OECD GUIDELINE FOR TESTING OF CHEMICALS

207

Adopted:
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"Earthworm, Acute Toxicity Tests"

1. INTRODUCTORY INFORMATION

• Prerequisites

– Water solubility
– Vapour pressure

• Guidance information

– Structural formula
– Purity of the test substance
– Chemical stability in water, soil and light
– n-Octanol/water partition coefficient
– Results of a ready biodegradability test (see Test Guideline 301)

• Qualifying statement

This Test Guideline can be used for substances that are either insoluble or soluble in water, although the method of application differs.

• Standard documents

There are no relevant international standards.

2. METHOD

A. INTRODUCTION, PURPOSE, SCOPE, RELEVANCE, APPLICATION AND LIMITS OF TEST

There are many methods of testing toxicity of chemicals to earthworms, including spot application, forced feeding and immersion tests. This Test Guideline includes two kinds of tests: a paper contact toxicity test and an artificial soil test.

A simple paper contact toxicity test is described as an optional initial screen to indicate those substances likely to be toxic to earthworms in soil and which will require further more detailed testing in an artificial soil.

The simple contact test is easy to perform and gives reproducible results with the recommended species. The artificial soil test gives toxicity data more representative of natural exposure of earthworms to chemicals.

- Definitions and units

LC50 in this Test Guideline is the median lethal concentration i.e. that concentration of the test substance which kills 50 per cent of the test animals within the test period.

For the contact test the concentration of the test substance is expressed in mg/cm^2. For the artificial soil test it is expressed in mg/kg (dry weight).

- Reference substances

The LC50 of a reference substance should be determined occasionally as a means of assuring that the laboratory test conditions are adequate and have not changed significantly. A suitable reference substance is chloracetamide.

- Principle of the test method

The screening test (filter paper contact test) involves exposing earthworms to test substances on moist filter paper in order to identify potentially toxic chemicals to earthworms in soil.

The artificial soil test involves keeping earthworms in samples of a precisely defined artificial soil to which a range of concentrations of the test substance has been applied. Mortality is assessed 7 and 14 days after application.

One concentration resulting in no mortality and one resulting in total mortality should be used.

- Conditions for the validity of the test

The mortality in the controls should not exceed 10 per cent at the end of either test.

B. DESCRIPTION OF THE TEST PROCEDURE

- Preparations

Equipment and materials

Normal laboratory equipment and especially the following equipment and materials are necessary:

– Earthworm cultures (see Experimental animals, below)

– Filter paper: 80 to 85 g/m^2, approximately 0.2 mm thick, medium grade

– *Artificial soil test substrate*, for example, as follows:

10 per cent sphagnum peat (as close to pH 5.5 to 6.0 as possible, no visible plant remains, finely ground, dried to measured moisture content)

20 per cent kaolin clay (kaolinite content preferably above 30 per cent)

70 per cent industrial sand (fine sand should be dominant with more than 50 per cent of the particles between 50 and 200 microns)

pH is adjusted to 6.0 ± 0.5 by addition of calcium carbonate [see reference (6)]

The dry constituents are blended in the correct proportions and mixed thoroughly, either in a large-scale laboratory mixer or small electric cement mixer. Moisture content is then determined by drying a small sample at 105°C and re-weighing. Deionised water is added to give an overall moisture content of about 35 per cent of the dry weight, and the medium is thoroughly mixed. The complete mixture should be moist but not so wet that water appears when the artificial soil is compressed. With some peats a moisture content of over 35 per cent may be suitable.

– All glass test containers, i.e. crystallising dishes or spoutless beakers, of approximately one litre covered with glass lids or perforated plastic film

– An illuminated cabinet or chamber controllable to ± 2°C with a light intensity of 400 to 800 lux.

• Experimental animals

Selection of species

The recommended test species is *Eisenia foetida* (Michaelsen). Although this is not a typical soil species, it occurs in soil rich in organic matter. Its susceptibility to chemicals resembles that of true soil-inhabiting species, it has a short life cycle, hatching from cocoons in 3 to 4 weeks, and reaching maturity in seven to eight weeks at 20°C. It is very prolific, each worm producing two to five cocoons per week from each of which emerge several worms. It is available commercially and can be bred readily in a wide range of organic waste materials. Cocoons can be purchased commercially or distributed from a central source to ensure the same strain is used (see Annex).

Eisenia foetida exists in two races which some taxonomists have separated into species [see referene (1)]. These are morphologically similar, but one, *E. foetida foetida*, has a 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 foetida* should be used. Other species may be used if the necessary methodology is available.

Worms should be adult (at least two months old with clitellum) with an individual weight of 300 to 600 mg.

• Performance of the test

Filter paper test

Flat-bottomed glass vials approximately 8 cm in length and 3 cm in diameter are recommended. Their sides are lined with filter paper cut to a suitable size so it does not overlap appreciably.

The test substance is dissolved in water (if soluble up to a concentration of 1000 mg/l) or in a suitable organic solvent (e.g. acetone, hexane or chloroform), as appropriate, to give a range of known concentrations. One ml of solution is pipetted into each vial and evaporated to dryness under a slow stream of filtered compressed air, the vial being rotated horizontally as it dries (for substances that are relatively insoluble in either water or organic solvents this may have to be repeated several times to obtain the larger deposits required). The control vial should be treated with 1 ml of deionised water or appropriate organic solvent. After drying, 1 ml of deionised water is added to each vial to moisten the filter paper. Each vial is sealed with a cap or plastic film with a small ventilation hole.

A preliminary range-finding test may be done optionally prior to a more precise screening test. This could be done as follows:

Amount applied to filter paper	Concentration of solution applied
1.0 mg/cm^2	7×10^{-2} g/ml
0.1 mg/cm^2	7×10^{-3} g/ml
0.01 mg/cm^2	7×10^{-4} g/ml
0.001 mg/cm^2	7×10^{-5} g/ml
0.0001 mg/cm^2	7×10^{-6} g/ml

For the main screening test five or more treatment levels in a geometric series should be used.

For each treatment, ten replicates, each consisting of one worm per vial, are the minimum requirement. More than one worm in a vial should not be used because the death of one worm may have adverse effects on others in the same vial. The precision of the test can be increased by using 20 replicates. In each test a range of treatment levels and ten control vials are used.

Worms should be kept on moist filter paper for three hours before being placed in test vials so they can void their gut contents. They are then washed and dried before use.

During the test, vials are laid on their sides on trays. The test temperature is 20° ± 2°C. Tests are done in the dark and for a period of 48 hours with a further optional mortality assessment after 72 hours.

Worms are classified as dead when they do not respond to a gentle mechanical stimulus to the front end. Any behavioural or pathological symptoms should be reported.

Artificial soil test

A preliminary range-finding test before a more precise main test is optional here as well. It could be based on treatments in the range 0.01, 0.1, 1.0, 10, 100, 1000 mg/kg (dry weight of artificial soil). For the test proper, five concentrations in a geometric series are used.

The artificial soil plus test substance should, whenever possible, be made up as follows: immediately before the start of the test, an emulsion or dispersion of the test substance in deionised water is mixed with the artificial soil 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 a suitable organic solvent (e.g. hexane, acetone or chloroform). The solvent should be allowed to evaporate. If the test substance is not soluble, dispersible or emulsifiable, 10 g of a mixture of fine ground quartz sand and quantity of test substance corresponding to 750 g wet weight of artificial soil are mixed with 740 g wet artificial soil for each test container. Only agents which volatilise readily may be used to solubilise, disperse or emulsify the test substance. The test medium must be ventilated before use. The amount of water evaporated should be replaced. The control should receive the same quantity of any additive agent.

For each test, 750 g weight of the test medium is placed into each glass container and ten earthworms, which have been conditioned for 24 hours in an artificial soil and then washed quickly before use, are placed on the test medium surface. The containers are covered with perforated plastic film to prevent the test medium from drying and kept under the test conditions for 14 days.

Four replicates for each treatment are recommended.

For each test, four control dishes, treated with the same solvent as that used in the test and containing ten worms, are used.

The test duration is 14 days (assessment of mortality at 7 and 14 days), and the test temperature is 20° ± 2°C. Testing is done in continuous light (to ensure that worms remain in the test medium throughout duration of test).

The mortality is assessed by emptying test medium onto a glass tray or plate, sorting worms from the medium and testing their reaction to a mechanical stimulus at the front end. After the 7-day assessment worms and medium are replaced in the test container. Any behavioural or pathological symptoms noted should be reported.

At the end of the test the moisture content of the test medium should be assessed and reported.

3. DATA AND REPORTING

- Treatment of results

The mortality/concentration data should be plotted on log probability graph paper and the median lethal concentration (LC50) and its confidence limits estimated (see reference 3). Other methods of probit analysis are also acceptable.

When two consecutive concentrations in a geometric series (at a ratio of at most 2.0) result in 0 and 100 per cent mortality, these two values are sufficient to indicate the range within which the LC50 falls.

- Test report

The test report should include the following information:

Test substance: chemical identification data, method of application

Test animals: age, keeping and breeding conditions, source of supply

Test conditions: description and details of any variation of test materials and recommended conditions

Information on preparation of the test medium.

Results:

- average live weight and number of live worms per treatment at start and end of test
- description of obvious physical or pathological symptoms or distinct changes in behaviour observed in the test organisms
- method used to determine the LC50 quoting all data used and test results
- graph showing concentration/effect curve
- mortality in control animals
- mortality with reference and test substance
- LC50 and all data used to calculate it
- moisture content of artificial soil at start and at end of test, pH value at start of test
- the highest concentration causing no mortality
- the lowest concentration causing 100 per cent mortality.

4. LITERATURE

1. M.B. Bouché, *Lombriciens de France, Ecologie et Systématique*. Publ. Institut National de la Recherche Agronomique (1972).
2. C.A. Edwards and J.R. Lofty, *Biology of Earthworms*, 2nd Edition, Chapman and Hall, London (1977).
3. J.T. Litchfield and F. Wilcoxon, *Journal of Pharmacol. Exper. Ther. 96*. 99-113 (1949).
4. C.E. Stephan, in *Aquatic Toxicology and Hazard Evaluation* (edited by F.L. Mayer and J.L. Hamelink) pp. 66-84, ASTM STP 634, American Society for Testing and Materials (1977).
5. C.A. Edwards, *Development of a Standardized Laboratory Method for Assessing the Toxicity of Chemical Substances to Earthworms*, Report EUR 8714EN, Commission of the European Communities (1983).

6. *The Analysis of Agricultural Materials*, Ministry of Agriculture, Fisheries and Food, Reference Book 427, HMSO, London (1981).

5. ANNEX

BREEDING OF TEST ORGANISMS

Eisenia foetida can be bred in a wide range of animal wastes. The recommended breeding medium is a 50:50 mixture of horse or cattle manure and peat, but other animal wastes are also suitable. The medium should be of pH about 7.0, have low ionic conductivity (less than 6.0 milli-Siemens) and not be contaminated excessively with ammonia or animal urine. Wooden breeding boxes of about 50 x 50 x 15 cm with tightly fitting lids are ideal for large-scale breeding and can produce more than 1000 worms in six weeks. To produce sufficient worms, such a medium will support up to 1 kg worms in 20 kg waste and each worm will weigh up to 1 g. To obtain worms of standard age and weight, it is best to start the culture with cocoons which take three to four weeks to hatch and seven to eight weeks to become mature worms at 20°C.