C. 8. TOXICITY FOR EARTHWORMS

ARTIFICIAL SOIL TEST

1. METHOD

1.1. Introduction

In this laboratory test, the test substance is added to an artificial soil in which worms are placed for 14 days. After this period (and optionally after seven days) the lethal effect of the substance on the earthworms is examined. The test provides a method for relatively short-term screening of the effect of chemicals on earthworms, by dermal and alimentary uptake.

1.2. Definition and unit

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

1.3. Reference substance

A reference substance is used periodically as a means of demonstration that the sensitivity of the test system has not changed significantly.

Analytical grade 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 Appendix) are kept in a defined artificial soil treated with different concentrations of the test substance. The content of the containers is spread on a tray 14 days (and optionally seven days) after the beginning of the test, and the earthworms surviving at each concentration counted.

1.5. Quality criteria

The test is designed to be as reproducible as possible with respect to the test substrate and organism. Mortality in the controls must not exceed 10% at the end of the test, or the test is 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 to pH 5,5 to 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 to 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 ($CaCO_3$), pulverized, 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 about 25 to 42% of the dry weight of the basic substrate. The water content of the substrate is determined by drying a sample to constant weight at 105 °C. 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.

(c) 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 seven days after starting the test.

1.6.3. Test procedure

Test concentrations

Concentrations of the test substance are expressed as weight of substance per dry weight of basic substrate (mg/kg).

Range finding test

The range of concentrations just causing mortalities of 0 to 100% 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/kilogram test substrate (dry weight).

If a full definitive test is to be carried out, one test batch per concentration and one for the untreated control, each with 10 worms, could be sufficient for the range-finding test.

Definitive test

The results of the range-finding test are used to choose at least five concentrations in a geometric series just spanning the range 0 to 100% mortality and differing by a constant factor not exceeding 1,8.

Tests using these series of concentration should allow the LC_{50} value and its confidence limits to be estimated as precisely as possible.

In the definitive test at least four test batches per concentration and four untreated controls, each with 10 worms, are used. 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).

Only agents which volatilize readily may be used to solubilize, 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.

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.

For each test batch, an amount of wet test substrate equivalent to 500 g dry weight is placed in 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 seven 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.

When the examination is made at seven days, the container is refilled with the substrate and the surviving earthworms are replaced on the same test substrate surface.

1.6.4. Test organisms

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

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 ($p = 0,05$) should be determined using standard methods (Litchfield and Wilcoxon, 1949, for equivalent method). The LC_{50} should be given as mg of test substance per kilogram 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 shall, if possible, contain the following:

-statement that the test has been carried out in accordance with the abovementioned 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 of 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 organisms,

-mortality in the controls,

-LC_{50} or highest tested concentration without mortality and lowest tested concentration with a mortality of 100%, 14 days (and optionally seven days) after setting up the test,

-plotting of the concentration/response curve,

-results obtained with the reference substance, whether in association with the present test or from previous quality control exercises.

4. REFERENCES

(1) OECD, Paris, 1981, Test Guideline 207, Decision of the Council C(81)30 final.

(2) Edwards, C. A. and lofty,]. R., 1977, Biology of Earthworms, Chapman and Hall, london, 331 pp.

(3) Bouche. M. B., 1972, Lombriciens de France, Ecologie et Systematique, Institut National de la Recherche Agronomique, 671 pp.

(4) Litchfield, J. T. andWilcoxon, F., A simplified method of evaluation dose effect experiments. I. Pharm. Exp. Therap., vol. 96, 1949, p. 99.

(5) Commission of the European Communities, Development of a standardized laboratory method for assessing the toxicity of chemical substances to earthworms, Report EUR 8714 EN, 1983.

(6) Umweltbundesamt/Biologische Bundesanstalt für land- und Forstwirtschaft, Berlin, 1984, Verfahrensvorschlag "Toxizitätstest am Regenwurm *Eisenia foetida* in künstlichem Boden', in: Rudolph/Boje, Ökotoxikologie, ecomed, Landsberg, 1986.

Appendix

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 maybe used for further breeding batches. The earthworms hatched from the cocoons are used for testing when mature (under the prescribed conditions after two to three months).

Keeping and breeding conditions

Climatic chamber: temperature 20 ± 2 °C preferably with continuous light (intensity 400 to 800 lux).

Breeding boxes: suitable shaI1ow containers of 10 to 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 (Bouche, 1972). These are morphologically similar but one, *Eisenia foetida foetida*, has typically transverse striping or banding on the segments and the other, *Eisenia foetida andrei*, lacks this and has a variegated reddish colour. Where possible *Eisenia foetida andrei* should be used. Other species may be used if the necessary methodology is available.