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)

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Published in Switzerland

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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.

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).

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

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

7.2.2 Testing substances added to the control soil

Control soil is used to prepare the test sample. For each test container (6.1), the mass of the substrate used shall be 500 g (dry mass). Add substances to the control soil and mix thoroughly.

For the introduction of 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 or a portion of it required to wet the soil substrate for the replicates of one concentration in order to meet the requirements of 5.3. Mix it thoroughly with the soil substrate before introducing it into the test containers.

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 standard soil and the water and mix it thoroughly before introducing it into the test containers.

Ultrasonic dispersion, organic solvents, emulsifiers or dispersants can 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 5.3) and the quantity of the test substance required to obtain the desired concentration. Add that mixture to the remainder of the standard soil and the water and mix thoroughly before introducing it into the test containers.

— Mix the test substance into the standard soil 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 1 000 mg/kg mass of test sample. Proceed simultaneously with all replicates per concentration and the control(s) required according to the selected approach.

Determine the pH for each test sample (one container per concentration) according to ISO 10390 at the beginning and end of the test.

7.2.3 Preparation of control container

The control container contains the control soil wetted with deionized water to generally reach 40 % to 60 % of the total water holding capacity (determined according to Annex E) and consistent with the rate used in the soil to be tested.

Prepare one control container for the preliminary test and at least four control containers for the definitive test.

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 7.2.2), use an additional control prepared with solvent but without the test substance. Cover the containers as indicated in 6.1.

7.3 Addition of the earthworms

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

Attention should be paid to the variation of mean biomass between containers. It is recommended that this variation does not exceed 150 mg.

Cover the containers as indicated in 6.1 and place them in the test enclosure (6.6.1).

7.4 Test conditions and measurements

One day after addition of the worms, spread 5 g per test container of air-dried finely ground food source (5.4) on the soil surface and moisten with potable water (about 5 ml to 10 ml per container). Feed once a week during the test period. If food consumption is low, reduce feeding to a minimum to avoid fungal growth or moulding. Record feeding activity 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. It is recommended that the water content does not differ by more than 10 % from that of the beginning of the test.

Keep the adult worms over a period of four weeks in the test sample. 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 four weeks in the test environment (6.6) 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 F gives examples of two suitable methods, including one which allows counting of cocoons.

7.5 Reference substance

The NOEC and/or the EC_x of a reference substance shall be determined to provide assurance that the laboratory test conditions are adequate and to verify that the response of the test organism does not change statistically over time. It is advisable to test a reference substance at least twice a year or, when testing is carried out in a lower frequency, in parallel to the determination of the toxicity of a test substance.

Boric acid is recommended as a reference substance. If the compound is mixed into the substrate, effects on reproduction should be observed (α = 0,05, where α is the level of significance) at concentrations of between 200 mg and 600 mg of boric acid per kilogram dry mass of substrate (see Annex G).

The test report on the performance of the reference compound shall be completed periodically and if the test conditions have changed.

8 Calculation and expression of results

8.1 Calculation

For each dilution or concentration, determine the percent mortality, the percent loss/increase in biomass of the adults after four weeks, and the number of offspring produced after another period of four weeks.

8.2 Expression of results

A graphical presentation of the mean values of the end points, including standard deviation of the measured values against the test soil(s), control soil(s) or the selected series of soil mixture ratios, should be prepared. This comparison or curve gives an impression of the quality of effects and their magnitudes. Express the mixture ratio as based on soil dry mass.

If dilution or concentration series were performed, indicate:

— in the EC_x approach, the percent soil mixture based on dry mass or in milligrams per kilogram of dried soil substrate, the median percent dilution of contaminated soil or median concentration of the test substance, which reduces the number of juvenile worms to 50 % (EC_{50}) compared to the control within the test period, or

— in the NOEC approach, the soil mixture ratio 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 and reduction of reproduction ($p < 0,05$).

9 Validity of the test

The results are considered to be valid if:

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

— the coefficient of variation of reproduction in the control does not exceed 30 %;

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

Annex G provides a summary of the performance of the method.

10 Statistical analysis

10.1 General

Most of the test methods with sub-lethal end points, e.g. growth, reproduction, involve quantitative effects, e.g. measuring the weight of the organisms or counting juvenile worms. Quantal effects may also be measured in the same test, such as mortality after four weeks exposure.

NOTE The results of quantal (binary) effects can express only two states (yes/no).

Guidance given here for statistical evaluation of test results aims to make the investigator aware of problems that can arise as a consequence of a test design selected. Computer programs do not necessarily guard against violations of rules that can cause erroneous analyses. It is strongly recommended to look for more information in specific guidance documents (e.g. Reference [24]) or contacting a statistician.

10.2 Single-concentration tests

Quantitative single-concentration tests (e.g. effects on reproduction or the biomass development) have different statistical methods. For sampling at several locations with field replication, ANOVA would be

a first step if results were suitable. If the null hypothesis of no difference was rejected, analysis would proceed to one of several multiple-comparison tests[29].

An example of a single-concentration test for quantitative effects can be counting juvenile worms as the end point of effects on reproduction or measuring the average biomass of earthworms after exposure to a sample of undiluted contaminated soil, compared to numbers of juvenile worms or biomass of earthworms exposed to a reference or standard soil. If there was only one sample tested, and one control sample, without any replicates, results cannot be compared by any statistical test. In a quantitative test with replication for the test soil and for the control soil, a standard *t*-test would be suitable for statistical analysis.

Analysis of variance (ANOVA) involving multiple comparisons of end-point data derived for undiluted test soils, including field replicates of field-collected soil from more than one sampling location, is commonly used for statistical interpretation of the significance of quantitative findings (e.g. biomass) from soil toxicity tests. This is a hypothesis-testing approach, and is subject to appreciable weaknesses.[24] The parametric analyses (e.g. ANOVA and multiple comparisons) for such data assume that the data are normally distributed, that the treatments are independent, and that the variance is homogeneous among the different treatments. These assumptions shall be tested. If the data satisfy these assumptions, analysis may proceed. If not, data may be transformed and tested again. As parametric tests are reasonably robust in the face of moderate deviations from normality and equality of variance, parametric analysis should proceed, even if moderate nonconformity continues after transformation[24] [29]. Data which fail to satisfy either test might be transformed to meet the requirements. If the original or transformed data do not satisfy either test for distribution of data, then analysis by nonparametric methods shall be carried out.

10.3 Multi-concentration tests

10.3.1 Preliminary test

If a clear dose-response is obvious, EC_x-values can be estimated by using regression techniques like logistic regression function or probit analysis. In other cases, the effect range should be determined by expert knowledge.

10.3.2 Definitive test

A point estimate (ER_x/EC_x approach) is recommended as the best quantitative end point. This is usually a specific degree of reduction in performance compared to the control. Linear and nonlinear regression methods are widely applied for statistical analysis. Operators should be aware of being able to understand the judgements in selecting appropriate mathematical models.

Hypothesis testing (NOEC approach) is commonly used to identify dilutions (concentrations) with significant effects compared to the control. As this method has many flaws, it is not recommended for future use.

Therefore, in cases where various dilutions (concentrations) of each sample of field-collected soil with negative control soil are tested, preference is given to the EC_x approach or, if required by legislation, the NOEC approach for data analysis:

a) ER_x/EC_x (effect concentration) approach

The ER_x/EC_x approach can only be used if a clear dose-response relationship is found. Wherever possible, the R^2 (where R is the regression coefficient) should be 0,7 or higher and the test mixtures used should encompass 20 % to 80 % effects. If these requirements are not fulfilled, expert knowledge is necessary for the interpretation of the test results.

To compute an ER_x/EC_x value, the treatment means are used for regression analysis after an appropriate dose-response function has been found (e.g. probit or logistic function). A desired ER_x/EC_x is obtained by inserting a value corresponding to x % of the control mean into the equation found by regression

analysis. Since EC_{50} values have smaller confidence limits compared with smaller effect concentrations (e.g. ER/EC_{20}), it is recommended that the ER/EC_{50} values be determined.

b) NOEC (no-observed-effect-concentration) approach

First of all, a statistical analysis of the homogeneity of the variances shall be made, e.g. by using Cochran's test. With homogeneous data, an appropriate statistical analysis, e.g. a "One-Way Analysis of Variance (ANOVA)", followed by a one-sided Dunnett test (α = 0,05), should be performed. If the homogeneity requirement is not fulfilled, it is recommended evaluating whether an appropriate transformation of the data can solve the problem. Otherwise nonparametric methods, e.g. the *U*-test by Mann and Whitney or the Bonferroni *U*-test can be used.

If a limit test has been performed and the pre-requisites (normality, homogeneity) of parametric test procedures are fulfilled, the Student-*t*-test, otherwise the unequal-variance *t*-test (Welch *t*-test) or a nonparametric test, such as the Mann-Whitney-U-test may be used.

In any case, the results of the statistical evaluation shall be biologically interpreted.

11 Test report

The test report shall include the following information:

a) a reference to this document, i.e. ISO 11268-2:2023;

b) the results expressed in accordance with 8.2;

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

d) the origin of the field soil used as a control and dilution soil (if appropriate);

e) any soil treatment prior to the test;

f) the method of preparation of the test sample, including the solvent used for a water-insoluble substance;

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

h) the conditions of the test environment;

i) the method used for calculation of EC_{50};

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

k) the total masses (e.g. range and mean) 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 four weeks;

l) the number of offspring per test container at the end of the test;

m) depending on the statistical approach selected, a list of the lowest concentration causing significant effects (LOEC), the highest concentration causing no observed effects (NOEC), EC_{10} and EC_{50} for the growth reduction and the method used for calculation;

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

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

p) all details not specified in this document or considered optional, as well as any effect which may have affected the results.

Annex A
(informative)

Determination of the chronic toxicity of chemicals on *Eisenia fetida/Eisenia andrei* under tropical conditions

A.1 General

Most of the data used in the risk assessment of pesticides applied in tropical countries are generated in North America or Europe with temperate species (e.g. maximum temperature around 20 °C and sandy to loamy soils). However, an extrapolation of temperate data to tropical conditions without a scientific basis can lead to erroneous results. Therefore, data used for the environmental risk assessment of chemicals in the tropics should be gained under tropical conditions (e.g. temperature of 26 °C to 28 °C and clayey soils). This annex describes the modifications to this guideline which are necessary in order to perform this test under tropical conditions (i.e. most of South America, Central Africa and Southern Asia). The information provided is based on recent work performed in Brazil[30][44] and Sri Lanka[17][18].

Basically, the test is performed as described in the main body of this document. Therefore, in the following, only those issues which shall be modified are listed (e.g. no changes are necessary as regards the test design, reference testing or validity criteria).

A.2 Modifications to Clauses 5, 6 and Annex D

A.2.1 Concerning Clause 5

5.1 Biological material

The same species (*Eisenia fetida* or *Eisenia andrei*) shall be used. However, the starting culture should originate from a tropical site, i.e. or with animals that are already adapted to a higher (e.g. 26 °C to 28 °C) temperature, for example. The compost worm has invaded many tropical soils following European settlements[6].

5.2.2 Artificial soil

When preparing artificial soil, the amount of organic matter (10 % or, if changed in general, 5 %) used originally shall be replaced by coir dust or composted coco peat[18][32][43]. Other changes are not necessary. Coir is the name given to the fibrous material that constitutes the thick mesocarp (middle layer) of the coconut fruit. Coconut peels extracted from green fruits shall be air-dried and finely ground. Before use in soil substrates, wet the resulting coconut powder and store it for a complete composting process for at least 30 days; after the fermentation activity ceases, air-dry and sieve the material. In contrast to sphagnum peat, the material already has a neutral pH (6,0 to 6,5), thus no further use of calcium carbonate is necessary. Due to the efforts needed to prepare coir material, it is recommended buying coir material that is already composted (e.g. in garden shops).

Do not buy material which – like sphagnum peat in temperate countries – has been amended with fertilizers. If in doubt about whether the material is composted or not, wet it for a few days in order to see whether fermentation is still occurring or not.

A.2.2 Concerning 6.6

6.6.1 Enclosure

According to the available literature[18],[19], tests can be performed at 27 °C ± 1 °C.

A.2.3 Concerning Annex D

Breeding should be performed at the same temperature as used in the test (i.e. 26 °C to 28 °C). The breeding substrate can vary according to local sources, but in general a 50:50 mixture of horse or cattle manure and coir/composted coco peat should be used.

NOTE Despite the fact that these species can be tested both under temperate as well as tropical conditions, in both cases, this compost-inhabiting species is not ecologically relevant in soil ecosystems, independently where these ecosystems are located.

Annex B
(informative)

Culturing and testing procedures for assessing the effects of contaminants on juvenile production using the earthworm, *Dendrodilus rubidus*

B.1 General

Eisenia spp. are not typical representatives suited to the assessment of northern temperate regions, such as boreal forest and taiga regions, thus questioning their use and ecological relevance.[50] Based on recommendations following an extensive review of potential test species suited to boreal, taiga and northern eco-zone regions,[50] *Dendrodrilus rubidus* was short-listed as a suitable earthworm test species. Canadian research efforts have produced a standardized test method for this species[22][25][26], which also has successful application to the assessment of contaminated site soils. A comparison between boreal (including *D. rubidus*) and standard test species demonstrated increased sensitivity for boreal species upon exposure to soil contaminated with petroleum hydrocarbons and excess salinity[49].

The test method using this species follows the majority of the ISO 11268 principles; however, test specifics have been modified slightly to suit the test species' specific requirements for the assessment of boreal and taiga soils. In addition, the test is suited to the assessment of particular forest soil horizons in contrast to test samples derived from arable regions, whereby the soil may be tilled, thus mixing horizons.[49] The performance of *D. rubidus* was assessed in different soil types collected from the boreal and taiga regions within Canada; the soils varied in their physical and chemical characteristics, with pH ranging from 4,2 to 7,7[22].

B.2 Geographic distribution and ecology

D. rubidus is a Holarctic species, widely distributed in most parts of Europe, Northern Asia including Japan as well as North America. By human settlers, *D. rubidus* has been introduced to South Asia, Central and South America and even on Pacific islands. It is typical for the boreal regions of Canada. The species typically occurs in woodlands, living in the moss layer and in particular under loose bark, most often in moist litter and at wet places.[21] The species is tolerant of acidic conditions associated with boreal soils, but sandy soils and sites with very low pH are usually avoided.[38] Finally, it can be found in composts or under manure, i.e. at places with accumulations of organic matter[55].

B.3 Culturing of *Dendrodrilus rubidus*

Dendrodrilus rubidus are dark red in colour and have a yellowish-orange tip (posterior 3 to 8 segments). They vary in length from 20 mm to 100 mm, and are approximately 2 mm to 5 mm in diameter.[54] The provenance of the species used for test method development was near Lethbridge, Alberta, Canada (49°69.17'N, 112°82.40'W).[49] [1)]

The test species are cultured in an artificial soil substrate (pH 7 ± 0,5) comprised of artificial and organic triple mix soil, peat, and $CaCO_3$ (for pH adjustment). The cultures are kept in plastic boxes, approximately 20 cm wide, 35 cm long and 12 cm deep. The cultures are fed biweekly (~ 8 g) with

1) Cultures of this test species may be acquired through contact with Environment and Climate Change Canada (methods@ec.gc.ca). This information is given for the convenience of users of this document and does not constitute an endorsement by ISO.

Magic® Worm Food (MWF)[2]. Alternate food sources (e.g. organic mixed grains or cooked oatmeal) may be used so long as culture health is unaffected.[22] Temperature in the culture area should be maintained at a constant 20 °C ± 2 °C (instantaneous 20 °C ± 3 °C). Cultures are monitored regularly for any illnesses (i.e. discoloration, lesions, pinching), contaminating organisms (e.g. mites, collembolan), and clitellated adults. The life cycle of the *D. rubidus* has been estimated at 75 to 76 days (confirmed by Reference [22] and Reference [20]). Cultures are refreshed as required (i.e. lack of clitellated adults or presence of significant mould).

The recommended food source, MWF, is composed of a mixture of shelled corn, oats, wheat middling's, lime, alfalfa meal, and soybean meal, ground to a fine powder; the specific proportion of these ingredients is proprietary. Upon reception of a new batch, MWF is dried in an oven at 60 °C for a minimum of 48 h; this ensures that potential biological contaminants (e.g. mites, enchytraeids) are eradicated. The dried MWF is then stored in a freezer (−30 °C to −10 °C) until required for testing or culturing purposes. Laboratory testing has shown that this food is effective at maintaining and increasing adult weights, as well as maintaining steady levels of juvenile production in control soils. Comparison studies performed with oatmeal as an alternative food source demonstrated significantly higher adult wet weight, general health (i.e. visual assessment of mobility, size, colour), and juvenile production in MWF fed replicates compared to oatmeal.

B.4 Procedure

B.4.1 General

The 56-day static (non-renewal) test is used to examine the effects of prolonged exposure of adult lab-cultured *D. rubidus* and, subsequent impacts on juvenile production, to one or more samples or concentrations of contaminated or potentially contaminated soil. This test can be performed utilizing one or more samples of contaminated or potentially contaminated soil or one or more concentrations of chemicals or chemical products added to control (or other) soil.

The test is initiated by placing four adult *D. rubidus* in each of a series of 250-ml test containers (five replicates per treatment and control) containing an approximate wet weight of soil equivalent to a volume of 200 ml. After 28 days of exposure, adults are removed from the test containers, and parameters of survival and clitellation are examined. Tests continue for an additional 28 days, after which point the test ends, and the number of live juveniles is assessed in each replicate and treatment, with ensuing statistical analyses as appropriate.

B.4.2 Biological material

Laboratory cultured *D. rubidus* should be used for this test, prior to which the species shall be taxonomically confirmed using appropriate methods. Organisms used in testing shall be sexually mature, which is signalled by the presence of a clitellum. The wet mass of individuals used for testing must range from 50 mg to 300 mg. In addition, organisms being used for testing should be visually assessed for good health (i.e. not pinched, no visible sores, no abnormal colouration) and mobility (i.e. actively moving and responsive to touch), and ideally, selected organisms should be similar in size.

B.4.3 Test containers

Test containers made of glass, or another chemically inert material, of 250 ml capacity should be used (e.g., 250-ml glass mason jars, cross-sectional area of 28,27 cm^2 and a depth of 9,5 cm). The opening of the test container is covered with 50 µm Nitex™[3] (nylon) mesh, cut into small squares (~ 12 cm by 12 cm). The mesh is placed over the opening and screwed tight to the lid with metal rings, ensuring

2) Magic® Worm Food (MWF) is the trademark of a product supplied by MAGIC® Products Inc. USA. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

3) Nitex is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

there are no folds or areas of separation between the edge of the ring and the mesh. The mesh is then covered with the metal caps of the mason jars. The mesh prevents adults and juveniles from escaping the test containers, as the test species may crawl through small perforations (e.g. size of a pin-hole). The Nitex™ mesh enables gas exchange and placing the metal cap overtop of the mesh helps regulate the soil moisture content throughout the test duration.

B.4.4 Test environment (conditions)

Conditions within incubator enclosures adhere to this test standard [i.e. (20 ± 2) °C] (see 6.6.1), with comparable light conditions (see 6.6.2). For boreal soils, the relative humidity is increased to >70 % to ensure that soils do not dry out during the test, which can affect test results.

B.4.5 Replicate requirement

Reproduction tests are conducted following the mixed approach described in the main portion of this method (6 to 8 concentrations in a geometric series); however, five replicates are used for both the control and treated soils, and each replicate contains four adult earthworms. Also, the different soil horizons collected from a site are treated as individual soils, and are not added together (e.g. layered) in a test container.

B.4.6 Soil requirement

The volume of soil must be sufficient to satisfy the replicate requirement (e.g. ~ 200 ml volume of soil per test container), minor sample loss during preparation, and physical and chemical measurements (e.g. moisture content, pH and/or chemical analysis).

B.4.7 Addition of test species to test containers

Before addition of test organisms, test food (1 g of MWF) is added to each test container. A number in excess of those required for the test should be collected from a culture and stored in a holding container (e.g. plastic bin) lined with moist paper towel. Collected earthworms should be active, healthy (i.e. no lesions, not pinching) and similar in size. *D. rubidus* produce a sheath of mucous like material that shall be gently removed prior to weighing with either a pair of blunt forceps or a paint brush. All worms are then weighed and transferred to a test container.

B.4.8 Feeding and soil moisture

Organisms are fed every two weeks throughout the test (i.e. days 0, 14, 28, and 42) with 1 g of MWF. A small depression is made in the centre of the soil in the test container (with a spoon or scoopula), the MWF is added to this hole, and a small amount of deionized water is used to hydrate the MWF. The hydrated food is then covered with a small amount of soil and lightly compressed. Care should be taken to not disturb the test organisms while making the small depression within the test soil (e.g. using a stainless-steel scoopula). Do not remove any old or uneaten food. Assess soil moisture, and if needed, moisten the surface of the soil with deionized water.

B.4.9 Test maintenance and measurements

Adults are removed from the test on day 28, and cocoons left to incubate another 28 days. On day 28, empty the contents of a test container onto a wax paper-lined tray. Sort through the test soil and record the number and health of surviving adults. Re-weigh any adults that were weighed at the test start. Any adult appearing dead should be touched on their anterior end, and if there is no response they are considered dead; missing adults are also counted as dead.

The test ends on day 56. Carefully sort through the contents of the tray, separating the clumps of soil, and remove and count all of the juveniles from the test soil. Other observations may include the number of hatched and unhatched cocoons and the presence of mould or other organisms (e.g. mites, collembola, etc.). The juveniles and cocoons of this species are quite small, and due to the production of the mucous sheath (collects soil particles and acts as a form of camouflage), small juveniles can be difficult to find. Experienced technologists should be used for the juvenile recovery step at test end. The

floatation method for collection of juveniles used for other earthworm tests is not recommended due to the small size of juveniles.

B.4.10 Validity of the test

The results are considered to be valid if:

— the percent mortality of the adults observed in the control(s) is ≤10 %;

— the rate of production of juveniles is at least 3 live juveniles per adult in the control(s).

B.4.11 Results and reporting

Calculation and expression of results (Clause 8), statistical analysis (Clause 10) and Test reporting (Clause 11) outlined for this document also apply for reproduction effects testing using *D. rubidus*.

Annex C
(informative)

Culturing and testing procedures for assessing the effects of contaminants on adult survival and cocoon production and hatchability using the earthworm, *Aporrectodea caliginosa* s.s.

C.1 General

Eisenia spp. are absent from agricultural soils[39] and were often found to be less sensitive to pesticides than other earthworm species found in mineral soils.[47] This questions their use and ecological relevance as a reference for a posteriori toxicity tests (after pesticide registration). Based on extensive review and meta-analysis, *Aporrectodea caliginosa* was short-listed as a suitable earthworm test species. *A. caliginosa* is one of the most dominant earthworm species in temperate regions and is highly represented in agroecosystems. Its wide distribution and sensitivity to pesticides makes it a relevant soil dwelling species for a posteriori pesticide risk assessment and more generally in soil-based ecotoxicological tests.

While *E. fetida* remains a suitable test species for initial screening of chemical effects, the use of *A. caliginosa* is relevant in complementing knowledge on the effects of pesticides, especially for pesticides already widely used on crops.

Efforts have been made to standardize test protocols for this species, with successful demonstrations of its use for the assessment of pesticide effects. The test method using this species is based on that developed for *E. fetida*/*E. andrei*. However, it has been slightly modified to suit *A. caliginosa* specific requirements and to assess adult survival and cocoon production and hatchability.

C.2 Geographic distribution and ecology of *A. caliginosa* s.s.

A. caliginosa is found in all temperate zones (i.e. in Europe, America, Asia, Oceania and South Africa). Moreover, it is one of the most abundant species in most soils of temperate zones[9][10][15][16][48]. *A. caliginosa* is numerically dominant in garden and most cultivated fields, including meadows. It lives in the first 15 cm of soil and is highly representative of agricultural soils, which is one of the two most relevant criteria for test organisms (i.e. the representativeness of the ecosystem to protect) according to the European Food Safety Authority.[27] Small individuals are common in the top 7 cm of the soil while larger individuals can go deeper (generally not more than 25 cm depth).

A. caliginosa displays high ecological plasticity and adaptability in agroecosystems[11][55], especially to agricultural practices such as soil tillage[14]. In addition, *A. caliginosa* is able to survive in soils with low organic matter (i.e. 1,4 % organic carbon[41]) and moisture content (i.e. at least three weeks under drought conditions[41]). They are present in moderately acid to alkaline soils (pH 5,9 to 11,1).

A. caliginosa plays several key ecological roles such as nutrient cycling, i.e. increasing nitrogen flux, lowering the C/N ratio[41][51] and enhancing nutrient availability for plants and microorganisms[52][53]. It can also increase microbial biomass[56] and, being an endogenic species, its relatively high burrowing activity can have a positive impact on water infiltration/discharge[28][42] and on soil aeration[31].

C.3 Taxonomy and morphological description

Aporrectodea caliginosa belongs to Annelida: Oligochaeta: Lumbricidae. The classification of this species is controversial and has changed several times. *A. caliginosa* s.s. (Savigny, 1826) is a complex of species that historically gathers three sub-species: *A. tuberculata*, considered as a species by Gates,[33]

A. trapezoides (Dugés, 1828) and *A. nocturna* (Evans, 1946). This classification has evolved since the last decades, and differs between studies and authors. For instance, Bouché[11] divided *A. caliginosa* into two species with another genus name: *Nicodrilus caliginosus* composed of three subspecies (i.e. *N. c. caliginosus*, *N. c. alternisetosus* and *N. c. meridionalis*) and *Nicodrilus nocturnus*.

Sims and Gerard[55] explained that these four taxa belong to a single species with phenotypic varieties: *A. caliginosa* s.s., *A. caliginosa* var. *trapezoides*, *A. caliginosa* var. *tuberculata*, *A. caliginosa* var. *nocturna*. More recently, Briones[13] proposed to consider two subspecies: *A. caliginosa caliginosa* and *A. calignosa trapezoides*. Finally, recent molecular analyses suggested that the *A. calignosa* complex of species is composed of at least five valid species: *A. caliginosa* s.s, *A. tuberculata*, *A. nocturna*, *A. trapezoides* and *A. longa*[48].

To summarize, *Aporrectodea caliginosa* that is proposed in this annex can be found under different names in the scientific literature: *Aporrectodea caliginosa* s.s (syn. *turgida*), *Aporrectodea caliginosa caliginosa* (or *Allolobophora caliginosa caliginosa*) and *Nicodrilus caliginosus caliginosus*.

According to Bouché,[11] *Aporrectodea caliginosa* s.s are non-pigmented earthworms. According to Sims and Gerard,[55] they are coloured pale pink anteriorly, whitish grey posteriorly. They vary in length from 60 mm to 85 mm, and in width between 3,5 mm and 4,5 mm. Their weight is comprised between 200 mg to 1 200 mg. They have a cylindrical body with 120 to 150 segments for Sims and Gerard [55] and 130 to 165 for Bouché[11].

C.4 Culturing of *Aporrectodea caliginosa* s.s.

The culture parameters are summarized in Table C.1. For maintenance and development of *A. caliginosa* s.s, a field loamy soil (pH 6 to 7) is more appropriate than standardized soils such as OECD and LUFA 2.2 soil.[12] Soil characteristics can be different as those presented in Table C.1 (e.g. natural soil type) but the culture can be less efficient. The different steps for the establishment of a culture of *A. caliginosa* s.s. in laboratory are summarized in Reference [7].

The cultures are kept in 1 L plastic containers, approximately 10 cm wide, 13 cm long and 8 cm deep. The supplied food should be animal dung (preferably from cattle or horse) previously frozen and defrosted twice, dried, milled (<1 mm), rewetted and mixed into the soil. For optimal growth, it is suggested to provide 2 g to 3 g and 4 g to 6 g of dried food per individual per month for juveniles and adults, respectively. The soil moisture shall be adjusted to 25 % to 30 % (or 60 % to 70 % of the water holding capacity), and the temperature must be around 15 °C (use of a temperature-controlled room or incubator is advisable). Cultures are monitored regularly for any illnesses (i.e. discoloration, lesions, pinching), contaminating organisms (e.g. mites, collembolan), and clitellated adults. The life cycle duration of *A. caliginosa* s.s. is between 4 and 6 months. The life span is unfortunately unknown but can be more than two years under laboratory conditions.

Table C.1 — Guidelines for sustained and optimized culture of *A. caliginosa* s.s

Culture parameters	
Soil type	Natural loamy/clay soil (pre-treated to remove macro- and meso-invertebrates
Soil depth (cm)	>3 cm
pH	6 to 7
Soil moisture (%)	25 % to 30 % or 60 % to 70 % of the water holding capacity
Food	Dried and rewetted animal dung (cattle or horse)
Food amount for juveniles < 300 mg (ind-1 month -1)	2 g to 3 g
Food amount for adults and juveniles > 300 mg (ind-1 month -1)	4 g to 6 g
Food location	Mixed into the soil
Food particle size (mm)	< 1
Temperature (°C)	15 ± 1
Light	24 h dark
Vessel type	Sealed, opaque, preferably plastic with ventilation holes in the lid
NOTE Reference [7] adapted from Reference [39].	

C.5 Procedures

C.5.1 Biological material

Laboratory cultured *A. caliginosa* s.s should be used for this test, prior to which the species shall be taxonomically confirmed using appropriate methods. The lab culture must be initiated with individuals sampled *in natura* but close to each other (in the same field). After morphological identification at the species level, some individuals should be barcoded to confirm that they belong to the same species. Organisms used in testing shall be sexually mature (presence of a clitellum), with a wet mass between 300 mg and 1 000 mg.

As far as possible, earthworms should be of similar size and mass for the test to have a homogenous population. Preferably, earthworms should be selected from a synchronized culture with a relatively homogeneous age structure. Before the test, wash them with potable water.

C.5.2 Test substrate and containers

The substrate for the tests must be a natural soil. Different soil types can be suitable, as far as they allow to fulfil the validity criteria, with preference for loamy/clay soils and pH within the range of 6 to 7). A positive correlation was reported in several studies between earthworm abundance and soil clay content while the number and mass of *A. caliginosa* were found to be negatively correlated with the sand content of soil (see Reference [12]). The soil must be adjusted to a moisture content of 25 % to 30 % (or 60 % to 70 % of the water holding capacity). The soil needs to be pre-treated (sieved or crushed to 2 min to 3 mm). Regarding the test duration (28 days to assess adult survival and cocoon production), animal dung (preferably from cattle or horse) should be added. To eliminate possible parasites, the dung can be frozen and defrosted twice, dried, milled (<1 mm), rewetted and mixed into the soil. For optimal growth, it is suggested to provide 4 g to 6 g of dried food per individual per month.

The containers can be (as for culturing) 1 l plastic containers, approximately 10 cm wide, 13 cm long and 8 cm deep.

C.5.3 Test environment (conditions)

Enclosure, capable of being controlled at a temperature of (15 ± 2) °C for the assessment of adult survival and cocoon production (28 days, in test substrate) and, (20 ± 2) °C for the assessment of cocoon hatchability (28 days, in Petri dishes).

Light cycle: 24 h dark.

C.5.4 Addition of the earthworms

Ten (10) individuals per vessel of 500 g dry soil should be used in the test.

C.5.5 Measurements

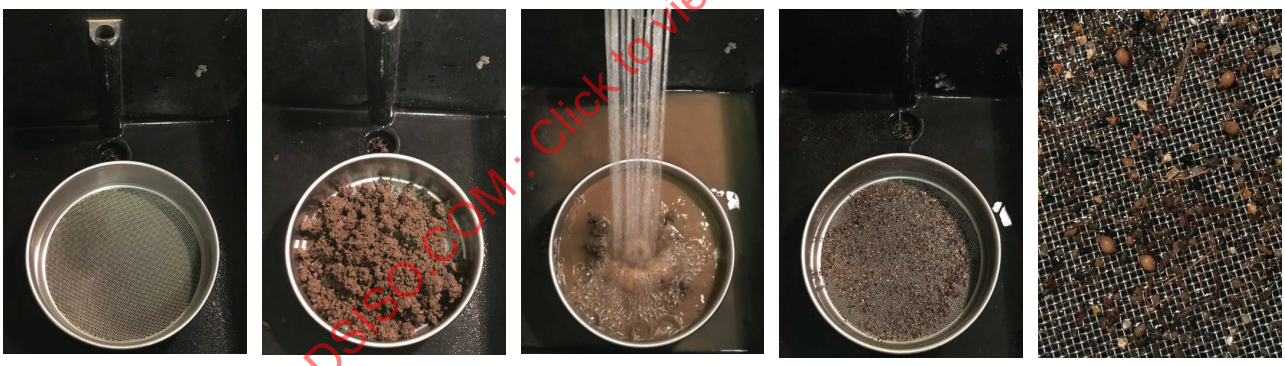
Adults are removed from the test on day 28. Cocoons are left in the soil for another period of 28 days at 20 °C (optimal temperature for the hatching of cocoons, see Reference [7]) and juvenile production is estimated through direct count on day 56. Alternatively, to measure cocoon production and hatchability (i.e. number and percentage of hatched cocoons, number of juveniles per cocoon), cocoons can also be collected on day 28 by wet sieving the soil through a 1 mm mesh size. This allows removal of the soil but retains the cocoons in the sieve (Figure C.1). The collected cocoons can then be placed onto wet filter paper in petri dishes[34] and incubated at 20 °C until they hatch (monitor every 2 days).

C.5.6 Validity of the test

The results are considered to be valid if:

— the percent mortality of the adults observed in the control(s) is ≤ 10 %;

— the rate of production of juveniles is at least 20 per control container.



A B C D E

Key

A empty sieve
B soil from test vessel
C wet sieving
D/E cocoons retrieval

Figure C.1 — Wet sieving method for cocoon retrieving (1 mm mesh size)

Annex D
(informative)

Culturing of *Eisenia fetida* and *Eisenia andrei*

This annex gives instructions on the breeding of test organisms that are used for the determination of reproductive toxicity. Breeding should preferably be carried out in a climatic chamber at (20 ± 2) °C. At this temperature and with the provision of sufficient food, the worms become mature after about two months to three months.

To obtain worms of standard age and size (mass), it is best to start the culture with cocoons. Once the culture has been established, it is maintained by placing adult worms in a breeding box with fresh substrate for 14 days to 28 days to allow further cocoons to be produced. The adults are then removed and the juveniles produced from the cocoons used as the basis for the next culture. The worms are fed continuously with animal waste and transferred into fresh substrate from time to time. The worms hatched from the cocoons are used for testing when they are between three months and 12 months old and considered to be adults.

Both species can be cultured in a wide range of animal wastes. The recommended breeding medium is a 50:50 mixture of horse or cattle manure and peat. 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 manure obtained from an organic source is recommended, since experience has shown that commercially available manure used as garden fertilizer can have adverse effects on the worms. The medium should have a pH value of approximately 6 to 7 (adjusted with calcium carbonate), a low ionic conductivity (less than 6 mS or 0,5 % salt concentration) and should not be contaminated excessively with ammonia or animal urine. The substrate should be moist but not too wet. Breeding boxes of 10 l to 50 l capacity are suitable.

Worms can be considered healthy if they move through the substrate, do not try to leave the substrate and reproduce continuously. Substrate exhaustion is indicated by worms moving very slowly and having a yellow posterior end. In this case, the provision of fresh substrate and/or a reduction in stocking density is recommended.

Annex E
(normative)

Determination of water holding capacity

E.1 General

The following method has been found to be appropriate for laboratory samples of test soils and standard soils.

E.2 Apparatus

E.2.1 **Glass tube**, approximately 20 mm to 50 mm in diameter and at least 100 mm in length.

E.2.2 **Water bath**, at room temperature.

E.2.3 **Filter paper.**

E.2.4 **Drying oven**, set to (105 ± 5) °C.

E.2.5 **Balance**, capable of weighing to an accuracy of ±0,1 g.

E.3 Method

Plug the bottom of the tube with filter paper and, after filling with the control soil or test sample to a depth of 5 cm to 7 cm, place the tube on a rack in a water bath. Gradually submerge the tube until the water level is above the top of the soil but below the upper edge of the tube. Leave the substrate sample in the water for about 3 h.

Since not all water absorbed by the substrate capillary can be retained, the tube containing the sample should be placed for a period of 2 h on very wet finely ground quartz sand for draining. The same quartz sand as is used for the soil substrate is satisfactory.

Weigh the sample, dry it to constant mass at 105 °C and reweigh it.

E.4 Calculation of the water holding capacity (C_{WH})

$$C_{WH} = \frac{m_S - m_T - m_D}{m_D} \times 100 \qquad \text{(E.1)}$$

where

C_{WH} is the water holding capacity, in percentage of dry mass, %;

m_S is the mass of the water-saturated substrate plus the mass of the tube plus the mass of the filter paper;

m_T is the tare (mass of tube plus mass of filter paper);

m_D is the dry mass of the substrate.

Annex F
(informative)

Techniques for counting juvenile worms hatched from cocoons

As hand-sorting is very time consuming, two faster methods for extracting the offspring from the soil substrate are recommended.

a) The test containers are placed in a water bath at a temperature of 50 °C to 60 °C. After a period of about 20 min, offspring appear at the substrate surface and can easily be collected and counted. The efficiency of the method should be checked. If offspring are collected by hand from the test sample, the inspection should be repeated.

b) If the peat and the cow dung were ground to a fine powder, the test soil can be washed through a sieve using the following method,[10] which can be used to determine the number of cocoons, as well as juvenile worms if required.

   — Two 0,5 mm sieves (diameter 30 cm) are placed on top of each other. The contents of a container are washed through these sieves with a powerful stream of tap water, leaving the young worms and cocoons mainly on the upper sieve. It is important to note that the whole surface of the upper sieve should be kept wet during this operation so that the juvenile worms float on a film of water, thereby preventing them from creeping through the sieve pores. The best results are obtained when a showerhead is used. After the soil substrate is washed through the sieve, juveniles and cocoons are rinsed from the sieve into a bowl.

   — Empty cocoons float on the water surface. Young worms sink to the bottom of the bowl. When the standing water is poured off, the young worms can be transferred to a petri dish with a little water. Using a needle or a pair of tweezers, worms can be picked out of the water one by one and counted.

Annex G
(informative)

Experience with performance criteria of the test

G.1 General

A summary of the performance of the method based on the results of 30 studies carried out at nine different testing facilities is given in Tables G.1 to G.3.

G.2 Validity criteria

Table G.1 — Validity criteria and percentage of tests fulfilling them

Criterion	Limit value	Accordance in % of studies (n = 30)
Mortality of adults in the control	≤ 10 %	100
Rate of production of juveniles per control container	≥ 30	83
Coefficient of variation of juveniles in the control	≤ 30 %	67 (33 between 30 and 50)

G.3 Sensitivity of the test system

The sensitivity of the test system is measured by counting all the results of different tests which show a significant difference in numbers of juveniles compared to a control. For a better comparison the percent decrease is divided into eight classes. Table G.2 shows the results for all tests and results with different dosages fulfilling the validity criteria.

Table G.2 shows that a reduction of 30 % to 40 % in the number of juveniles from the control is detected by the test system successfully.

Table G.2 — Sensitivity of the test system based on 45 results of 19 tests fulfilling the validity criteria

Percentage reduction of juveniles compared to control	Number of results	Significant results in % of class results (Williams-test)
< 5	6	0
5 – 10	2	0
10 – 20	10	30
20 – 30	5	60
30 – 40	4	100
40 – 50	2	100
50–60	1	100
> 60	**15**	**100**

G.4 Results of tests using boric acid as reference substance

Reproduction and biomass of *Eisenia fetida* are given for the control and concentrations of boric acid tested [mg/kg soil (dry mass)]. Effects of the concentrations applied are expressed by the mean and the standard deviation (SD) (mean ± SD) of the number of juveniles, as well as the percentage of the respective rate of reproduction compared to the control (= 100 %) after 56 days of test duration (n = 2 to 6, where n is the number of samples) and the mean adult biomass [%] after 28 days. The EC_{50} for reproduction was calculated as 484 mg boric acid/kg soil dry mass.

Table G.3 — Example of an earthworm reproduction test with boric acid as reference substance

Concentration mg/kg soil (dry mass)	**Number of juveniles** Mean ± SD	**% Control**	**Mean adult biomass** %
Control	357 ± 45,2	100	149
75,0	320 ± 7,07	89,6	155
100	431 ± 80,6	121	160
133	454 ± 15,6	127	161
178	407 ± 19,1	114	156
237	446 ± 50,9	125	157
316	418 ± 75,0	117	167
422	298 ± 21,2	83,5	157
562	52,5 ± 9,19	14,7	154
750	1,00 ± 1,41	0,3	143
1 000	**0,00 ± 0,00**	**0,00**	**114**
NOTE See Reference [8].			

In a literature review (see ISO 15799), geometrical mean EC_{50} values for the two species *Eisenia fetida* and *Eisenia andrei* were calculated as 588 mg boric acid/kg soil dry mass and 420 mg boric acid/kg soil dry mass, respectively. It should be noted that some of these results were formally performed according to OECD [46] and Environment Canada [23] guidelines. However, due to the small differences between these guidelines, the results are considered to be comparable.

The information compiled here (see Table G.3 and Table G4) shows that the reference substance boric acid reduces reproduction by 50 % at about 200 mg/kg to 600 mg/kg dry mass soil.

Table G.4 — Compilation of results obtained in different laboratories on Boric acid

Species	Test method	Substrate	Response	Endpoint	Duration	Value (mg/kg soil dry weight)	95 % confidence interval	Source (Laboratory)	Date	Comments	Reference
Eisenia fetida	OECD 222 / ISO 11268-2	Artificial soil (5 % peat)	Reproduction (number of juveniles)	EC 50	56 days	**206**	179 - 231	INERIS	February 2013		
Eisenia fetida	ISO 11268-2	Artificial soil (5 % peat)	Reproduction (number of juveniles)	EC 50	56 days	**412**	375 - 475	Eurofins France	March 2015		
Eisenia fetida	ISO 11268-2	Artificial soil (5 % peat)	Reproduction (number of juveniles)	EC 50	56 days	**456**	402 - 487	Eurofins France	March 2016		
Eisenia fetida	ISO 11268-2	Artificial soil (5 % peat)	Reproduction (number of juveniles)	EC 50	56 days	**486**	412 - 525	Eurofins France	March 2017		
Eisenia fetida	ISO 11268-2	Artificial soil (5 % peat)	Reproduction (number of juveniles)	EC 50	56 days	**512**	474 - 571	Eurofins France	January 2018		
Eisenia fetida	ISO 11268-2	Artificial soil (5 % peat)	Reproduction (number of juveniles)	EC 50	56 days	**553**	514 - 596	Eurofins France	March 2019		
Eisenia fetida	OECD 222	Artificial soil (5 % peat)	Reproduction (number of juveniles)	EC 50	56 days	**484**	465 - 504	ECT	2009?	Result also mentioned in ECHA Boric acid dossier	Becker et al (2011)
Eisenia fetida	OECD 222 / ISO 11268-2	Artificial soil (5 % peat)	Reproduction (number of juveniles)	EC 50	56 days	**259**	234 - 286	ECT	2016		ECHA 6,6'-di-tert-butyl-2,2'-methylenedi-p-cresol dossier
Eisenia fetida	OECD 222 / ISO 11268-2	Artificial soil ?	Reproduction (number of juveniles)	EC50	56 days	**325**	315 - 350	ECT	2017		ECHA Hexamethylene-diamine dossier
Eisenia fetida	OECD 222	Artificial soil (10% peat)	Reproduction (number of juveniles)	EC50	56 days	**354**	Not Determinable	ECT	2017		

Table G.4 *(continued)*

Species	Test method	Substrate	Response	Endpoint	Duration	Value (mg/kg soil dry weight)	95 % confidence interval	Source (Laboratory)	Date	Comments	Reference
Eisenia fetida	OECD 222	Artificial soil (10 % peat)	Reproduction (number of juveniles)	EC50	56 days	**330**	289 - 383	ECT	2018		
Eisenia fetida	OECD 222	Artificial soil (10 % peat)	Reproduction (number of juveniles)	EC50	56 days	**250**	213 - 293	ECT	2019		
Eisenia fetida	ISO 11268-2	Artificial soil (10 % peat)	Reproduction (number of juveniles)	EC 50	56 days	**529**	481 - 585	Eurofins France	October 2014		
Eisenia fetida	ISO 11268-2	Artificial soil (10 % peat)	Reproduction (number of juveniles)	EC 50	56 days	**> 300**	NA	SGS France	April 2019	Limit test: 300 mg/kg. < 10 % inhibition	
Eisenia fetida	ISO 11268-2	Artificial soil (10 % peat)	Reproduction (number of juveniles)	EC 50	56 days	**315**	274-355	INERIS	August 2019		
Eisenia fetida	ISO 11268-2	LUFA 2.3	Reproduction (number of juveniles)	EC 50	56 days	**< 400**	NA	SGS France	February 2018	Tested concentrations: 400 and 600 mg/kg. 100 % inhibition at 400 mg/kg	
Eisenia fetida	ISO 11268-2	LUFA 2.3	Reproduction (number of juveniles)	EC 50	56 days	**< 400**	NA	SGS France	December 2018	Tested concentrations: 400 and 600 mg/kg. 100 % inhibition at 400 mg/kg	