INTERNATIONAL STANDARD

ISO 11268-2

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

Part 2:
Determination of effects on reproduction

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

Partie 2: Détermination des effets sur la reproduction

Contents

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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 11268-2 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 to G of this part of ISO 11268 are for information only.

Introduction

This part of ISO 11268 describes a method for testing the effects of chemicals on earthworm reproduction and mortality in an artificial soil. It can also be adapted for testing or comparing soils to assess, for example, the effects of remediation treatments, and for assessing sublethal effects and no-effect levels for pesticides or other added chemicals.

This part of ISO 11268 has been drawn up taking into consideration test procedures recommended by the Organization for Economic Cooperation and Development and by the European Union (see references in annex G).

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

Part 2:
Determination of effects on reproduction

1 Scope

This part of ISO 11268 describes a method for determining the effects of substances on the reproduction, mortality, and growth of *Eisenia fetida fetida* and *Eisenia fetida andrei* by dermal and alimentary uptake using an artificial soil 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 0,0133 Pa at 25 °C.

NOTE 1 No provision is made in the test method for monitoring the persistence of the substance under test.

NOTE 2 Recommendations for adapting the method for comparing or monitoring soil quality or agricultural practice, e.g. for assessing the effect of pesticides, are given in annexes D and E.

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 10390:1994, *Soil quality — Determination of pH.*

ISO 11268-1:1993, *Soil quality — Effects of pollutants on earthworms (Eisenia fetida) — Part 1: Determination of acute toxicity using artificial soil substrate.*

ISO 11274:—[1)], *Soil quality — Determination of water retention characteristics — Laboratory methods*

ISO 11465:1993, *Soil quality — Determination of dry matter and water content on a mass basis — Gravimetric method.*

3 Definitions

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

1) To be published.

3.1
growth
increase in biomass (i.e. the fresh mass of organisms) expressed as a percentage of the fresh mass of organisms at the start of the test

3.2
reproduction
increase in the mean number of offspring per test vessel after 8 weeks incubation under the specified test conditions

3.3
reproduction rate
mean numbers of offspring hatched from the cocoons and surviving until the end of the test period per adult earthworm remaining alive after four weeks

3.4
lowest observed effect concentration
LOEC
lowest concentration of the test substance which is observed to have a significant effect when compared with the control, expressed as mass of the test substance per dry mass of the test substrate

3.5
no observed effect concentration
NOEC
highest tested concentration of a test substance at which no lethal or other effect (such as mass alteration) is observed, expressed as mass of test substance per dry mass of the test substrate

3.6
EC50
concentration estimated to reduce the reproduction rate at the end of the test to 50 % compared to the control, expressed as mass of test substance per dry mass of the test substrate

4 Principle

The effects on reproduction, growth, and percent mortality of adult earthworms (species *Eisenia fetida fetida or E. fetida andrei*) placed in a defined artificial soil substrate containing the test substance in different concentrations, are determined. A single application of test substance is made and the effects on mortality and biomass are determined after 4 weeks. The effect on reproduction is measured by counting the number of offspring hatched from the cocoons after an additional period of 4 weeks.

All test concentrations above the LOEC must have a harmful effect equal to, or greater than, that observed at the LOEC. Where there is no prior knowledge of the concentration of the test substance likely to have an effect, then it is useful to conduct the test in two steps:

— a preliminary acute toxicity test is carried out, as described in ISO 11268-1, to give an indication of the effect concentration, and the concentration giving total mortality. Concentrations to be used in the final test can then be selected;

— the final test on reproduction to determine the concentration of a chemical which, when evenly mixed into the artficial soil, causes no significant effects on numbers of offsprings hatched from cocoons compared with the control (NOEC), and the lowest concentration causing effects (LOEC).

5 Reagents

5.1 Biological material

The biological material consists of adult earthworms of the species *Eisenia fetida fetida* or *Eisenia fetida andrei* (see references in annex F), which are between two months and 1 year old, with a clitellum, and a wet mass between 300 mg and 600 mg.

Synchronization of breeding is achieved by placing adult worms in breeding containers (see annex A) and removing them after not more than 4 weeks. Offspring of the remaining cocoons reach an adult stage after at least 2 months.

Condition the selected worms for 1 d to 7 d in an artificial soil before use.The food, which is also used as food source in the test (see 5.3), shall be given in a sufficient amount (see 5.2.1).

5.2 Test substrate

5.2.1 Soil substrate

The substrate used to make artificial soil (5.2.2) shall have the following composition:

Artificial soil component	Percentage expressed on dry mass basis
Sphagnum peat, air-dried, finely ground and with no visible plant remains	10%
Kaolinite clay, air-dried, containing not less than 30 % kaolinite	20%
Industrial quartz sand, air-dried, (predominantly fine sand with more than 50 % by mass of particle size 0,05 mm to 0,2 mm), dependent on $CaCO_3$ needed	70%

Before the experiment, mix the test substrate with the food. When dried cow manure (see 5.3) is used, an amount of 5 g per 500 g dry mass of soil is recommended.

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

NOTE The amount of calcium carbonate required will depend on the components of the soil substrate, including food, and should be determined by measurements on subsamples immediately before the test.

Use 500 g to 600 g dry mass of soil substrate per test container (6.1) to make the artificial soil.

5.2.2 Artificial soil

Prepare the artificial soil by wetting the soil substrate (5.2.1) with deionized or distilled water to reach a water content of between 40 % and 60 % by mass of the total water-holding capacity determined in accordance with ISO 11274.

NOTE The artificial soil should be wetted to a point where there is no standing water or free water appearing when the soil is compressed. If ISO 11274 has not yet been published, use the interim method given in annex C to determine water-holding capacity .

Determine the water content and the pH in the presence of 1 mol/l KCl, in accordance with ISO 11465 and ISO 10390 respectively, of the artificial soil in a mixed sample, immediately before the start of the test and at the end of the test in one sample of the control and one sample of each concentration tested (when acid or basic substances are tested, do not adjust the pH).

5.3 Food

Any food source of a quality shown to be capable of at least maintaining the earthworm population shall be used.

To achieve sufficient reproduction it is essential to feed the earthworms during the experiment. Air-dried finely ground cow manure has been found to be a suitable food. Each charge of food shall be tested to determine whether the earthworms will feed on it, and whether any adverse effects (e.g. by ammonia and other potentially harmful additives) occur. Growth and cocoon production should not be reduced compared to worms kept in a substrate without food. (Conditions as described in ISO 11268-1).

6 Apparatus

Standard laboratory equipment, and:

6.1 Containers, of 1 litre to 2 litre capacity with a cross-sectional area of about 200 cm^2, such that a depth of 5 cm to 6 cm of the soil substrate contains 500 g to 600 g dry mass. Test containers shall permit gaseous exchange between the medium and the atmosphere and access of light (e.g. by means of a perforated transparent cover), and shall have provisions to prevent worms from escaping (e.g. by using a tape to fix the cover).

6.2 Apparatus capable of measuring the pH and water content of a substrate.

6.3 Test environment

6.3.1 Enclosure, capable of being controlled to a temperature of 20 $^0C \pm 2\ ^0C$.

6.3.2 Light source, 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.

7 Procedure

7.1 Preliminary test

If it is necessary to determine the range of concentrations for use in the final test, perform a preliminary acute test in accordance with ISO 11268-1 for four concentrations of the test substance and a control (for example 0 mg/kg, 1 mg/kg, 10 mg/kg, 100 mg/kg and 1000 mg/kg, the concentrations being expressed in milligrams of test substance per kilogram of dried soil substrate (5.2.1)) using ten worms per concentration and per container.

7.2 Final test

7.2.1 Introduction of the test substances

Use either method a), b) or c), as appropriate.

a) Water-soluble substance

Immediately before starting the test, dissolve the quantity of the test substance in the water required for the replicates of one concentration in water (or that portion of it necessary to wet the soil substrate) in order to meet the requirements of 5.2.2, and mix it thoroughly with the soil substrate before introducing it into a test container.

b) 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) and mix it with a portion of the quartz sand required. After evaporating the solvent by placing the container under a fume hood, add the remainder of the basic substrate and the water and mix it thoroughly before introducing it into the test containers. Continue as described in 7.2.3.

NOTE Ultrasonic dispersion, organic solvents, emulsifiers or dispersants may be used to disperse substances with low aqueous solubility. When such auxiliary substances are used, all test concentrations and an additional control should contain the same minimum amount of auxiliary substance.

WARNING: Take appropriate precautions when dealing with solvent vapour to avoid danger from inhalation or explosion, and to avoid damage to extraction equipment, pumps etc.

c) 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 5.2.1) and the quantity of the test substance required to obtain the desired concentration. Place the mixture, the remainder of the soil substrate (5.2.1) and the water into the test container (6.1) and mix thoroughly.

Mix the test substance into the artificial soil substrate in accordance with ISO 11268-1 before the earthworms are added.

Base the concentrations selected to provide the LOEC/NOEC on the results of the preliminary test. Space the concentrations by a factor not exceeding 2.

Substances mixed into the substrate do not need to be tested at concentrations higher than 1000 mg/kg mass of test substrate.

Proceed simultaneously with at least four replicates per concentration and the control(s).

7.2.2 Control container

Prepare control containers in the same way as the test containers but without the test substance. If the preparation of the test requires the use of auxiliary substances (see 8.2.1) use additional control containers similar to the test containers without the test substance. Treat these containers in the same way as those without the test substance.

7.2.3 Addition of the biological material

For each test container and the control container prepare, wash and wipe (using absorbent paper) ten worms (5.1). To avoid systematic errors in distributing the worms to the test containers, the homogeneity of the test population shall be determined by weighing a sample of 20 worms individually. Having ensured homogeneity, batches of 10 worms shall then be selected, weighed and placed in each test container. Batches of worms shall be assigned using a randomization procedure.

Cover the containers as indicated in 6.1

Place the containers in the test chamber or in the test enclosure (6.3.1).

7.2.4 Determination

One day after addition of the worms spread 5 g per test container of air dried finely ground food source (5.3) on the soil surface and moisten with potable water (about 5 ml to 6 ml per container). Feed once a week during the test period. If food consumption is low, reduce feeding to a minimum. Record feeding behaviour and the quantity of food applied over the test period for each test container.

Maintain the water content of the soil substrate in the test containers during the test period by reweighing the test containers periodically and if necessary by replenishing lost water. At the end of the test the water content shall not differ by more than 10 % from that at the beginning of the test.

Keep the adult worms over a period of 4 weeks in the test substrate. At the end of this period, remove the adults and for each container record the total number and mass of living adult worms. Keep the test containers for another period of 4 weeks in the test environment (6.3) to allow offspring to develop. At the beginning of this period juveniles are fed once with 5 g of food per test container carefully mixed by hand into the substrate. After this period count the number of offspring per test container hatched from the cocoons using a suitable method.

NOTE Annex B gives examples of two suitable methods, including one which allows counting of cocoons.

7.3 Reference substance

Carbendazim is recommended as a reference substance. If the compound is mixed into the substrate, observe the effects on reproduction (α = 0,05) at concentrations of between 1 mg and 5 mg ai carbendazim per kilogram dry mass of substrate. (For surface application see annex D)

8 Calculation and expression of results

8.1 Calculation

For each concentration, determine the percent mortality, the percent loss/increase in biomass of the adults, and number of offspring produced in the final test.

Compare means by suitable statistical methods, e.g. Williams, Dunnett`s or Student`s *t* test and test for significance (α = 0,05) of differences from control(s).

8.2 Expression of results

Indicate, in milligrams per kilogram dry mass of soil substrate, the highest concentration tested without mortality, significant changes in biomass of adults, significant reduction in numbers of offspring (NOEC), the lowest concentration showing an effect (LOEC), and, if possible, an LC/EC50.

9 Validity of the test

The results are considered to be valid if:

a) the rate of production of juveniles is at least 30 per control container;

b) the coefficient of variance of reproduction in the control does not exceed 30 %;

c) the percent mortality of the adults observed in the control(s) is ≤ 10 %.

10 Test report

The test report shall refer to this part of ISO 11268 and in addition to the results expressed as in 8.2, shall provide the following information:

– a detailed description of the test substance and information on physical and chemical properties if helpful for the interpretation of the test results;

- a complete description of the biological material employed (species, age, mass range, breeding conditions, supplier);
- the method of preparation of the test substrate, and any auxiliary substances used for a low-/non-water-soluble substance;
- the results obtained with the reference substance, if used;
- the detailed conditions of the test environment;
- a table giving the per cent mortality obtained for each container, for each concentration and for the control;
- the total masses of live adult worms at the beginning of the test, and the total mass of the surviving worms per test container after a period of 4 weeks;
- the number of offspring per test container at the end of the test;
- the highest concentration causing no observed effects (NOEC), and the lowest observed concentration causing effects (LOEC);
- a description of obvious or pathological symptoms or distinct changes in behaviour (e.g. reduced feeding activity) observed in the test organisms per test container;
- the water content and pH of artificial soil at start and at the end of the test, for the control and each concentration;
- all operating details not specified in this part of ISO 11268, and any occurrences liable to have affected the results.