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Second edition
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Soil quality — Effects of pollutants on earthworms —

Part 1: Determination of acute toxicity to *Eisenia fetida/Eisenia andrei*

Qualité du sol — Effets des polluants vis-à-vis des vers de terre — Partie 1: Détermination de la toxicité aiguë vis-à-vis de Eisenia fetida/Eisenia andrei

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. 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.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

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

This second edition cancels and replaces the first edition (ISO 11268-1:1993), which has been technically revised.

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

— *Part 1: Determination of acute toxicity to* Eisenia fetida/Eisenia andrei

— *Part 2: Determination of effects on reproduction of* Eisenia fetida/Eisenia andrei

— *Part 3: Guidance on the determination of effects in field situations*

Introduction

Ecotoxicological test systems are applied to obtain information about the effects of contaminants in soil and are proposed to complement conventional chemical analysis (see ISO 15799 [33] and ISO 17616 [34]). ISO 15799 includes a list and short characterization of recommended and standardized test systems, and ISO 17616 gives guidance on the choice and evaluation of the bioassays. Aquatic test systems with soil eluate are applied to obtain information about the fraction of contaminants potentially reaching the groundwater by the water path (retention function of soils), whereas terrestrial test systems are used to assess the habitat function of soils. As standardized test systems using earthworms as indicator organisms for the habitat function of soil, an acute test for survival and a chronic test for reproduction are available.

This part of ISO 11268 describes a method that is based on the determination of the acute toxicity of contaminated soils to adult earthworms of the species *Eisenia fetida* (Savigny 1826) and *Eisenia andrei* (André 1963). Optionally, the method can be used for testing chemicals added to a standard soil (e.g. artificial soil) for their acute toxic potential to earthworms. Finally, information is provided on how to use this method for testing chemicals under tropical conditions (see Annex A).

Eisenia fetida and *Eisenia andrei* are considered to be representatives of soil fauna and earthworms in particular. Background information on the ecology of earthworms and their use in ecotoxicological testing is available. Other species, e.g. *Aporrectodea caliginosa*, *Lumbricus rubellus* and *Lumbricus terrestris*, have also been used as test organisms. These or other species have not been proven to be more sensitive in general, and the database and experience in testing soils is small [15][16][23].

This part of ISO 11268 has been drawn up taking into consideration test procedures adopted by the Organization for Economic Cooperation and Development [26][27] and by the European Union [9].

Soil quality — Effects of pollutants on earthworms —

Part 1:
Determination of acute toxicity to *Eisenia fetida/Eisenia andrei*

WARNING — Contaminated soils may contain unknown mixtures of toxic, mutagenic, or otherwise harmful chemicals or infectious microorganisms. Occupational health risks may arise from dust or evaporated chemicals during handling and incubation. Precautions should be taken to avoid skin contact.

1 Scope

This part of ISO 11268 specifies one of the methods for evaluating the habitat function of soils and determining the acute toxicity of soil contaminants and chemicals to *Eisenia fetida*/*Eisenia andrei* by dermal and alimentary uptake. It is applicable to soils and soil materials of unknown quality, e.g. from contaminated sites, amended soils, soils after remediation, agricultural or other sites concerned, and waste materials.

Effects of substances are assessed using a standard soil, preferably a defined artificial soil substrate. For contaminated soils, the effects on survival are determined in the test soil and in a control soil. According to the objective of the study, the control and dilution substrate (dilution series of contaminated soil) should be either an uncontaminated soil comparable to the soil sample to be tested (reference soil) or a standard soil (e.g. artificial soil).

Information is provided on how to use this method for testing chemicals under temperate as well as under tropical conditions.

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 substances or contaminants during the test.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 10381-6, *Soil quality — Sampling — Part 6: Guidance on the collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory*

ISO 10390, *Soil quality — Determination of pH*

ISO 10694, *Soil quality — Determination of organic and total carbon after dry combustion (elementary analysis)*

ISO 11260, *Soil quality — Determination of effective cation exchange capacity and base saturation level using barium chloride solution*

ISO 11277, *Soil quality — Determination of particle size distribution in mineral soil material — Method by sieving and sedimentation*

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

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
contaminant
substance or agent present in the soil as a result of human activity

[ISO 15176:2002]

3.2
survival
percentage of living worms at the end of the test period

3.3
mortality
percentage of dead or missing worms at the end of the test period

3.4
LC_{50}
lethal concentration
median lethal percentage of a test sample in a reference or a standard control soil, or concentration of a substance in the test sample, which kills 50 % of the test animals within the test period

NOTE The LC_{50} is expressed as a percentage of test-soil dry mass per test-mixture dry mass.

3.5
limit test
single concentration test consisting of at least four replicates each, the test sample without any dilution or the highest concentration of test substance mixed into the control soil and the control

3.6
lowest observed effect rate (LOER) or effect concentration (LOEC)
lowest tested percentage of a test sample in a control soil or concentration of a substance at which a statistically significant effect is observed

NOTE The LOEC is expressed as a percentage of test-soil dry mass per test-mixture dry mass. All test mixtures above the LOEC have a harmful effect equal to or greater than that observed at the LOEC. If this condition cannot be satisfied, an explanation should be given for how the LOEC and **NOEC** (3.7) have been selected.

3.7
NOEC
no observed effect concentration
test soil percentage immediately below the LOEC or, highest tested concentration of a test substance which, when compared to the control, has no statistically significant lethal or other effect such as mass alteration (error probability: $p < 0{,}05$)

NOTE The NOEC is expressed as a percentage of test-soil dry mass per test-mixture dry mass.

3.8
reference soil
uncontaminated site-specific soil (e.g. collected in the vicinity of a contaminated site) with similar properties (nutrient concentrations, pH, organic carbon content and texture) to the test soil

3.9
standard soil
field-collected soil or artificial soil whose main properties (e.g. pH, texture, organic matter content) are within a known range

EXAMPLE Euro-Soils [21], artificial soil [26] LUFA Standard Soil [24].

NOTE The properties of standard soils can differ from those of the test soil.

3.10
control soil
reference or standard soil used as a control and as a medium for preparing dilution series with test samples or a reference substance which fulfils the validity criteria

NOTE In the case of natural soil, it is advisable to demonstrate its suitability for a test and for achieving the test validity criteria before using the soil in a definitive test.

3.11
test mixture
mixture of contaminated soil or the test substance with a **control soil** (3.10)

NOTE Test mixtures are given in percentage of contaminated soil based on soil dry mass.

3.12
test mixture ratio
ratio of test soil to control soil in a test mixture

NOTE Different ratios may be applied in a dilution series to establish a dose-response relationship.

4 Principle

The percent mortality of adult earthworms (species: *Eisenia fetida* or *Eisenia andrei*) exposed to the test soil are compared to those observed for samples exposed to a control soil. If appropriate, effects based on exposure to a dilution range of contaminated soil or range of concentrations of a test substance are determined after seven days and 14 days. Test mixtures are prepared at the start of the test and are not renewed within the test period.

The results obtained from the tests are compared with a control and are used to determine the dilutions or concentrations which cause no effects on biomass and survival (NOEC) and the mortality of 50 % of earthworms (LC_{50}, 14 days).

The test is conducted in two steps:

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

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

If the preliminary test shows no mortality, a limit test (see 7.1.3) may be performed as the definitive test.

NOTE The use of a reference soil is an essential requirement to demonstrate the present status of the test population, and to avoid misinterpretation of results.

5 Reagents and material

5.1 Biological material, consists of adult earthworms of the species *Eisenia fetida* or *Eisenia andrei* [12][17][19] at least three months old, with a clitellum and a wet mass between 300 mg and 600 mg (*E. fetida*) and between 250 mg and 600 mg (*E. andrei*).

Select worms used for the test to form, as far as is practicable, a homogeneous population from the standpoint of size and mass. Worms should preferably be selected from a synchronized culture with a relatively homogeneous age structure. Before the test, wash them with potable water.

NOTE An example of culturing *Eisenia fetida*/*Eisenia andrei* is given in Annex B.

5.2 Test sample, which may consist of field-collected soil or control soil amended by the test substance.

5.2.1 Field-collected soils, soil or waste materials

The sample(s) can be field-collected soil from an industrial, agricultural or other site of concern, or waste materials (e.g. dredged material, municipal sludge from a wastewater treatment plant, composed material, or manure) under consideration for possible land disposal.

Test samples shall be sieved by 4 mm mesh and thoroughly mixed. If necessary, soil may be air-dried without heating before sieving. Storage of test samples should be as short as possible. Store the soil in accordance with ISO 10381-6 using containers that minimize losses of soil contaminants by volatilization and sorption to the container walls. Soil pH should not be corrected as it can influence bioavailability of soil contaminants.

For interpretation of test results, the following characteristics shall be determined for each soil sampled from a field site:

a) pH in accordance with ISO 10390,

b) texture (sand, loam, silt) in accordance with ISO 11277,

c) water content in accordance with ISO 11465,

d) water holding capacity according to Annex C,

e) cationic exchange capacity in accordance with ISO 11260,

f) organic carbon in accordance with ISO 10694.

According to the objective of the study, the control and dilution substrate (dilution series of contaminated soil) should be either an uncontaminated soil comparable to the soil sample (reference soil) to be tested or standard soil, preferably the artificial soil substrate.

NOTE It is important to measure the water holding capacity of all mixtures used in the test.

5.2.2 Control soil, either a) reference soil (3.8) or b) standard soil (3.9) that allows the presence of earthworms.

a) If reference soils from uncontaminated areas near a contaminated site are available, they should be treated and characterized like the test samples. If a toxic contamination or unusual soil properties cannot be ruled out, standard control soils should be preferred.

b) For testing the effects of substances mixed into soil or making dilutions of the test sample, standard soils shall be used to prepare the test sample. The properties of the field-collected standard soil shall be reported.

The substrate called artificial soil can be used as a standard soil and has the following composition:

	Percentage expressed on a 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 sizes 0,05 mm to 0,2 mm)	69 %

Approximately 0,3 % to 1,0 % calcium carbonate ($CaCO_3$, pulverized, analytical grade) are necessary to get a pH of 6,0 ± 0,5.

NOTE 1 Taking the properties of highly non-polar [log $K_{ow} > 2$, where K_{ow} is the partitioning coefficient (octanol/water)] or ionizing substances into account, 5 % of peat have proven to be sufficient for maintaining the desired structure of the artificial soil.

NOTE 2 It has been demonstrated that *Eisenia fetida* can comply with the validity criteria, even as regards reproduction, when tested in field soils with lower organic carbon content (e.g. 2,7 %) [17], and experience shows that this can be achieved in artificial soil with 5 % peat. It is therefore not necessary, before using such a soil in a definitive test, to demonstrate the suitability of the artificial soil in complying with the validity criteria, unless the peat contents lowered more than specified above [27].

Prepare the artificial soil at least three days prior to starting the test, by mixing the dry constituents listed above thoroughly in a large-scale laboratory mixer. A portion of the deionized water required is added while mixing is continued. Allowance should be made for any water that is used for introducing the test substance into the soil. The amount of calcium carbonate required can vary, depending on the properties of the individual batch of sphagnum peat and should be determined by measuring sub-samples immediately before the test. Store the mixed artificial soil at room temperature for at least two days to equilibrate acidity. To determine pH and the maximum water holding capacity, the dry artificial soil is pre-moistened one or two days before starting the test by adding deionized water to obtain approximately half of the required final water content of 40 % to 60 % of the maximum water holding capacity.

The total water holding capacity is determined according to Annex C; the pH is determined according to ISO 10390.

5.2.3 Boric acid, as a reference substance (see Annex D).

6 Apparatus

Usual laboratory equipment and the following.

6.1 Test containers, made of glass or another chemically inert material, of about one to two litres in capacity, should be used. The containers should have a cross-sectional area of approximately 200 cm^2 so that a moist substrate depth of about 5 cm to 6 cm is achieved when 500 g dry mass of substrate are added. 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 earthworms from escaping (e.g. by using a tape to fix the cover).

6.2 Apparatus to determine the dry mass of the substrate, in accordance with ISO 11465.

6.3 Large-scale laboratory mixer, for the preparation of the test sample (5.2).

6.4 Precision balance, with an accuracy of at least 1 mg.

6.5 Polyethylene-membrane, perforated with small holes allowing exchanges between the sample and the atmosphere.

6.6 Test environment.

6.6.1 Enclosure, capable of being controlled at a temperature of (20 ± 2) °C.

6.6.2 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.

7 Procedure

7.1 Experimental design

7.1.1 General

A sample of field-collected test soil can be tested at a single concentration (typically 100 %) or evaluated for toxicity in a multi-concentration test, whereby a series of dilutions are prepared by mixing measured quantities with a control soil (5.2.2). When testing substances a series of concentrations is prepared by mixing quantities