207

Adopted:
4 April 1984

OECD GUIDELINE FOR TESTING OF CHEMICALS

"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.

208

Adopted:
4 April 1984

OECD GUIDELINE FOR TESTING OF CHEMICALS

"Terrestrial Plants, Growth Test"

1. INTRODUCTORY INFORMATION

• Prerequisites

- Water solubility
- Vapour pressure

• Guidance information

- Structural formula
- Solubility in organic solvents
- n-Octanol/water partition coefficient
- Absorption behaviour
- Purity of the test substance
- Chemical stability in water and light
- Results of a ready biodegradability test (see Test Guideline 301)

• Qualifying statement

This Test Guideline does not give a separate indication of possible damage resulting from vapour action of the test substance, nor does it measure damage which could result from direct contact with the foliage.

• Standard documents

There are no relevant international standards.

2. METHOD

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

This Test Guideline is designed to determine possible toxic effects of soil-incorporated solid or liquid chemical substances on the emergence of seedlings and the early stages of growth of a variety of terrestrial plants after a single application.

• Definitions

– EC 50 in this Test Guideline is the concentration at which the change in growth is 50 per cent of that of the control.

– Emergence in this Test Guideline refers to the appearance of the seedling above the soil surface.

– LC 50 in this Test Guideline is the concentration at which the change in emergence is 50 per cent of that of the control.

– Growth is expressed in terms of plant weight.

• Reference substances

No reference substances are recommended for this test. However, if a reference substance has been tested, the results should be given.

• Principle of the test method

The test substance is incorporated at various concentrations into soil in which the seeds are sown. The number of seedlings that emerge is recorded. At least two weeks after 50 per cent of the seedlings have emerged in the control, the plants are harvested and weighed.

• Conditions for the validity of the test

A minimum of 80 per cent of the control seeds should produce healthy seedlings. The control seedlings should exhibit normal growth throughout the test.

B. DESCRIPTION OF THE TEST PROCEDURE

• Preparations

Equipment and materials

Suitable facilities for plant testing are necessary, including phytotrons, glasshouses or plant growth chambers. Planting containers must be non-porous plastic or glazed pots.

It is not necessary to use sterile soil. The soil should be sieved (0.5 cm) to remove coarse fragments. Carbon content should not exceed 1.5 per cent (3 per cent organic matter). Fine particles (under 20 µm,) should make up between 10 and 20 per cent. The pH should be between 5.0 and 7.5.

Any method of soil treatment can be used which results in even dispersion of the test substance throughout the soil. Surfactants should not be used.

- Experimental plants

Selection of species

A minimum of three species should be selected for testing, at least one from each of the categories below:

Category	Tests species	
1	ryegrass	*Lolium perenne*
	rice	*Oryza sativa*
	oat	*Avena sativa*
	wheat	*Triticum aestivum*
	sorghum	*Sorghum bicolor*
2	mustard	*Brassica alba*
	rape	*Brassica napus*
	radish	*Raphanus sativus*
	turnip	*Brassica rapa*
	Chinese cabbage	*Brassica campestris var. chinensis*
3	vetch	*Vicia sativa*
	mung bean	*Phaseolus aureus*
	red clover	*Trifolium pratense*
	fenugreek	*Trifolium ornithopodioides*
	lettuce	*Lactuca sativa*
	cress	*Lepidium sativum*

Other species may be used if the rationale for their selection is justified in the test report.

- Test conditions

Temperature, humidity and light conditions should be suitable for maintaining normal growth of each species for the test period.

- Performance of the test

A control and three concentrations should be tested in a randomised block design with a minimum of four replicates per treatment. A minimum of five seeds should be planted in each replicate within 24 hours of incorporation of the test substance. All seeds of each species for each test should be of the same size class. The seed should not be imbibed.

The test substance can be incorporated into the soil as follows:

1. Dissolve the chemical in a volatile solvent.
2. Mix the solution with sand.
3. Sit the sand slurry while the solvent evaporates.
4. Mix the sand with soil.
5. Maintain a constant sand-soil ratio for all treatments including controls.

Application rates should be equivalent to 0.0 (control), 1.0, 10.0 and 100.0 mg substance per kg of oven dried soil. The seeds are then planted.

The size of pots or containers should be adequate to allow un restricted growth of the selected species. Plants should be watered as needed.

The test should be terminated no sooner than 14 days after 50 per cent of the control seedlings have emerged.

3. DATA AND REPORTING

- Treatment of the results

The number of plants that emerge per replicate is recorded and the average weight per replicate determined (wet weight immediately after harvest or dry weight after oven drying at

approximately 70°C) and expressed on a per plant basis. The effect of the test substance on emergence should be expressed as LC50, and the effect on growth as EC50.

- Test report

The test report should include the following information:

Test substance: chemical identification data

Test organisms: species/varieties tested, seed source, weight and viability

Test conditions:

- pot dimensions and amounts of soil
- method of incorporation of the test substance
- growth conditions (e.g. light intensity, photo period, day/night temperature, watering schedule) and type of testing facility (e.g. phytotron, glasshouse, growth chamber)
- soil characteristics (pH, per cent organic matter, per cent particles smaller than 20 µm, sterilised or not)

Results:

- all data in tabular form
- graphical presentation of concentration-effect relationship
- LC 50 values for emergence
- EC 50 values for growth
- phytotoxic effects noted for each concentration and control
- any photographs of plants, phytotoxic effects, etc.
- any deviation from this Test Guideline

4. LITERATURE

1. A. Nyffeler, H.R. Gerber, K. Hurle, W. Pestemer and R.R. Schmidt, *Weed Research 22*, 213-222 (1982).

209

Adopted:
4 April 1984

OECD GUIDELINE FOR TESTING OF CHEMICALS

"Activated Sludge, Respiration Inhibition Test"

1. INTRODUCTORY INFORMATION

• Prerequisites

- Water solubility
- Vapour pressure

• Guidance information

- Structural formula
- Purity of the test substance

• Qualifying statements

- This test guideline is most readily applied to substances which, due to their water solubility and low volatiliy, are likely to remain in water.

- For test substances with limited solubility in the test media, it may not be possible to determine the EC 50.

- Results based on oxygen uptake may lead to erroneous conclusions when the test substance has the propensity to uncouple oxidative phosphorylation.

• Recommendation

Activated sludge may contain potentially pathogenic organisms and should be handled with care.

• Standard documents

See Section 4, Literature.

2. METHOD

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

The method described in this test guideline assesses the effect of a test substance on micro-organisms by measuring the respiration rate under defined conditions in the presence of different concentrations of the test substance. The method is based on that described by ETAD (Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry), in which activated sludge obtained from a sewage treatment plant is used as the microbial source.

The purpose of this test guideline is to provide a rapid screening method whereby substances which may adversely affect aerobic microbial treatment plants can be identified and to indicate suitable non-inhibitory concentrations of test substances to be used in biodegradability tests.

A range-finding test may precede a definitive test. It provides information about the range of concentrations to be used in the main test.

Two controls without test substance are included in the test design, one at the start and the other at the end of the test series. Each batch of activated sludge should also be checked using a reference substance.

- Definitions

The respiration rate is the oxygen consumption of aerobic sludge or waste-water micro-organisms expressed generally as mg O_2 per litre per hour.

EC 50 in this Test Guideline is the concentration of the test substance at which the respiration rate is 50 per cent of that shown by the control under conditions described in this guideline.

- Reference substances

It is recommended that 3,5-dichlorophenol as a known inhibitor of respiration be used as a reference substance and tested for EC 50 on each batch of activated sludge as a means of checking that the sensitivity of the sludge is not abnormal.

- Principle of the test method

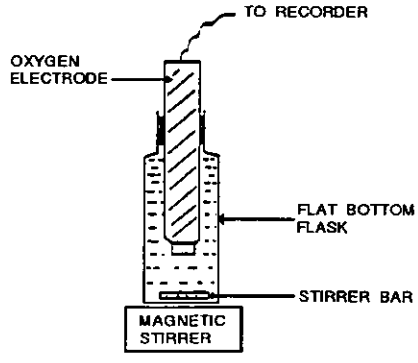
The respiration rate of an activated sludge fed with a standard amount of synthetic sewage feed is measured after a contact time of 30 minutes or 3 hours, or both. The respiration rate of the same activated sludge in the presence of various concentrations of the test substance under otherwise identical conditions is also measured The inhibitory effect of the test substance at a particular concentration is expressed as a percentage of the mean respiration rates of two controls. An EC 50 value is calculated from determinations at different concentrations.

- Conditions for the validity of the test

The test results are valid if

- the two control respiration rates are within 15 per cent of each other ;
- the EC 50 (3 hours) of 3,5-dichlorophenol is in the accepted range 5 to 30 mg/l.

Figure 1 : Measuring Apparatus*



* The precise design is not critical. However, there should be no head space and the probe should fit tightly in the neck of the measuring flask.

B. DESCRIPTION OF THE TEST PROCEDURE

- Preparations

Equipment

Normal laboratory equipment and especially the following is necessary :

- Measuring apparatus (see Figure 1)
- Aeration device
- pH-electrode and measuring equipment
- O_2-electrode.

Solutions of the test substance

Solutions of the test substance are freshly prepared at the start of the study using a stock solution. A stock solution concentration of 0.5 g/l is appropriate if the procedure recommended below is followed.

[Note : A solution of 3,5-dichlorophenol can be conveniently prepared by dissolving 0.5 g 3,5-dichlorophenol in 10 ml of 1N NaOH, diluting to approximately 30 ml with distilled water, adding under stirring 1N H_2SO_4 to the point of incipient precipitation – approximately 8 ml of 1N H_2SO_4 will be required – and finally diluting the mixture to one litre with distilled water. The pH should then be in the range 7 to 8].

Test concentrations

At least five concentrations, spaced by a constant factor preferably not exceeding 3.2, should be used.

Synthetic sewage feed

A synthedic sewage feed is made by dissolving the following amounts of substances in 1 litre of water :

- 16 g peptone
- 11 g meat extract
- 3 g urea
- 0.7 g NaCl

- 0.4. g $CaCl_2.2H_2O$
- 0.2 g $MgSO_4.7H_2O$
- 2.8 g $K_2 HPO_4$

[Note : This synthetic sewage is a 100 fold concentrate of that described in the OECD Technical Report "Proposed method for the determination of the biodegradability of surfactants used in synthetic detergents" June 11, 1976, with moreover dipotassium hydrogen phosphate added.]

- Test system

Microbial innoculum

Activated sludge from a sewage treatment plant is normally used as the microbial innoculum for the test. Where possible, activated sludge should be obtained from a sewage work treating predominantly domestic sewage. If this is not possible, the activated sludge may be obtained from sewage works treating predominantly industrial waste water but used only following de-adaptation. Even so, results obtained with activated sludge from works treating industrial waste waters may be atypical.

On return to the laboratory the sludge is washed, if necessary, with tap water or an isotonic solution. After centrifuging the supernatant is decanted. This procedure is repeated three times. A small amount of the washed sludge is weighed and dried. From this result the amount of wet sludge can be calculated which must be suspended in water in order to obtain an activated sludge with a mixed liquor suspended solids level of 4 g/l (± 10 per cent). This level gives a concentration of 1.6 g/l in the test medium if the procedure recommended below is followed.

If the sludge cannot be used on the day of collection, 50 ml synthetic sewage is added to each litre of the activated sludge prepared as described above ; this is then aerated overnight at 20 ± 2°C. It is then kept aerated for use during the day. Before use the pH is checked and buffered, if necessary, to pH 6.0 to 8.0 using sodium bicarbonate solution. The mixed liquor suspended solids should be determined as described in the preceeding paragraph.