BS EN ISO 11268-2:2015

BSI Standards Publication

Soil quality — Effects of pollutants on earthworms

Part 2: Determination of effects on reproduction of Eisenia fetida/Eisenia andrei (ISO 11268-2:2012)

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National foreword

This British Standard is the UK implementation of EN ISO 11268-2:2015. It is identical to ISO 11268-2:2012. It supersedes BS 7755-4.2.2:1998 which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee EH/4, Soil quality.

A list of organizations represented on this committee can be obtained on request to its secretary.

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Bodenbeschaffenheit - Wirkungen von Schadstoffen auf Regenwürmer - Teil 2: Bestimmung der Wirkung auf die Reproduktionsleistung von Eisenia fetida/Eisenia andrei (ISO 11268-2:2012)

This European Standard was approved by CEN on 6 August 2015.

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EUROPÄISCHES KOMITEE FÜR NORMUNG

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European foreword

The text of ISO 11268-2:2012 has been prepared by Technical Committee ISO/TC 190 “Soil quality” of the International Organization for Standardization (ISO) and has been taken over as EN ISO 11268-2:2015 by Technical Committee CEN/TC 345 “Characterization of soils” the secretariat of which is held by NEN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by February 2016, and conflicting national standards shall be withdrawn at the latest by February 2016.

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Endorsement notice

The text of ISO 11268-2:2012 has been approved by CEN as EN ISO 11268-2:2015 without any modification.

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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ISO 11268-2 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

This second edition cancels and replaces the first edition (ISO 11268-2:1998), which has been technically revised.

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

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

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

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

Introduction

Ecotoxicological test systems are applied to obtain information about the effects of contaminants in soil and are proposed to complement conventional chemical analysis (see ISO 15799 [34] and ISO 17616 [35]). ISO 15799 includes a list and short characterization of recommended and standardized test systems and ISO 17616 gives guidance on the choice and evaluation of the bioassays. Aquatic test systems with soil eluate are applied to obtain information about the fraction of contaminants potentially reaching the groundwater by the water path (retention function of soils), whereas terrestrial test systems are used to assess the habitat function of soils. As standardized test systems using earthworms as indicator organisms for the habitat function of soil, an acute test for survival and a chronic test for reproduction are available.

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

Eisenia fetida and *Eisenia andrei* are considered to be representatives of soil fauna and earthworms in particular. Background information on the ecology of earthworms and their use in ecotoxicological testing is available. Other species, e.g. *Aporrectodea caliginosa*, *Lumbricus rubellus* and *Lumbricus terrestris*, have also been used as test organisms. These or other species have not been proven to be more sensitive in general, and the database and experience in testing soils is small [16][17].

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

INTERNATIONAL STANDARD **ISO 11268-2:2012(E)**

Soil quality — Effects of pollutants on earthworms —

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

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

1 Scope

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

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

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

The method is not applicable to volatile substances, i.e. substances for which H (Henry's constant) or the air/water partition coefficient is greater than 1, or for which the vapour pressure exceeds 0,013 3 Pa at 25 °C.

This method does not take into account the persistence of the substance during the test.

2 Normative references

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

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

ISO 10390, *Soil quality — Determination of pH*

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

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

ISO 11268-1, *Soil quality — Effects of pollutants on earthworms — Part 1: Determination of acute toxicity to* Eisenia fetida/Eisenia andrei

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

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

3 Terms and definitions

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

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

[ISO 15176:2002]

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

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

3.3
reproduction
mean number of offspring per test vessel after eight weeks' incubation under the specified test conditions

3.4
ER_x (effective rate) or EC_x (effective concentration)
x % effect rate or concentration of the test sample or test substance at which reproduction is reduced by x % compared to the control

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

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

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

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

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

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

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

EXAMPLE Euro-Soils [11], artificial soil [27], LUFA standard soil [23].

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

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

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

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

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

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

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

4 Principle

The effects on reproduction of adult earthworms (species: *Eisenia fetida* or *Eisenia andrei*) exposed to the test soil are compared to those observed for samples exposed to a control soil. If appropriate, effects based on exposure to a dilution range of contaminated soil or range of concentrations of a test substance are determined. In addition, observations on growth and survival of adult earthworms are recorded. Test mixtures are prepared at the start of the test and are not renewed within the test period.

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

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

— a preliminary test carried out, in accordance with ISO 11268-1, to give an indication of the effect dilution/concentration and of the dilution/concentration giving no mortality (NOER/NOEC). Dilutions/concentrations to be used in the definitive test can then be selected.

— the definitive test to determine sublethal effects of (dilutions of) contaminated soil or the concentration of a chemical which, when evenly mixed into the standard soil, causes no significant effects on numbers of offspring hatched from cocoons compared with the control (NOER/NOEC), and the lowest concentration causing effects (LOER/LOEC).

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

5 Reagents and material

5.1 Biological material, consists of adult earthworms of the species *Eisenia fetida* or *Eisenia andrei* [15], [19], [20], which are between two months and one year old, with a clitellum, and a wet mass between 300 mg and 600 mg (*E. fetida*) and between 250 mg and 600 mg (*E. andrei*).

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

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

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

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

5.2.1 Field-collected soils, soil or waste materials

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

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

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

— pH in accordance with ISO 10390,

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

— water content in accordance with ISO 11465,

— water holding capacity according to Annex C,

— cationic exchange capacity in accordance with ISO 11260,

— organic carbon in accordance with ISO 10694.

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

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

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

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

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

	Percentage expressed on a dry-mass basis
— Sphagnum peat finely ground and with no visible plant remains	10 %
— Kaolinite clay containing not less than 30 % kaolinite	20 %
— Industrial quartz sand (dominant fine sand with more than 50 % of particle sizes 0,05 mm to 0,2 mm)	69 %

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

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

NOTE 2 It has been demonstrated that *Eisenia fetida* can comply with the validity criteria, even as regards reproduction, when tested in field soils with lower organic carbon content (e.g. 2,7 %) [18], and experience shows that this can be achieved in artificial soil with 5 % peat. It is therefore not necessary, before using such a soil in a definitive test, to demonstrate the suitability of the artificial soil in complying with the validity criteria, unless the peat contents lowered more than specified above [28].

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

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

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

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

5.4 Boric acid, as a reference substance.

6 Apparatus

Usual laboratory equipment and the following.

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

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

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

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

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

6.6 Test environment.

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

6.6.2 Light source (e.g. white fluorescent tubes), capable of delivering a constant light intensity of 400 lx to 800 lx on the containers at a controlled light/dark cycle of between 12 h:12 h and 16 h:8 h.

7 Procedure

7.1 Experimental design

7.1.1 General

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

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 (3.10) (single-concentration tests). If a reference soil (3.8) 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 [18]. 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 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 controls 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 controls are recommended. This mixed approach allows a NOEC as well as an EC_x evaluation.

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 mixture

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 C. 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 (5.2.2) 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 5.2.2. 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 5.2.2) 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 (5.2.2) wetted with deionized water to reach 40 % to 60 % of the total water holding capacity (determined according to Annex C).

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. The range of mean biomass between vessels should not exceed 100 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 (5.3) on the soil surface and moisten with potable water (about 5 ml to 6 ml per container). Feed once a week during the test period. If food consumption is low, reduce feeding to a minimum 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. At the end of the test, the water content shall not differ by more than 10 % from that at the beginning of the test.

Keep the adult worms over a period of 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 D 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, observe the effects on reproduction ($\alpha = 0{,}05$, where α is the level of significance) at concentrations of between 400 mg and 600 mg of boric acid per kilogram dry mass of substrate. 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 %.

See Annex E.

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.

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. as provided by Reference [10]) 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 [11].

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 [10]. 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 [10][11]. 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 ($\alpha = 0{,}05$), 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.

Guidance given here for statistical evaluation of test results aims to make the investigator aware of problems that can arise in 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 seeking additional information in specific guidance documents (e.g. as provided by Reference [10]) or contacting a statistician in any case where a special design is used.

11 Test report

The test report shall include the following information:

a) a reference to this part of ISO 11268;

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, list 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 part of ISO 11268 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 earthworms 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 (i.e. with temperate species). 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. This Annex describes the modifications to this guideline which are necessary in order to perform this test under tropical conditions. The information provided is based on recent work performed in Brazil [13][26] and Sri Lanka [4][5].

Basically, the test is performed as described in the main body of this part of ISO 11268. Therefore, in the following, only those issues which shall be modified are listed (for example, no changes are necessary as regards the test design, reference testing or validity criteria).

A.2 Modifications to Clauses 3, 5, 6 and Annex B

A.2.1 Concerning Clause 3, Terms and definitions

3.13
temperate conditions
⟨soil ecotoxicological testing⟩ abiotic conditions considered to be typical for the temperate regions of the world (i.e. most of Europe and North America)

EXAMPLE Maximum temperature of 20 °C and sandy to loamy soils.

3.14
tropical conditions
⟨soil ecotoxicological testing⟩ abiotic conditions considered to be typical for the tropical regions of the world (i.e most of South America, Central Africa and Southern Asia)

EXAMPLE A higher temperature of 26 °C to 28 °C and clayey soils.

A.2.2 Concerning Clause 5, Reagents and material

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. with animals that are already adapted to a higher temperature, for example. The compost worm has invaded many tropical soils following European settlements [1].

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 [6][15][21]. 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.2.3 Concerning Subclause 6.6, Test environment

6.6.1 Enclosure

According to the available literature, tests can be performed at 26 °C to 28 °C.

A.2.4 Concerning Annex B

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.

Annex B
(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 C
(informative)

Determination of water holding capacity of artificial soil

C.1 General

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

C.2 Apparatus

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

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

C.2.3 **Filter paper**.

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

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

C.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.

C.4 Calculation of the water holding capacity (WHC)

$$\mathrm{WHC} = \frac{m_S - m_T - m_D}{m_D} \times 100 \qquad \text{(C.1)}$$

where

WHC 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 D
(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 [8], 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 E
(informative)

Performance of the method

E.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 E.1 to E.3.

E.2 Validity criteria

Table E.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)

E.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 E.2 shows the results for all tests and results with different dosages fulfilling the validity criteria.

Table E.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 E.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**

E.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 E.3 — Example of an earthworm reproduction test with boric acid as reference substance [2]

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**

In a literature review [3], 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[28] and Environment Canada [9] guidelines. However, due to the small differences between these guidelines, the results are considered to be comparable.

The information compiled here (including Table E.3) shows that the reference substance boric acid reduces reproduction by 50 % at about 400 mg/kg to 600 mg/kg dry mass soil.

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