INTERNATIONAL STANDARD

ISO 11268-2

Third edition
2023-01

Soil quality — Effects of pollutants on earthworms —

Part 2: Determination of effects on reproduction of *Eisenia fetida/Eisenia andrei* and other earthworm species

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 *et d'autres espèces de vers de terre*

Reference number
ISO 11268-2:2023(E)

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

Published in Switzerland

Contents

Page

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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 190, *Soil quality,* Subcommittee SC 4, *Biological characterization,* in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 444, *Environmental characterization,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This third edition cancels and replaces the second edition (ISO 11268-2:2012), which has been technically revised.

The main changes are as follows:

— modification of the concentration for the reference substance (boric acid);

— inclusion of alternative species of earthworms – *Dendrodrilus rubidus, Aporrectodea caliginosa* – in informative annexes; information on their taxonomy and ecology as well as their specific testing requirements have also been added.

A list of all the parts in the ISO 11268 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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 and ISO 17616). 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.

This document 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 or test soil under tropical conditions (see Annex A).

Eisenia fetida and *Eisenia andrei* are considered to be representatives of soil fauna and earthworms in particular in temperate regions. Background information on their earthworm ecology and their use in ecotoxicological testing is available. However, these species do not occur regularly in agricultural lands (crop sites and grasslands) or forests in these regions. In addition, they are not representative of boreal or tropical regions. Therefore, other species such as *Dendrodilus rubidus* (an epigeic litter inhabitant in boreal regions) and *Aporrectodea caliginosa* (an endogeic mineral dweller in temperate regions) have been added as potential alternative test species (see Annexes B and C). These alternative earthworm species have been used as ecotoxicological test species for some time, however, testing experience has been limited to specific countries.

This document has been drawn up taking into consideration test procedures adopted by the Organization for Economic Cooperation and Development[45][46] and by the European Union[29].

Soil quality — Effects of pollutants on earthworms —

Part 2: Determination of effects on reproduction of *Eisenia fetida/Eisenia andrei* and other earthworm species

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 document 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.

This method is designed mainly for determining the effects of soil contaminants and chemicals on the reproduction of *Eisenia fetida/Eisenia andrei*. Technical information is also provided on how to use *Eisenia fetida/andrei* for testing chemicals under tropical conditions (see Annex A). Finally, this method also includes technical information on how to use it with other environmentally relevant earthworm species: e.g. *Dendrodrilus rubidus* and *Aporrectodea caliginosa* (see Annexes B and C).

This method does not apply to substances for which the air/soil partition coefficient is greater than one, or to substances with vapour pressure exceeding 300 Pa, at 25 °C. This method does not take into account the persistence of the substance during the test.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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 10390, *Soil, treated biowaste and sludge – 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*

ISO 18400-206, *Soil quality — Sampling — Part 206: Collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory*

3 Terms and definitions

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

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

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

[SOURCE: ISO 15176:2019, 3.2.6]

3.2
growth
increase in biomass (i.e. the fresh mass of organisms)

Note 1 to entry: 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 container after eight weeks' incubation under the specified test conditions

3.4
ER_x
EC_x
effective rate
effective concentration
x % effect rate or concentration of the test sample or test substance at which *reproduction* (3.3) 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* (3.10) and the control

3.6
LOER
LOEC
lowest observed effect rate
lowest observed effect concentration
lowest tested percentage of a test sample in a *control soil* (3.10) or concentration of a substance at which a statistically significant effect is observed

Note 1 to entry: The LOEC is expressed as a percentage of test-soil dry mass per test-mixture dry mass. All *test mixtures* (3.11) 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
NOEC
no observed effective rate
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* (3.3) or mass alteration (error probability: $p < 0,05$)

Note 1 to entry: 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,[29] artificial soil,[46] LUFA standard soil[40].

Note 1 to entry: The properties of standard soils can differ from those of the test soil.

3.10
control soil
reference (3.8) or *standard soil* (3.9) 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 1 to entry: 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 1 to entry: Test mixtures are given in percent of contaminated soil based on soil dry mass.

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 recommended 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;

— a 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 A reference soil is used to demonstrate the appropriate status of the test population, and to avoid misinterpretation of results.

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).

Alternative species of earthworms and their respective requirements are added as specific annexes in this document:

— *Dendrodrilus rubidus* (see Annex B);

— *Aporrectodea caliginosa* (see Annex C).

5 Reagents and material

5.1 Biological material, consists of adult earthworms of the species *Eisenia fetida* or *Eisenia andrei*[32][36][37], which are between three 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*). It is recommended to check the identity of the strain used to avoid species misidentifications. DNA barcoding described in ISO 21286 is suitable for that purpose.

NOTE 1 During the ring test performed to validate ISO 21286, only 17 of the 28 laboratories involved (61 %) provided correct identification of their laboratory culture of compost worms. Most laboratories with wrong or unknown assignments had *E. andrei* in culture, or a mixture of both species[57].

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 2 An example of culturing *Eisenia fetida*/*Eisenia andrei* is given in Annex D.

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 5.4), 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.

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, composted 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 18400-206 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;

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

— water content in accordance with ISO 11465;

— water holding capacity according to Annex E;

— cationic exchange capacity in accordance with ISO 11260;

— organic carbon in accordance with ISO 10694.

The water holding capacity of all mixtures used in the test should also be measured.

5.3 Control soil, either a) reference soil or b) standard soil 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 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 for adult survival and juvenile reproduction when tested in field soils with lower organic carbon content (e.g. 2,7 %),[20] 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 content is lower than 5 %[30].

Prepare the artificial soil at least three days prior to starting the test, by mixing the dry constituents 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 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 the 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 water holding capacity is determined according to Annex E; the pH is determined according to ISO 10390.

5.4 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,[37] 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 can 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 [45]).

5.5 Boric acid, as reference substance.

6 Apparatus

The usual laboratory equipment and the following shall be used.

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. When testing substances, a series of concentrations is prepared by

mixing quantities of the test substance with a standard soil (e.g. artificial soil). The concentrations are expressed in milligrams of test substance per kilogram of dried control soil.

Depending on the knowledge of relevant response levels, a preliminary test may precede the definitive test. Each definitive test consists of a series of soil mixtures (treatments).

7.1.2 Preliminary test

A preliminary test to find the range of mixture ratios affecting earthworms is optional, e.g. 0 %, 1 %, 5 %, 25 %, 50 %, 75 %, 100 %, or of the test substance, e.g. 0 mg/kg, 1 mg/kg, 10 mg/kg, 100 mg/kg and 1 000 mg/kg. The preliminary test is conducted without replication.

When no effects are observed, even at 100 % contaminated soil or at concentrations of 1 000 mg test substance/kg standard soil (dry mass), the definitive test can be designed as a limit test.

7.1.3 Definitive test

The design of the definitive test depends on the test objectives. Typically, the habitat properties of samples of a field-collected test soil are characterized by comparing the biological effects found in the test soil(s) with those found in the control soil (single-concentration tests). If a reference soil to be used as a control is not available or not appropriate due to toxicity or atypical physicochemical characteristics, effects are compared to a standard soil instead. If a reference soil is available to be used as a control soil, it is recommended that a standard soil exhibiting a typical known response be included, and that the results be used to judge the validity and acceptability of the test.[35] Results found for the standard soil assist in distinguishing contaminant effects from non-contaminant effects caused by soil physicochemical properties of the test soil and/or the control soil.

If, for characterization purposes, a test design including a dilution series is required, three designs are possible [the concentrations shall be spaced by a factor not exceeding two (2)].

— For the NOEC/NOER approach, at least five concentrations in a geometric series should be used. Four replicates for each concentration plus eight controls are recommended.

— For the EC_x approach, 12 concentrations should be used. Two replicates for each concentration plus six control replicates are recommended. The spacing factor can be variable: smaller at low concentrations and larger at high concentrations.

— For the mixed approach, six to eight concentrations in a geometric series should be used. Four replicates for each concentration plus eight control replicates are recommended. This mixed approach allows a NOEC as well as an EC estimate to be calculated.

A limit test can be sufficient if, in the preliminary test, no toxic effect was observed. In the limit test, only the test soil without any dilution and the control shall be tested with at least four replicates each.

7.2 Preparation of test mixtures

7.2.1 Testing of contaminated soil

Mix the test soil with the reference soil or the standard soil thoroughly (either manually or by using a hand mixer) according to the selected dilution range. Check the homogeneity of the mixture visually. The total mass of the test soil and the reference soil or the standard soil shall be 500 g to 600 g (dry mass) in each test container (6.1). Wet the test mixture with deionized water to reach an appropriate water content of usually 40 % to 60 % of the total water holding capacity determined according to Annex E. In some cases, e.g. when testing waste materials, higher percentages are required. A rough check of the soil moisture content can be obtained by gently squeezing the soil in the hand; if the moisture content is correct, small drops of water should appear between the fingers.

Determine the pH for each test mixture (one container per concentration) according to ISO 10390 at the beginning and end of the test (when acid or basic substances are tested, do not adjust the pH).