 10% finely ground dried sphagnum peat [pH 2.6 (±0.1)], about 1% calcium carbonate [pulverised and chemically pure, to adjust the pH of the substrate to 7 (±0.5)], and 0.5% finely ground dried cattle manure (all proportions on a dry weight basis). Sufficient water was added to bring the moisture content to 35% of the dry weight of the substrate, equivalent to 25.9% of the wet weight of the substrate.

The chemical to be tested was added in 200 ml of a solution, emulsion or dispersion of deionised water, with stirring, to moist artificial soil equivalent to 1.0 kg dry wt. Of this wet substrate, 1.55 kg (equivalent to 1.0 kg dry wt) was added to each glass container. Each test batch was mixed separately in a household mixer. Chemicals that were not soluble, emulsifiable or dispersible in water (Table 1) were ground with powdered quartz sand in a mortar, and 10 g of this mixture was added to the substrate while stirring in a mixer. One of the test substances (Table 1) was added to the substrate in acetone solution while stirring. Before adding the earthworms to the substrate, it was ventilated for about 15 min, after which, the water lost by evaporation was replaced. In the main tests, three or four replicates each with ten earthworms (in some cases six earthworms) were prepared for each container.

Treated substrate was poured into glass containers (capacity 1.5 to 2 litres), and ten earthworms, that had been washed in water, were placed on top of the substrate in each container. The containers were covered with fine mesh gauze and stored in an incubation chamber. The chamber was held at 22 (±1)°C and 70–90% relative humidity, and had a light-dark cycle of 12:12 h. After 7, 14 and 21 days, finely ground dried cattle manure (5 g) was mixed into each test container, and the water lost by evaporation was replaced. After 28 days, the numbers of living earthworms were determined by washing the substrate with flowing water through a 1-mm sieve.

A few substances (see Table 1) were tested as described by an EEC proposal for an EEC test guideline 'Toxicity for Earthworms; Artificial Soil Test'.[7] The conditions described in this proposal are only slightly different from those in the method given above. The main differences are: the test duration of 14, instead of 28 days; 20% kaolin (with about 50% kaolinite) in the substrate, instead of 5% bentonite; and no addition of cattle manure during the course of the experiment.

2.3. Artisol Test

The Artisol Test, devised by M. B. Bouché, was performed as described by the EEC proposal for an EEC test guideline 'Toxicity for Earthworms; Artisol Test'.[7] The substrate (Artisol) consisted of 90 g (dry wt) of silica (trade name 'Levilite Standard') per test container, and 215 ml

of deionised water. This medium was kneaded with about 1425 g of glass balls (diameter 15–20 mm), and this mixture was added to glass containers (1.5-litre) which were then covered with glass lids. If the substance was soluble, emulsifiable or dispersible in deioinised water, it was added to the silica with the water while stirring. If the substance was insoluble (Table 1), it was ground with 10 g of silica in a mortar, and this mixture was added to 80 g of silica plus water while stirring. One of the test substances (Table 1) was dissolved in acetone, and this solution was spread on the silica with the water. In this case, the silica was aerated for about 15 min before replacement of the water that had evaporated. The silica-water mixture was then combined with the glass balls described. In the main tests, three or four replicates, each with ten earthworms, were prepared for each test concentration.

The silica-coated glass balls were placed into glass containers and ten earthworms were added to each. The worms, which had been washed with water, were placed on top of the silica. The containers were covered with glass lids and placed in an incubation chamber at 20 (±1)°C in the dark. After 14 days, the number of living earthworms was determined by pouring the contents of a container on to a 1-mm sieve and removing the silica with flowing water.

2.4. Test organisms

The earthworm species used was *Eisenia foetida andrei*.[10] It has been bred in this laboratory for several years under the conditions described in the BBA proposal for an Organisation for Economic Cooperation and Development (OECD) guideline.[9] The earthworms used in the tests were adult and more than 2 months old. Their average weight was between 300 and 400 mg.

2.5. Determination of LC_{50} values

The LC_{50} values were calculated by a probit analysis.[11,12] In some cases, it was not possible to calculate an LC_{50} by a mathematical method; for those compounds, the geometric mean, of the logarithmic values of the highest concentration tested with less than 50% mortality, and the lowest concentration with more than 50% mortality, was taken as the LC_{50}. Where formulations of pesticides were tested, the concentrations given are in terms of the active ingredient (a.i.).

2.6. Chemicals

The pesticides and other chemicals used were as follows (w.p.=wettable powder; e.c.=emulsion concentrate): benomyl w.p. (50% a.i.); bupirimate e.c. (25% a.i.); captafol w.p. (80% a.i.); captan w.p. (50% a.i.); carbaryl (technical) e.c. (85% a.i.); chlordane (technical); chloroacetamide; copper (II) sulphate; dialifos e.c. (43% a.i.); DNOC w.p. (40% a.i.); endosulfan e.c. (35% a.i.); ethiofencarb soluble concentrate (10% a.i.); fentin acetate+maneb w.p. (54+16% a.i.); potassium bromate; mercaptodimethur (methiocarb) w.p. (50% a.i.); methaphenamiphos e.c. (40% a.i.); methidathion w.p. (40% a.i.); pentachlorophenol; and propoxur e.c. (20% a.i.).

3. Results

The main characteristics of the three methods are summarised in Table 2. Table 1 compares the results obtained with these methods. The LC_{50} values in the Contact Filter Paper Test ranged

Table 2. Main characteristics of the three test methods

	Contact Filter Paper Test	Artificial Soil Test	Artisol Test
Substrate	Filter paper	Mixture of sand, mineral clay and peat	Silica
Duration (days)	2	28	14
Temperature (°C)	20	22	20
Light	Constant darkness	Light-dark (12:12 h)	Constant darkness

from 0.0008 to 0.53 mg cm^{-2}; in the Artificial Soil Test they ranged from 3.6 to 625 mg kg^{-1}; and in the Artisol Test from 3.0 to 1930 mg kg^{-1}.

In Figure 1, the LC_{50} values of the Contact Filter Paper Test (log values) are plotted against the results of the Artificial Soil Test, and the figure shows only a weak correlation; the correlation coefficient (r)=0.55 (n=18). The deviation of single points from the regression line was relatively great. While some test substances tended to be highly toxic in the Contact Filter Paper Test, they were relatively non-toxic in the Artificial Soil Test (for example, compounds **9** and **12**). Some were highly toxic in the Artificial Soil Test but of relatively low toxicity in the Contact Filter Paper Test (for example, compounds **3**, **5** and **7**).

The Contact Filter Paper Test and the Artisol Test were also poorly correlated (Figure 2). The correlation coefficient (r=0.48, n=12) was very weak. The relationship of the toxicity of the substances between these methods was about the same as the corresponding relationship between

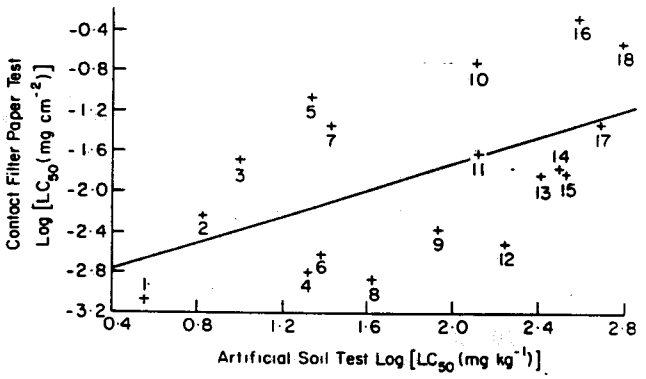

Figure 1. Correlation between results from the Contact Filter Paper Test and those from the Artificial Soil Test (r=0.55). The numbers (1–19) refer to the compounds listed in Table 1.

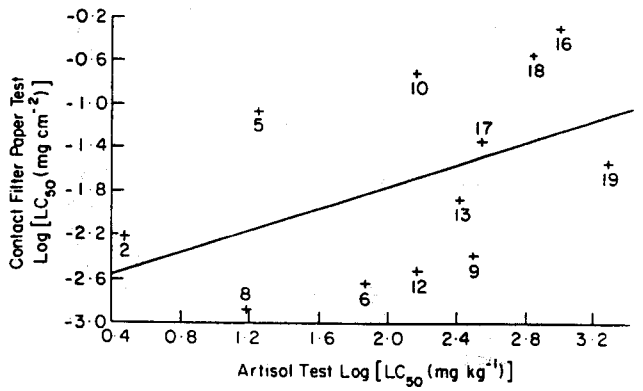

Figure 2. Correlation between results from the Contact Filter Paper Test and those from the Artisol Test (r=0.48). The numbers (1–19) refer to the compounds listed in Table 1.

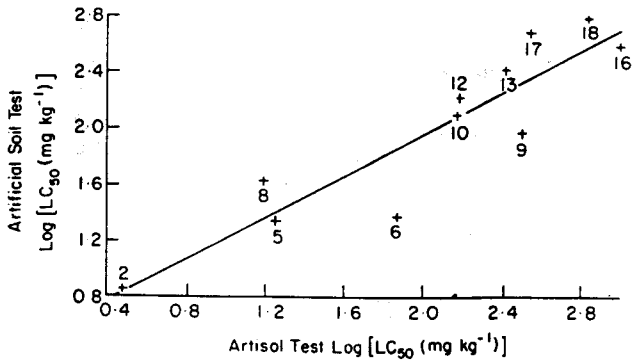

Figure 3. Correlation between results from the Artificial Soil Test and those from the Artisol Test (r=0.91). The numbers (1–19) refer to the compounds listed in Table 1.

Table 3. Correlations between the three methods

Test methods correlated	Regression equation	n	Correlation coefficient (f)	P(%)
Filter Paper—Artificial Soil	$y=0.65x-3.02$	18	0.55	1 (approx.)
Filter Paper—Artisol	$y=0.50x-2.76$	12	0.48	>5
Artificial Soil—Artisol	$y=0.72x+0.50$	11	0.91	<0.1

the Contact Filter Paper Test and the Artificial Soil Test (see, for example, compounds **5**, **9** and **12**).

Figure 3 is a plot of the results of the Artificial Soil Test and the Artisol Test. The correlation coefficient (r)=0.91 (n=11). Thus, the correlation between the results of these two methods was very strong (P<0.1%).

Table 3 summarises the correlations between the three methods.

4. Discussion

In these experiments, the earthworms did not feed on the filter paper in the 48-h Contact Filter Paper Test. Thus, the results show only the contact toxicity of the chemicals to the earthworms. In contrast to this, uptake of the chemicals in the other two methods was through skin and gut contact. In the Artisol Test, *E. foetida* fed on the silica, although it was of no nutrient value to them. In the Artificial Soil Test, the earthworms also fed on the substrate. The strong correlation between these two test methods probably indicates a similar mode of uptake of the chemicals by the earthworms.

The lengths of the tests differ greatly. Thus, it is possible that the poor correlation between the Contact Filter Paper Test (48 h) and the two other tests (14 and 28 days) was due to the different lengths of exposure to the chemicals. However, it is not practical to lengthen the Contact Filter Paper Test significantly. Hence, use of the Contact Filter Paper Test may lead to conclusions that do not reflect the hazards of chemicals to earthworms in soil.

The degree of adsorption of the substances to the substrates must also be considered. Currently there is little information on the adsorption capacity of the silica ('Levilite') used in these experiments. The adsorptive characteristics of the Artificial Soil are mainly determined by the peat and mineral contents. In spite of the organic content (10% peat) in this substrate, it is believed to have about the same adsorptive capacity as a typical arable soil.[7]

In this context it must be clearly stated that the reasons for the high correlation between the Artifical Soil Test and the Artisol Test are not fully understood. It can be speculated that the mode of uptake of the chemicals, namely through skin contact and/or gut uptake, is the same, and that this is responsible for the good correlation. On the other hand, it can also be speculated that the degree of adsorption of the chemicals to the artifical soil and silica are the same, and that this gives the good correlation. F. Heimbach (1982, unpublished data), for example, has demonstrated with two pesticides that there was an inverse correlation between pesticide toxicity and the peat content (2.5, 5 and 10%) of the artificial soil. Finally, depending on the characteristics of the chemicals, their uptake by the gut of the earthworms may be independent of their adsorption to the substrate. Further experiments are needed to resolve these speculations.

The Artificial Soil Test presents the same possibility of assessing the toxicity of chemicals to earthworms as the Artisol Test. As experience has shown that the Artisol Test is easy to handle and to standardise, results obtained in different laboratories using this method could be of a reliable degree of comparability. This fact should be strongly considered in the selection of methods of legislative purposes.

Acknowledgement

The author thanks Ms G. Pesch for capable technical assistance.

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