
**Soil quality — Effects of pollutants on
earthworms —**

Part 2:

**Determination of effects on reproduction
of *Eisenia fetida*/*Eisenia andrei***

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*Qualité du sol — Effets des polluants vis-à-vis des vers de terre —
Partie 2: Détermination des effets sur la reproduction 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 11268-2 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 11268-2:1998), 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*

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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 ^[34] and ISO 17616 ^[35]). 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 sublethal effects of contaminated soils on 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 standard soils (e.g. artificial soil) for their sublethal hazard 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 ^{[16][17]}.

This part of ISO 11268 has been drawn up taking into consideration test procedures adopted by the Organization for Economic Cooperation and Development ^{[27][28]} and by the European Union ^[11].

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Soil quality — Effects of pollutants on earthworms —

Part 2: Determination of effects on reproduction of *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 effects of soil contaminants and chemicals on the reproduction of *Eisenia fetida*/*Eisenia andrei* by dermal and alimentary uptake. This chronic test 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 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 persistence of the substance 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 11268-1, *Soil quality — Effects of pollutants on earthworms — Part 1: Determination of acute toxicity to Eisenia fetida/Eisenia andrei*

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

growth

increase in biomass (i.e. the fresh mass of organisms)

NOTE It is expressed as a percentage of the fresh mass of organisms at the start of the test.

3.3

reproduction

mean number of offspring per test vessel after eight weeks' incubation under the specified test conditions

3.4

ER_x (effective rate) or EC_x (effective concentration)

x % effect rate or concentration of the test sample or test substance at which reproduction is reduced by x % compared to the control

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

NOER (no observed effective rate) or NOEC (no observed effect concentration)

test soil percentage immediately below the LOER/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 reduced reproduction or 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 [11], artificial soil [27], LUFA standard soil [23].

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 percent 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 effects on reproduction 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. In addition, observations on growth and survival of adult earthworms are recorded. Test mixtures are prepared at the start of the test and are not renewed within the test period.

After four weeks, adult worms are removed from the test containers and effects on mortality and biomass are measured by counting and weighing. The effect on reproduction as the definitive end point is measured by counting the number of offspring hatched from the cocoons after an additional period of four weeks. The results obtained from the tests are compared with a control soil or, if appropriate, are used to determine the dilutions or concentrations which cause no effects on biomass, mortality and reproduction (NOER/NOEC) and the dilution (concentration) resulting in x % reduction of juveniles hatched from cocoons compared to the control (ER_x/EC_x , 56 d), respectively.

If testing a dilution or concentration series, all test dilutions/concentrations above the LOER/LOEC shall have a harmful effect equal to, or greater than, that observed at the LOER/LOEC. Where there is no prior knowledge of the dilution/concentration of the test soil/test substance likely to have an effect, then it is useful to conduct the test in two steps:

- a preliminary test carried out, in accordance with ISO 11268-1, to give an indication of the effect dilution/concentration and of the dilution/concentration giving no mortality (NOER/NOEC). Dilutions/concentrations to be used in the definitive test can then be selected.
- the definitive test to determine sublethal effects of (dilutions of) contaminated soil or the concentration of a chemical which, when evenly mixed into the standard soil, causes no significant effects on numbers of offspring hatched from cocoons compared with the control (NOER/NOEC), and the lowest concentration causing effects (LOER/LOEC).

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* [15], [19], [20], which are between two months and one year 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 age, 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.

Condition the selected worms for one day to seven days in standard or control soil before use. The food, which is also used as a food source in the test (see 5.3), shall be given in sufficient amount (see 7.4).

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

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:

- pH in accordance with ISO 10390, [ISO 11268-2:2012](https://standards.iteh.ai/catalog/standards/sist/2134989e-39f8-47a8-89d9-1fda4cc74098/iso-11268-2-2012)
- texture (sand, loam, silt) in accordance with ISO 11277
- water content in accordance with ISO 11465,
- water holding capacity according to Annex C,
- cationic exchange capacity in accordance with ISO 11260,
- organic carbon in accordance with ISO 10694.

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 $6,0 \pm 0,5$.

NOTE 1 Taking the properties of highly non-polar ($\log K_{ow} > 2$, where K_{ow} is the octanol/water coefficient) 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 %) [18], 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 [28].

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 mixture into the soil. The amount of calcium carbonate 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 G; the pH is determined according to ISO 10390.

5.3 Feeding, any food of a quality shown to be suitable for at least maintaining worm mass during the test is considered acceptable. Experience has shown that oatmeal, mashed potato powder [20], cow or horse manure is a suitable food. Checks should be made to ensure that cows or horses from which manure is obtained are not subject to medication or treatment with substances, such as growth promoters, nematicides or similar veterinary products that could adversely affect the worms during the test. Self-collected cow manure is recommended, since experience has shown that commercially available cow manure used as garden fertilizer can have adverse effects on the worms. The manure should be air-dried, finely ground and pasteurized before use.

Each fresh batch of food should be fed to a non-test worm culture before use in a test, to ensure that it is of suitable quality. Growth and cocoon production should not be reduced compared to worms kept in a substrate that does not contain the new batch of food (conditions as described in OECD 207 [27]).

5.4 Boric acid, as a reference substance.

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 cm² 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).