INTERNATIONAL STANDARD

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Soil quality — Effects of pollutants on earthworms (*Eisenia fetida*) —

Part 1:
Determination of acute toxicity using artificial soil substrate

Qualité du sol — Effets des polluants vis-à-vis des vers de terre (Eisenia fetida) —

Partie 1: Détermination de la toxicité aiguë en utilisant des substrats de sol artificiel

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 11268-1 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

ISO 11268 consists of the following parts, under the general title *Soil quality — Effects of pollutants on earthworms (Eisenia fetida)*:

— *Part 1: Determination of acute toxicity using artificial soil substrate*

— *Part 2: Determination of effects on reproduction*

— *Part 3: Guidance on field testing*

Annexes A and B of this part of ISO 11268 are for information only.

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Introduction

This International Standard, describing a method using artificial soil, has been drawn up taking into consideration test procedures recommended by the Organisation for Economic Co-operation and Development (OECD).

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Soil quality — Effects of pollutants on earthworms (*Eisenia fetida*) —

Part 1:
Determination of acute toxicity using artificial soil substrate

1 Scope

This International Standard describes a method for determining the acute toxicity of substances to *Eisenia fetida* by dermal and alimentary uptake using an artificial substrate. The method is not applicable to volatile substances i.e. substances for which H (Henry's constant) or the air/water partition coefficient is greater than 1, or for which the vapour pressure exceeds 0,013 3 Pa at 25 °C.

This method does not take into account the possible degradation of the test substance.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this part of ISO 11268. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this part of ISO 11268 are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 11274:—[1], *Soil quality — Determination of the water retention characteristic — Laboratory methods.*

ISO 11461:—[1], *Soil quality — Determination of soil water content on a volume basis — Gravimetric method.*

1) To be published.

3 Definitions

For the purposes of ISO 11268, the following definitions apply.

3.1 LC 50: The median lethal concentration, i.e. that concentration of test substance initially present which kills 50 % of the test animals within the test period.

3.2 NOEC (no observed effect concentration): The highest tested concentration of a test substance at which no lethal or other effect (such as weight alteration) is observed.

NOTE 1 Both LC 50 and NOEC are expressed in milligrams of test substance per kilogram (dry mass) of the test substrate (5.2).

4 Principle

The percent mortality of adult earthworms (species *Eisenia fetida*) placed in a defined substrate containing the test substance in different concentrations is determined after 7 d and 14 d. The test substance is added in a single step and the test is completed without replenishing the test substances. Test concentrations are measured as milligrams of substance per kilogram of dry test substrate.

The results obtained from the tests are compared with a control and are used to estimate the concentration which causes the mortality of 50 % of earthworms (LC 50, 14 d).

NOTE 2 Chloroacetamide has been found to be a suitable reference substance.

The test is conducted in two steps:

— a preliminary test, which gives an approximate indication of the concentrations responsible for total mortality and for the absence of mortality, which serves to determine the range of concentrations for the final test;

— the final test to determine the concentrations causing between 10 % and 90 % mortality, which yields the definitive result.

Substances are not tested at concentrations higher than 1 000 mg per kg of dry substrate.

If the preliminary test shows no mortality, it is not necessary to perform the final test.

5 Reagents

5.1 Biological material

The biological material consists of adult earthworms of the species *Eisenia fetida* at least two months old, with a clitellum and a wet mass between 300 mg and 600 mg.

NOTE 3 *Eisenia fetida* exists in two sub-species which some taxonomists regard as distinct species: *Eisenia fetida* and *Eisenia andrei*. These are morphologically similar, but one, *E. fetida fetida (E. fetida)* has a typically transverse striping or banding on the segments, and the other, *E. fetida andrei (E. andrei)* lacks this and has a variegated reddish colour. Either sub-species (species) may be used.

Select worms used for the test to form as far as is practicable a homogenous population from the standpoint of size and weight. Before the test, wash them with potable water.

NOTE 4 An example of a breeding technique for *Eisenia fetida* is given in annex A.

5.2 Test substrate

For each glass container (6.1), the quantity of substrate used per glass container shall be equivalent to 500 g (dry mass).

2) To be published.

The substrate called "artificial soil" shall have the following composition:

	Percentage expressed on dry mass basis
Sphagnum peat finely ground and with no visible plant remains	10 %
Kaolinite clay containing not less than 30 % kaolinite	20 %
Industrial quartz sand (dominant fine sand with more than 50 % of particle size 0,05 mm to 0,2 mm)	70 %

Add sufficient calcium carbonate ($CaCO_3$), pulverized and of recognized analytical grade, to bring the pH of the wetted substrate to 6,0 ± 0,5 (commonly about 0,5 % of the mass of the dry ingredients).

The substrate shall be wetted with deionized or distilled water to reach 40 % to 60 % of the total water holding capacity determined in accordance with ISO 11274[2].

Determine the water content of the substrate in accordance with ISO 11461[2].

NOTE 5 Allowance should be made for any water that is to be used for introducing the test substance into the soil.

6 Apparatus

Usual laboratory equipment and

6.1 Glass containers, one for each test, of capacity about 1 litre to 2 litres, not tightly closed, to allow exchanges between the medium and the atmosphere.

6.2 Apparatus to determine the dry mass of the substrate in accordance with ISO 11461.

7 Test environment

7.1 Enclosure, capable of being controlled at a temperature of 20 °C ± 2 °C.

7.2 Light source (e.g. white fluorescent tubes), capable of delivering a constant light intensity of 400 lx to 800 lx on the containers at a controlled light/dark cycle of between 12 h: 12 h and 16 h: 8 h.

8 Procedure

8.1 Preparation of the test

The concentrations of the test substance shall be expressed as mass of substance per dry mass of soil substrate (mg/kg) (see 5.2).

8.1.1 Introduction of the test substances

8.1.1.1 Water soluble substances

Immediately before starting the test, dissolve the quantity of test substance required for the replicates of one concentration in the water (or that portion of it necessary to wet the soil substrate in order to meet the requirements of 5.2) and mix it thoroughly with the test substrate (5.2) described before introducing it into a glass container (6.1).

Continue as described in 8.1.2.

8.1.1.2 Substances insoluble in water but soluble in organic solvents

Dissolve the quantity of test substance required to obtain the desired concentration in a volatile solvent (such as acetone or hexane).

Add it to a glass container (6.1) with the test substrate (5.2). Mix thoroughly and evaporate the solvent by placing the container under a fume hood. Then add the water in accordance with 5.2. Mix thoroughly and continue as described in 8.1.2.

WARNING — Appropriate precautions should be taken when dealing with solvent vapour to avoid danger from inhalation or explosion, and to avoid damage to extraction equipment, pumps etc.

8.1.1.3 Substances insoluble in water or organic solvents

For a substance insoluble in a volatile solvent, prepare a mixture of 10 g of finely ground industrial quartz sand (see 5.2) and the quantity of the test substance required to obtain the desired concentration. Transfer the mixture, the remainder of the test substrate (5.2) and the water (see 5.2) to the glass container (6.1).

Mix thoroughly and continue as described in 8.1.2.

8.1.2 Introduction of the biological material

Determine the water content and the pH of the artificial soil at the beginning and end of the test (when acid or basic substances are tested, do not adjust the pH).

Take 10 worms prepared as described in 5.1, dry them on absorbent paper, weigh them and add them to the test container.

Cover the container as indicated in 6.1 and place it in the test enclosure (7.1).

8.1.3 Control container

Prepare the control containers in the same way as the test containers. If the preparation of the test requires the use of a solvent (see 8.1.1.2), use an additional control prepared with solvent but without the test substance. Cover the containers as indicated in 6.1.

8.2 Preliminary test

Perform a preliminary test for five concentrations of the test substance [for example: 0,1 mg/kg, 1 mg/kg, 10 mg/kg, 100 mg/kg and 1 000 mg/kg, the concentrations being expressed in milligrams of test substance per kilogram of dried test substrate (5.2)] and for a control using 10 worms per concentration and per container.

Prepare the test containers as indicated in 8.1.1 and 8.1.2.

Place the test containers in the test environment described in clause 7.

After 7 d, count the live worms and remove the dead worms if visible.

After 14 d, count the live worms and dead worms in each container (a worm is considered to be dead if it displays no reaction to a pin prick applied to its anterior side).

For each container and each concentration, calculate the percent mortality. Note the symptoms observed on the animals.

8.3 Final test

Based on the results of the preliminary test, perform the final test on five concentrations of the test substance, providing a geometric progression between the highest concentration causing no mortality and the lowest concentration causing total mortality.

Proceed simultaneously with four replicates per concentration and for a control test without the test substance (see 8.1.3) and, if necessary, for another control with solvent, placing each container in the test environment (clause 7). During the test period and after 14 d, proceed as indicated in 8.2.

At the end of the test, for each container, determine the total number and mass of living worms, the water content in one control container and the pH in one container per test concentration.

8.4 Reference substance

Conduct a test with chroloroacetamide analytical grade according to the procedure described in 8.1.1.1.

NOTE 6 The LC 50 (3.1) should be between 20 mg/kg and 80 mg/kg.

9 Calculation and expression of results

9.1 Calculation

For each concentration, determine the percent mortality obtained in the final test.

NOTE 7 When the data are adequate, the LC 50-14 d value and the confidence limits ($T = 0,95$) should be determined using standard methods (Litchfield and Wilcoxon[6] or equivalent methods, see annex B).

When two consecutive concentrations at a ratio less than or equal to 1,8 (for example 10,18) give only 0 % and 100 % mortality, the two values are sufficient to indicate the range within which the LC 50 falls.

9.2 Expression of results

Indicate, in milligrams per kilogram of dried soil substrate, the LC 50-14 d (or more if the test is continued beyond this period, see clause 9), the highest concentration tested causing 0 % mortality and the lowest concentration tested causing 100 % mortality.

10 Validity of the test

The results are considered to be valid if

the percent mortality observed in the control is < 10 %;

the average loss of biomass of the worms in the control does not exceed 20 %.

11 Test report

The test report shall include the following information:

a) a reference to this International Standard;

b) the results expressed as in 9.2;

c) the complete description of the biological material employed (species, age, weight range, breeding conditions, supplier);

d) the method of preparation of the test substrate, including the solvent used for a water insoluble substance;

e) the identity of the reference substance and the results obtained when using it;

f) conditions of the test environment;

g) the method used for calibration of LC 50;

h) a table giving the percent mortality obtained for each container, for each concentration and for the control;

i) the mass of live worms at the end of the test;

j) if the data is available, the highest concentration causing no observed effects (NOEC);

k) a description of obvious or pathological symptoms or distinct changes in behaviour observed in the test organisms;

l) the water content and pH of artificial soil at the start and end of the test;

m) all operating details not specified in this International Standard, and any occurrences likely to have affected the results;

n) a plot of the concentration/response curve.

Annex A
(informative)

Example of breeding technique for *Eisenia fetida*

This annex gives instructions for the breeding of test organisms that are used for the determination of acute toxicity.

Eisenia fetida 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. The medium should have a pH of about 5 to 7 (regulated with calcium carbonate), a low ionic conductivity (less than 6 mS) or less than 0,5 % salt concentration and should not be contaminated excessively with ammonia or animal urine. The substrate should be moist but not too wet. Wooden breeding boxes or any other shallow containers of 10 litres to 50 litres volume are suitable.

To obtain worms of standard age and mass, it is best to start the culture with cocoons. Place adult worms in a breeding box with fresh substrate to produce cocoons and remove them after 14 d to 21 d. These animals may be used for further breeding batches. The earthworms hatched from the cocoons are used for testing when mature.

Breeding is preferably carried out in a climatic chamber at 20 °C ± 2 °C. At this temperature, worms become mature after 2 to 3 months.