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Soil quality — Effects of pollutants on earthworms —

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

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

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Foreword

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

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

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

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

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

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

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

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

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

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

Introduction

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

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

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

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

Soil quality — Effects of pollutants on earthworms —

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

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

1 Scope

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

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

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

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

This method does not take into account the possible degradation of the substances or contaminants during the test.

2 Normative references

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

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

ISO 10390, *Soil quality — Determination of pH*

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

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

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

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

3 Terms and definitions

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

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

[ISO 15176:2002]

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4 Principle

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

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

The test is conducted in two steps:

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

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

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

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

5 Reagents and material

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

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

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

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

5.2.1 Field-collected soils, soil or waste materials

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

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

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

a) pH in accordance with ISO 10390,

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

c) water content in accordance with ISO 11465,

d) water holding capacity according to Annex C,

e) cationic exchange capacity in accordance with ISO 11260,

f) organic carbon in accordance with ISO 10694.

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

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

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

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

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

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

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

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

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

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

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

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

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

6 Apparatus

Usual laboratory equipment and the following.

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

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

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

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

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

6.6 Test environment.

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

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

7 Procedure

7.1 Experimental design

7.1.1 General

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

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 test 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 [17]. Results found for the standard soil assist in distinguishing contaminant effects from non-contaminant effects caused by soil physicochemical properties.

If, for characterization purposes, a test design including a dilution series is required, a minimum of five test mixtures shall be prepared. Therefore, a geometric series of mixture rates with a factor not exceeding 2 shall be selected based on the preliminary test. At least four replicates of each treatment are prepared. 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 soil shall be tested with at least four replicates each.

When testing chemicals added to standard soil, perform the definitive test on five concentrations of the test substance, providing a geometric progression between the highest concentration causing no mortality and the lowest concentration causing total mortality. Proceed simultaneously with four replicates per concentration and for a control without the test substance and, if necessary, for another control with solvent, placing each container in the test environment (6.6).

To increase the precision and power of statistical testing when analysing quantitative single-concentration tests (e.g. effects on biomass development), it is recommended that eight replicates be prepared for the control.

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 samples 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 at least four replicates per concentration and the control(s).

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

Place the containers at random in the test enclosure (6.6.1) for 14 days.

After seven days, count the live worms and remove the dead worms if visible (a worm is considered to be dead if it displays no reaction to a pin prick applied to its anterior side). Note the symptoms observed on the animals.

At the end of the test after 14 days, for each container, determine the total number and mass of living worms, the water content in one control container and the pH in one container per test concentration.

7.5 Reference substance

The NOEC and/or the LC_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 used as a reference substance. Significant effects on mortality should be observed between 3 000 mg and 4 500 mg of boric acid per kilogram of artificial soil (dry mass). According to the literature, the mean LC_{50} ($n = 7$, where n is the number of samples) in artificial soil is 3 500 mg of boric acid per kilogram (confidence limits 3 000 mg/kg to 4 500 mg/kg).

8 Calculation and expression of results

8.1 Calculation

For each dilution or concentration, determine the percent mortality obtained in the definitive test. Data are combined for replicate containers at a given concentration.

For tests of acute lethality or other quantal effects, percent effects are transformed to probits or logits allowing the estimation of a straight-line model and limiting the number of parameters to be estimated.

When two consecutive dilutions or concentrations at a ratio less than or equal to 2 (for example 10, 18) give only 0 % and 100 % mortality, the two values are sufficient to indicate the range within which the LC_{50} falls.

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 test mixture ratio, 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 dose response relationship, the percent test mixture based on dry mass or in milligrams per kilogram of dried soil substrate, the median lethal percent dilution of contaminated soil or median lethal concentration of the test substance, which kills 50 % of the test animals within the test period according to the selected model (LC_{50}); or

— in the NOEC approach, the test 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 (NOEC).

9 Validity of the test

The results are considered to be valid, if

— the percent mortality observed in the control is < 10 %, and

— the average loss of biomass of the worms in the control does not exceed 20 %.

10 Statistical analysis

10.1 General

Testing for a statistically significant effect in single concentration quantal tests (e.g. mortality) depends on the type of investigative programme and its design. For a sample from one location with “field” replicates (e.g. a survey of contaminated soil), results can be tested with Fisher’s exact test. For a survey of several locations with field replicates, results can be assessed by logistic regression carried out by, or under supervision of, a statistician; analysis of variance (ANOVA) might sometimes be feasible.

Quantitative single-concentration tests (e.g. effects on 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 [8].

NOTE Guidance given here for statistical evaluation of test results aims to make the investigator aware of problems that may 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 that additional information be sought in specific guidance documents (e.g. as provided by Reference [8]) or to contact a statistician.

10.2 Single-concentration tests

10.2.1 Quantal effects

In a quantal test, each organism either shows an effect or does not show it. The effect is binary. Binary and quantal are synonymous. The effect can be lethal or sublethal (e.g. an earthworm dies or lives or shows an avoidance reaction or does not). Thus tests are based on the proportion of organisms that showed the effect, after exposure to a fixed concentration of test material and a defined period of time. Quantal results follow a binomial distribution, which determines the choice of the appropriate statistical tests.

Mortality is the most common end point in single concentration tests, and the resulting data are quantal. A test might assess mortality of earthworms exposed to full-strength contaminated soil. The choice of the appropriate statistical test depends on the test design. The test design can include testing one sample without replication and a control or using field replicates from a single location, i.e. several samples collected at the same time and place. In both cases, Fisher’s exact test is recommended as a first choice. If single-soil samples from a number of locations were tested at one concentration with a control, no opportunities for statistical testing exist. Such a study would be just exploratory, potentially inducing further sampling and testing with replication. If field replicates were taken, i.e. several samples at each location, useful statistical analysis becomes feasible, even for quantal data at one concentration. A possible approach would be logistic regression. This regression would be “categorical”, i.e. based on control, location 1, location 2, etc., rather than the familiar regression on a continuous independent variable such as concentration. This approach is considered particularly fruitful if a gradient of effect was expected.

10.2.2 Quantitative effects

An example of a single-concentration test for quantitative effects can be measuring the average biomass of earthworms after exposure to a sample of undiluted contaminated soil, compared to the biomass of earthworms exposed to a reference or standard soil. If there was only one sample tested, and one control material, without any replicates, results cannot be compared by any statistical test. In a quantitative test with replication for the test soil (material) 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 (single-concentration test), 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 [8]. 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 [8]. 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 General

For a multi-concentration quantal test, the end point is the Effective Concentration, usually the median Effective Concentration (EC_{50}). Lethal tests are a sub-category, and the usual end point is the median Lethal Concentration (LC_{50}). The exposure time shall be given, e.g. the 14-d-LC_{50}.

10.3.2 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.

10.3.3 Definitive test

In cases where various dilutions (concentrations) of each sample of field-collected soil with negative control soil or clean reference soil are tested, data can be analysed in two ways:

a) Preferably by the EC_x (effect concentration) approach

The 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 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 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. EC_{20}), it is recommended that EC_{50} values be determined.

b) Alternatively, e.g. if required by legislation, by the 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 ($\alpha = 0{,}05$, where α is the level of significance), should be performed. If the homogeneity requirement is not fulfilled, it is recommended to evaluate if an appropriate transformation of the data can solve the problem. Otherwise non-parametric methods, e.g. the U-test by Mann & 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 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 LC_{50};

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

k) the mass (e.g. range and mean) of live worms at the end of the test;

l) if the data are available, the highest concentration causing no observed effects (NOEC);

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

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

o) all details not specified in this part of ISO 11268 or considered as optional, as well as any effect which may have affected the results.

Annex A
(informative)

Determination of the acute toxicity of chemicals (in particular pesticides) on earthworms under tropical test conditions

A.1 General

Most of the data used in the risk assessment of chemicals, in particular 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] [14] [27] 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][13][20]. 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 to buy 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, it shall be wetted 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*

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 to be 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)

Background information on the acute effect of boric acid on earthworms

Background information on the acute effect of boric acid on earthworms is given in Tables D.1 and D.2. See Reference [2].

Table D.1 — Background information on the acute effect of boric acid on earthworms (*Eisenia andrei*) in artificial soil

Test method or guideline (substrate)	Duration	End point	LC_{50}	95 % lower conf. limit	95 % upper conf. limit	Reference
	d		mg boric acid/kg soil dry mass			
EC's Biol. Test method EPS 1/RM/43 2004b, resp. Draft 2002b (artificial soil)	14	adult mortality	3978 3320	3607 3011	4386 3661	[30]
EC's Biol. Test method EPS 1/RM/43 2004b, resp. Draft 2002b (artificial soil)	14	mortality	3503	3113	3942	[30]
EC's Biol. Test method EPS 1/RM/43 2004b, resp. Draft 2002b (artificial soil)	14	adult mortality	3397	3026	3815	[30]
EC's Biol. Test method EPS 1/RM/43 2004b, resp. Draft 2002b (artificial soil)	14	mortality	3524	3127	4014	[28]
EC's Biol. Test method EPS 1/RM/43 2004b (artificial soil)	14	mortality	3236	3020	3467	[18]

Table D.2 — Background information on the acute effect of boric acid on earthworms (*Eisenia andrei*) in natural soil

Test method or guideline (substrate)	Duration	End point	LC_{50}	95 % lower conf. limit	95 % upper conf. limit	Reference
	d		mg boric acid/kg soil dry mass			
EC's Biol. Test method EPS 1/RM/43 2004b, resp. Draft 2002b (Alberta Black Chernozem)	14	adult mortality	3938 3245	3577 2954	4334 3565	[30]

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