INTERNATIONAL STANDARD

ISO 17512-1

First edition
2008-12-15

Soil quality — Avoidance test for determining the quality of soils and effects of chemicals on behaviour —

Part 1: Test with earthworms (*Eisenia fetida* and *Eisenia andrei*)

Qualité du sol — Essai d'évitement pour contrôler la qualité des sols et les effets des produits chimiques sur le comportement —

*Partie 1: Essai avec des vers de terre (*Eisenia fetida *et* Eisenia andrei*)*

Reference number
ISO 17512-1:2008(E)

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

Published in Switzerland

Contents

Page

Foreword

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

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 17512-1 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

ISO 17512 consists of the following parts, under the general title *Soil quality — Avoidance test for determining the quality of soils and effects of chemicals on behaviour*:

— *Part 1: Test with earthworms (*Eisenia fetida *and* Eisenia andrei*)*

The following part is under preparation:

— *Part 2: Test with collembolans (*Folsomia candida*)*

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). ISO 15799 includes a list and short characterisation of recommended and standardised test systems. 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 standardised test systems, a mortality test (ISO 11268-1) and a reproduction test (ISO 11268-2) exist to investigate the habitat function of a soil with respect to earthworms as representatives of the soil biocenosis.

The reproduction test with earthworms (ISO 11268-2) is applied to detect effects resulting from sublethal concentrations. Such endpoints are preferably applied to obtain information on environmental effects. However, the reproduction test is very labour-intensive and time-consuming, needing long incubation periods with results obtained only after 56 days. As the test period and the work expense dictate the costs of a given test, it is preferable to obtain the results within a short test period and at a high level of sensitivity. That is especially the case for the assessment of remediated soils. This feature is offered by the avoidance test with *Eisenia fetida* and *Eisenia andrei*. Experiences gained in a laboratory comparison test with eight contaminated soils in three laboratories point out that the avoidance test is as sensitive as the reproduction test (Reference [5]). However, it is not intended to use this test to replace the earthworm reproduction test.

NOTE The results were compared with those of the earthworm acute and reproduction tests carried out with the same soils. The results showed that with a criterion of > 80 % avoidance response, a 72 % agreement of the results was achieved.

Soil quality — Avoidance test for determining the quality of soils and effects of chemicals on behaviour —

Part 1:
Test with earthworms (*Eisenia fetida* and *Eisenia andrei*)

1 Scope

This part of ISO 17512 specifies a rapid screening method for evaluating the habitat function of soils and the influence of contaminants and chemicals on earthworm behaviour.

The sublethal test is a rapid method that reflects the bioavailability of contaminant mixtures in natural soils and substances spiked into soils to *Eisenia fetida* and *Eisenia andrei*. The avoidance behaviour of the worms is the measurement endpoint of the test. This test is not intended to replace the earthworm reproduction test.

Two different designs (a two section unit and a six section unit) have been developed and successfully applied. Both designs are applicable to either single-concentration (e.g. for assessing the quality of a field soil) or multi-concentration (e.g. for assessing the toxicity of a spiked chemical) tests. In both cases, the earthworms are allowed to make the initial choice on which compartment, control and a treatment [in the two section test vessel between right and left side; in the six section test vessel between the (3 + 3) alternating compartments], to enter.

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 10390, *Soil quality — Determination of pH*

ISO 11268-2:1998, *Soil quality — Effects of pollutants on earthworms (*Eisenia fetida*) — Part 2: Determination of effects on reproduction*

ISO 11269-2, *Soil quality — Determination of the effects of pollutants on soil flora — Part 2: Effects of chemicals on the emergence and growth of higher plants*

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

ISO 15799, *Soil quality — Guidance on the ecotoxicological characterization of soil and soil materials*

3 Terms and definitions

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

3.1
avoidance behaviour
tendency (of an organism) to avoid the test soil while preferring the control soil

3.2
habitat function
ability of soils/soil materials to serve as habitat for micro-organisms, plants and soil-living animals and their interactions

[ISO 15799:2003]

NOTE Ecotoxicological tests as indicators for the habitat function provide information concerning the respective test parameter, e.g. acute test for survival, or chronic tests for reproduction.

3.3
limited habitat function
habitat function (3.2) is limited if on average > 80 % of worms are found in the control soil (indication as an impact on behaviour)

3.4
effective concentration
EC_x
concentration at which a specific effect is detected [where x is a percentage (10, 25, 50) of this effect; e.g. avoidance]

EXAMPLE In this part of ISO 17512, an EC_{50} means the concentration of a substance or mixture of substances in soil that is estimated to cause a behavioural response in 50 % of the test earthworms.

4 Principle

Ten adult earthworms (species *Eisenia fetida* or *Eisenia andrei)* are exposed at the same time to a control soil and a contaminated soil or a soil containing test substances. Test soil and control soil are placed into each test vessel and the earthworms are thus presented with a choice between the test soil and the control soil. Two test-vessel designs are available:

a) a two section test vessel; and

b) a six section test vessel.

After an incubation period of two days, the number of worms is determined in all sections of the vessels.

Individual studies (e.g. testing boric acid in one of the two designs in different laboratories) or comparative investigations (testing the same chemical or soil in the same laboratory, e.g. Reference [8]) have in some cases shown different results. Recently, both designs were validated in interlaboratory tests in Canada (Reference [2]) and France; however, no international ring test using both designs in parallel has been performed so far. Therefore, for the time being, the choice of the design is up to the experimenter. When doing so, practical considerations like costs of the units as well as the amount of waste produced should also be taken into consideration.

5 Reagents and materials

5.1 Boric acid reference toxicant, recommended. H_3BO_3 has been used historically as a soil chemosterilant and is an effective non-selective biocide (relative molecular mass: 61,81). Earthworms can detect and avoid sublethal concentrations that adversely affect reproduction. Boric acid satisfies the following criteria that attest to its suitability as a reference toxicant:

a) it is effective at relatively low concentrations that are not strongly influenced by the nature of the substrate;

b) it is relatively stable and persistent so that concentrations do not change rapidly over the duration of the test;

c) it is reasonably water soluble or miscible in water, does not volatilise readily, and can be readily mixed with soils;

d) there is a standard method for measuring boric acid concentrations in soil;

e) it represents a minimal hazard to technicians and it is free of disposal problems.

5.2 Biological material, consisting of adult earthworms of the species *Eisenia fetida* or *Eisenia andrei* (individual mass: between 300 mg and 600 mg). Synchronisation of breeding of the organisms for this test is not necessary. An example of how to breed compost worms is given in Annex B.

Condition the selected worms for at least one day in the selected control soil (5.4).

NOTE *Eisenia fetida* and *Eisenia andrei* are compost worms. Ecologically, these species are not the most important in soils (Reference [7]). On the other hand, from a practical point of view, compost worms are much more suitable than any other lumbricid species due to the fact that they reproduce very quickly and easily in the laboratory (i.e. mass cultures can be obtained). In addition, the sensitivity of these species is more or less of the same order of magnitude in comparison to other earthworm species. In most cases, the differences between species are — depending on the chemical or contaminant mixture tested — not larger than a factor of 10 in acute or chronic tests (References [6], [7]). Despite the fact that other earthworm species have already successfully been used in avoidance tests (see Annex C), a factor describing their range of avoidance response is not yet known.

5.3 Test substrate. The soil to be tested should be sieved (size of openings, 2 mm) adjusted to about 60 % of the maximum water holding capacity. The optimum water content is achieved, if there is no standing water or free water appearing when the soil is compressed.

NOTE For highly silty and loamy soils, it can be difficult to get the necessary amount of soil sieved to $\leqslant$ 2 mm with an acceptable expenditure of work. The holes of the sieves may plug up within several minutes. Frequent cleaning is necessary. In this case, it is acceptable to sieve the amount of soil needed for the test to $\leqslant$ 4 mm.

Determine the water content and the pH in the presence of 1 mol/l KCl, in accordance with ISO 11465 and ISO 10390, respectively, immediately before the start of the test. In addition, the maximum water holding capacity shall be determined according to Annex F.

5.4 Control soil: three choices are possible (see also ISO 15799). Option a) is preferred, but since such a soil is often not available either a standard soil, b), or an artificial soil, c), is possible (potential influences of these soils are covered by the 80 % assessment criterion, see Clause 8).

a) A control soil as similar as the test soil in all characteristics other than the presence of contaminants.

b) A soil with the characteristics according to ISO 11269-2 [$C_{org} \leqslant$ 1,5 %, sand (0,063 mm to 2 mm) content of 50 % to 75 %, $<$ 20 % of particles less than 0,02 mm; pH of 5 to 7,5].

c) Artificial soil in accordance with ISO 11268-2.

Natural soils should be sieved and the water content adjusted according to 5.3.

6 Apparatus

Usual laboratory equipment and in particular the following.

6.1 Containers (see Annex A).

6.1.1 Two section chamber: containers of capacity 1 l to 2 l with a cross-sectional area of about 0,02 m^2, such that a depth of 50 mm to 60 mm of the soil is achieved.

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 worms from escaping (e.g. by using a tape to fix the cover). To avoid lateral effects of light, glass vessels shall be wrapped.

Two section chambers are commercially available[1)].

NOTE Due to the short test period and the proportionally large volume of soil in the vessels, a reduction of chemical concentration in the soil resulting from sorption to the vessel walls is negligible. Nevertheless, inert material (e.g. glass or stainless steel) is preferred.

6.1.2 Six section chamber (circular test units or vessels):

1) stainless steel for testing soil contaminated with organic compounds;

2) plastic (high density inert material) for testing soil contaminated with metals or metalloid compounds.

The circular test unit has a central chamber with six cut pie-shaped interconnecting compartments into which the test soil is placed; interconnecting holes are located along the bottom of the compartment walls (three per side) and along the bottom of the central chamber (two per side) so that the worms can move freely between compartments. The plastic test unit should be wrapped in an opaque material (tin foil) to eliminate light. Provisions to prevent worms from escaping are necessary.

The six section chamber is not commercially available. Therefore all details necessary to construct such chambers are presented in the figures and in the text.

6.2 Divider (e.g. plastic or thin sheets of metal):

a) for the two section chamber, to divide the containers vertically into two identical sides;

b) for the six section chamber, to slide along the walls of the compartments at the end of a test to isolate each section.

6.3 Equipment for measuring the water content of a substrate (according to ISO 11465).

6.4 Test environment.

6.4.1 Enclosure or environmental chamber, capable of being maintained at (20 ± 2) °C.

6.4.2 Light source, 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.

NOTE A day/night cycle was chosen so that the conditions are comparable to the acute and reproduction test.

1) Bellaplast No. 597 is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

7 Procedure

7.1 Appropriate concentration range

The avoidance test is designed to detect sublethal effects. Therefore, the test is invalid if more than one worm per vessel (i.e. one out of 10) is dead or missing at the end of the test (see 7.5). In order to avoid mortality, the performance of a range-finding test is recommended.

7.2 Testing of soils

7.2.1 Two section chamber

At the beginning of the test, the vessels (6.1.1) are divided into two equal sections by means of a vertically introduced divider. Vessels are filled with sieved soil up to a height of 50 mm to 60 mm. One half of the vessel is filled with test soil (section A), the other half is filled with control soil (section B). Then the separator is removed and 10 worms are placed on to the separating line of each test vessel (from there they have the possibility to dig quickly into the soil, using the slit left by the divider as a starting point). The containers are covered according to 6.1.1 and placed in the environmental chamber or in the test enclosure (6.4.1).

No feeding of the animals is required during the test.

The test is run with five replicates per treatment (test soils, controls or reference substance). To obtain a more precise quantification of the behavioural effect, a dilution series may be prepared. For dilution of the contaminated soil, the control soil should be used.

At the end of the test period (48 h) the control and test soils in each vessel are separated by inserting the dividers. The dividers shall be inserted before the test units are moved from the environmental chamber. The number of worms is determined for both sections of the vessels. Worms divided due to the introduction of the divider are counted as 0,5 independent of the length of the remaining body. Missing worms are considered to have either escaped from the test chamber or to have died and disintegrated during the test (see 7.1).

7.2.2 Six section chamber

The test soil and control soils are prepared (sieved, hydrated and mixed) and placed to a depth of 50 mm to 60 mm (350 ml soil) in each of three compartments in an alternating pattern (e.g. compartments 1, 3, and 5 have test soil and compartments 2, 4, and 6 have control soil) (see also Annex E). There is no soil in the central chamber. Ten earthworms are added to the central chamber, one at a time, and the compartment entered by each individual is recorded. The containers are covered (6.1.2) and placed in an environmental chamber (6.4.1).

No feeding of the animals is performed during the test.

The test is run with five replicates for a single concentration test and at least with duplicates for a multi-concentration test. For multi-concentration tests, the test soil consists of the site soil diluted with the appropriate control soil.

At the end of the test period (48 h) the dividers are positioned to prevent further movement of the earthworms between compartments. The dividers shall be inserted before the test units are moved from the environmental chamber. The number of worms in each compartment is recorded and the total number in each treatment within a test unit determined. Individual earthworms sliced inadvertently by the dividers are to be recorded as 0,5 independent of the length of the remaining body. Missing worms are considered to have either escaped from the test chamber or to have died and disintegrated during the test (see 7.1).

NOTE The hypothesis tested is that at the beginning of the test the worms are randomly distributed among sections and at the end of the test for a true avoidance response the earthworms are not distributed randomly among the sections in a vessel. If, at the beginning of the test, the worms are non-randomly distributed, then there might not be an avoidance response. Alternatively, there might be an avoidance response at the beginning of the test by worms refusing to enter sections with contaminated soil that they instantly avoid. This rarely happens when the levels in soil are sublethal.

7.3 Testing of chemical

While the main use of avoidance tests is testing of potentially contaminated soils, it is also possible to use this test for the assessment of the effects of single chemicals after they have spiked into a soil (examples of chemicals detected by earthworms are given Annex D). Modifications to test single chemicals (including statistical procedures) are specified in Annex E.

7.4 Reference substance

Boric acid is recommended as the reference toxicant. An avoidance behaviour response should be obtained at a concentration of 750 mg H_2BO_3 per kilogram of soil measured on the dry mass basis when artificial soil or another control soil is used. Testing by the soil toxicology laboratory of Environment Canada generated a boric acid EC_{50} of 618 mg/kg in a six section chamber test for avoidance behaviour using a chernozem clay loam control soil spiked with boric acid (Reference [8]). When reporting EC_{50} values, also state the main soil properties (i.e. pH, texture and organic matter content).

7.5 Validity criteria

The test is invalid if the number of dead or missing worms is > 10 % per treatment.

To validate the test set, check the homogeneity of distribution of the worms. For this purpose, fill the whole test vessel with the same soil and ensure that the orientation of the test vessels in the room is the same. On average, the ratio of worms should be within the range 60 % : 40 % for a two section chamber. More information concerning the distribution of worms in such dual tests using different soils is provided in Annex I.

8 Calculation and expression of results

The mean plus or minus standard deviation of live individuals in the test soil is determined for each treatment at the end of the test. For tests using the two section vessel, as well as for the six section vessel, the results are presented as the number of individuals in the test soil per test vessel.

If the test soil and the control soil differ only regarding the contamination, statistical calculations may be performed as follows.

For a single concentration test, the mean number of individuals at the end of the test in the test soil is compared to the mean of the control soil treatment using Fisher's exact test or another statistic appropriate for pairwise comparisons (Reference [15]). Results showing a significantly lower mean number of surviving worms in the test soil, relative to those in the control soil, indicate an avoidance response (or preference for the control soil) to the test soil. This result suggests that the habitat function of the test soil is limited.

For calculation of the percentage effect of a substance concentration, the mean number of worms in the test soil is compared with the mean number of worms in the control soil [negative responses (= the worms prefer the test soil) are considered as 0 % of avoidance]) in accordance with Equation (1).

$$x = \left(\frac{n_c - n_t}{N} \right) \times 100 \qquad (1)$$

where

x is avoidance, expressed as a percentage;

n_c is the number of worms in the control soil (either per vessel or in the control soil of all replicates);

n_t is the number of worms in the test soil (either per vessel or in the test soil of all replicates);

N is the total number of worms (usually 10; either per vessel or in the control soil of all replicates).

Using these data, any median effective concentration, EC_x, for a specified percentage effect (EC_{50} or EC_{20}) and its associated confidence limits can be calculated.

For statistical analysis of ecotoxicity data see Reference [15].

A comparison of results obtained in two and six chamber systems is given in Annex G.

If control soil and test soil differ in more of the main properties than just contaminants, statistical calculations are not appropriate. In this case, the application of a fixed threshold value instead of a statistical significant difference between the number of worms in the control and the test soil is recommended. Test soils with less than 20 % of the total number of worms are classified as having limited habitat function.

Data on the influence of soil properties on avoidance behaviour are given in Annex H.

If an attraction of > 80 % by the test soil is observed, the presence of chemical substances cannot be excluded. The result should also be assessed as an effect.

9 Test report

The test report shall contain at least the following information:

a) a reference to this part of ISO 17512;

b) the results expressed in accordance with Clause 8;

c) detailed description of the characteristics of the test soil and of the control soil;

d) if chemicals are tested, a detailed description of the test substance and method of application or incorporation;

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

f) full description of the experimental design and procedure;

g) description of the test conditions, including moisture content and pH value;

h) number of adults in the test soil and in the control soil at the beginning (only relevant for the six section chamber test) and end of the test;

i) mortality of the adults;

j) description of obvious morphological symptoms observed in the test organisms;

k) assessment with respect to habitat function limited and not limited, respectively or statistically calculated values (lowest observed effective concentration, no observed effective concentration and/or EC_x) including 95 % confidence limits, method of calculation, plot of the exposure concentration-response relationship;

l) discussion of the results;

m) all details not specified in this part of ISO 17512 or considered as optional, as well as any effect which may have affected the results.

Annex A
(informative)

Test chambers

A.1 Two section chamber

Round or rectangular containers (glass or plastic) of capacity 1 l to 2 l with a cross-sectional area of about 0,02 m^2.

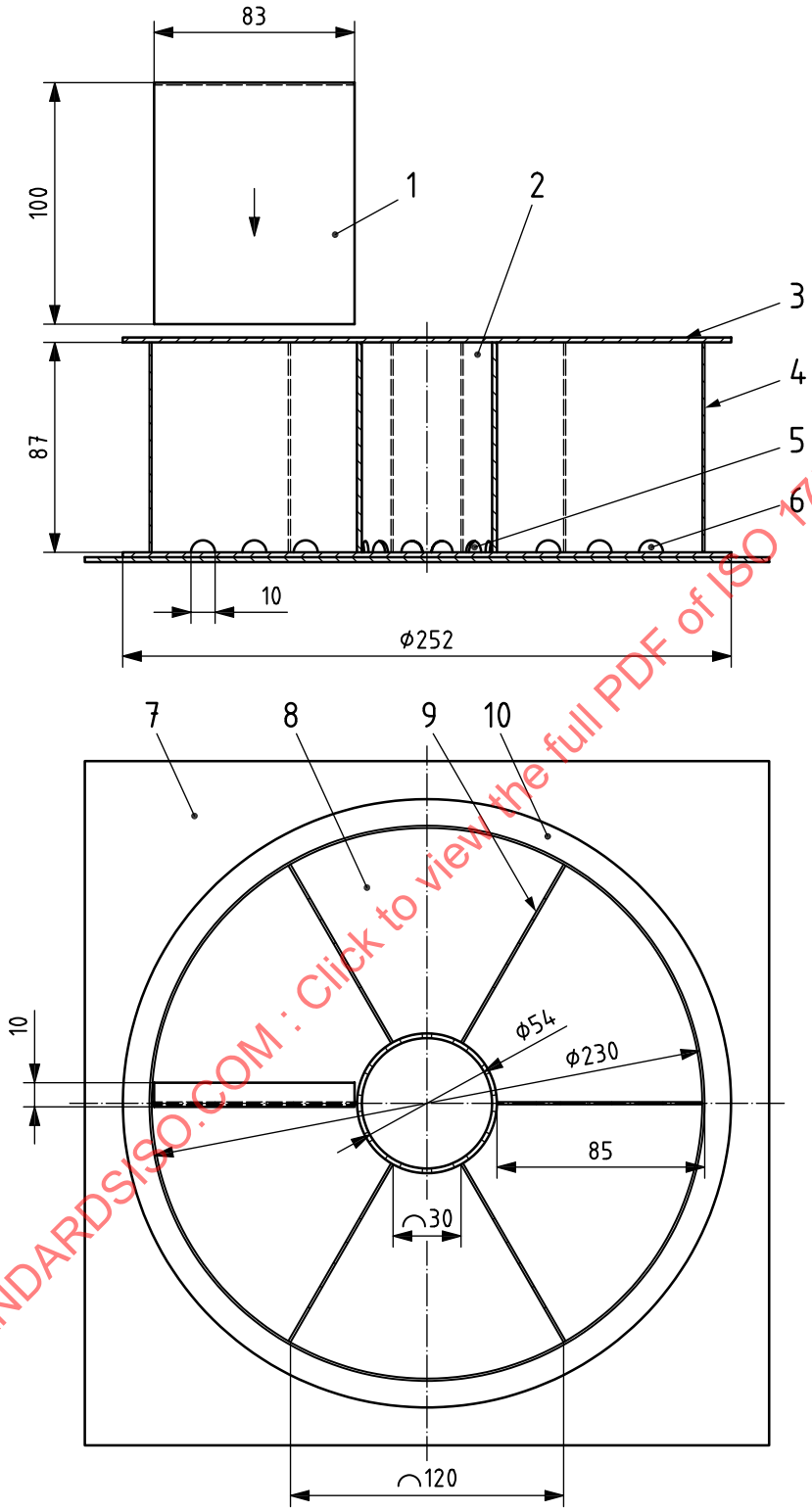
Figure A.1 — Example of a two section chamber

A.2 Six section chamber

Circular six section vessel with a central soil-free chamber. The design of the test unit is circular with an outer diameter of 230 mm. Each test unit is partitioned into six cut pie-shaped compartments that surround a central, circular (54 mm diameter) compartment. A series of holes of diameter 10 mm (two per compartment) connect the central compartment to each of the six compartments. The six compartments are also connected to adjacent compartments by three holes along the bottom of the section walls separating the compartments. The test unit is constructed of either high-quality stainless steel (1 mm to 4 mm thick) for use with soils contaminated with organic compounds, or plastic (5 mm to 6 mm thick) for use with soils contaminated with metal compounds.

Figure A.2 — Example of a six section chamber

Dimensions in millimetres



Key

1 removable partition (six per test unit)
2 inner chamber without soil where worms are placed at start of test
3 lid
4 outer wall of test unit
5 holes between inner chamber and compartment
6 holes between compartments
7 wooden support stand
8 cut pie-shaped test compartments
9 wall separating compartments
10 steel base of test unit

Figure A.3 — Details of a six section chamber

Annex B
(informative)

Example of a breeding technique for *Eisenia fetida* and *Eisenia andrei*

This method is in accordance with ISO 11268-1:1993, Annex A and ISO 11268-2:1998, Annex A.

Both species can be bred in a wide range of animal wastes. The recommended breeding medium is a mixture of one volume of horse or cattle manure and one volume of peat. The medium should have a pH value of about 6 to 7 (regulated with calcium carbonate), a low ionic conductivity (less than 6 mg/kg soil dry mass or less than 0,5 % common salt concentration) and should not be contaminated excessively with ammonia or animal urine. The substrate should be moist, but not too wet. In cases of doubt, the moisture should be checked as follows. When the soil is gently squeezed by hand, only small drops of water should appear between the fingers. Breeding boxes of capacity 10 l to 50 l are suitable.

To obtain worms of standard age and mass, it is best to start the culture with cocoons. Therefore adult worms are put into a breeding box with fresh substrate to produce cocoons and remove them after 14 days to 28 days. These individuals can be used for further breeding batches. The earthworms hatched from the cocoons are used for testing when mature (after at least two months, but less than 12 months).

Breeding is preferably carried out in an environmental chamber or enclosure at (20 ± 2) °C. At this temperature, worms become mature after two months to three months.

Annex C
(informative)

Further test organisms

The test has also been performed with *Lumbricus terrestris,* an organism with a high ecological relevance. In this case some modifications of the described test design are necessary.

Individual mass	3 g to 10 g
Incubation period	72 h
Temperature	(18 ± 2) °C
Reference substance	Boric acid
Test vessels	so far, the test has only been performed in the six section chamber; although *L. terrestris* is larger than *E. fetida*, the same test vessels were successfully applied (Reference [10])

In addition, mineral soil dwelling species like *Aporrectodea caliginosa* (Reference [3]) or *Aporrectodea tuberculata*, (References [19], [20]) as well as epigeic species like *Lumbricus rubellus* and *Dendrobaena octaedra* were used in two section chambers (References [19], [20]). Other test species might be suitable. Necessary modifications (e.g. size of test chamber, test duration, temperature) should be defined for each of the additional test species.

For breeding methods of common soil dwelling earthworm species, see Reference [18].

Annex D
(informative)

Contaminants that earthworms can detect and avoid in the avoidance test

The avoidance test is suitable for assessing those contaminants that can be detected by earthworms (e.g. *Eisenia fetida*) via sensory receptors (e.g. chemoreceptors). This seems to apply for a broad range of contaminants. Up to now it has been shown that the test is suitable for mineral oil, polyaromatic hydrocarbons, 2,4,6-trinitrotoluene (TNT), manganese, zinc, copper sulfate, petroleum hydrocarbons (crude oil), and mixtures consisting of several heavy metals, KCl, NH_4Cl, benomyl, carbendazim, lambda-cyhalothrin, mancozeb and complex hydrocarbon mixtures such as amines and glycol products and condensate (References [4], [5], [8], [9], [10], [11], [12]).

If testing volatile compounds such as low-molecular petroleum hydrocarbons (BTEX, C_5 to C_{10}), they may impact the results of the avoidance test due to transport to the control soil (section B) via the gaseous phase.

Annex E
(normative)

Testing of chemicals in the avoidance test

E.1 General

To test chemicals, modifications to the test procedures are required.

E.2 Test substrate

If the test is used for substance testing, prepare artificial soil according to ISO 11268-2 or use a sandy soil with the characteristics specified in ISO 11269-2 (organic carbon content $\leqslant$ 1,5 % mass fraction, sand content of 50 % to 75 %, $<$ 20 % in the fine particle fraction; pH of 5 to 7,5). The sandy soil should be sieved to $\leqslant$ 2 mm. Adjust the water content according to 5.3. The incubation corresponds to the testing of soils.

E.3 Control soil

Use the same soil as control soil and as test soil (see 5.3). However, do not add test substance to the control soil. Adjust the water content according to 5.3.

E.4 Procedure

E.4.1 General. For the testing of chemicals use one of the methods specified in E.4.2 to E.4.5.

E.4.2 Water-soluble test substances. Immediately before starting the test, an emulsion or dispersion of the test substance in deionised water is prepared in a quantity sufficient for all replicates of one concentration. The emulsion or dispersion is mixed thoroughly with one batch of (artificial) soil before introducing it into a test vessel.

E.4.3 Test substances insoluble in water but soluble in organic solvents. The quantity of test substance required to obtain the desired concentration is dissolved in a volatile solvent (such as acetone or hexane) and it is mixed with quartz sand (10 g/kg). After evaporation of the solvent by placing the container in a fume hood for at least 1 h, the portion of quartz sand required is mixed thoroughly with the soil. If artificial soil is used, the amount of quartz sand used for application of the test substance shall be considered when preparing the substrate. In this case, after evaporation of the solvent, the remainder of the basic substrate (allowing for the amount of sand used to prepare the test substance) and the water is added, and it is mixed thoroughly before introducing it into the test containers.

NOTE Ultrasonic dispersion, organic solvents, emulsifiers or dispersants can be used to disperse substance with low aqueous solubility. When such auxiliary substances are used, all test concentrations and the control soil should contain the same minimum amount of auxiliary substance.

E.4.4 Test substances insoluble in water or organic solvents. A mixture of 10 g of finely ground quartz sand and the quantity of the test substance required to obtain the desired concentration is prepared. Afterwards, this mixture is mixed thoroughly with the pre-moistened artificial soil and with the amount of deionised water in order to get the final moisture required before introducing it into the test vessels.

E.4.5 Test substances at high concentrations (e.g. mineral oil). Those substances which need to be tested in high concentrations to simulate the conditions of contaminated sites may be added directly to the soil. A homogenous distribution of the test substance in the soil shall be demonstrated.

E.4.6 Practical details. Different concentrations of the test substance are investigated. Preliminary tests using four concentrations (e.g. 1 mg/kg, 10 mg/kg, 100 mg/kg and 1 000 mg/kg) as well as final tests in accordance with ISO 11268-2, may be performed.

When using a two section chamber, the mixture is filled in section A of the vessels; the control soil without chemicals is filled in section B.

When using a six section chamber, the test soil with the test substance is added to three compartments within each test unit and the control soil is added to the remaining three compartments within each test unit, in an alternating pattern as described in 7.2.2. The different concentrations are placed into separate test units and, each test substance concentration-control soil combination is replicated two or three times (i.e. there are two or three test units per combination of treatments). Earthworms have not been observed to remain in the central chamber during the test.

E.5 Calculation and expression of the results

For each concentration calculate the mean number of worms in the test soil of one vessel. For calculation of the percent effect per substance concentration, the mean number of worms in the test soil is compared with the mean number of worms which are expected to be present in the control soil assuming a random distribution of the animals among sections.

The avoidance, x, expressed as a percentage, is calculated according to Equation (E.1):

$$x = \left(\frac{n_c - n_t}{N} \right) \times 100 \qquad \text{(E.1)}$$

where

n_c is the number of worms in the control soil (either per vessel or in the control soil of all replicates);

n_t is the number of worms in the test soil (either per vessel or in the test soil of all replicates);

N is the total number of worms (usually 10; either per vessel or in the control soil of all replicates).

Negative responses (in other words, the worms prefer the test soil) are considered as 0 % of avoidance.

Using these data, any median effective concentration, EC_x, for specified percent effect (EC_{50} or EC_{20}) and its associated confidence limits can be calculated. To estimate the EC_{50}, Spearman-Kärber or probit procedures, with no trimming of the data, are applied to the avoidance values, expressed as percentages, for each concentration.

If the test was performed following a limit test design (control versus one treatment), the number of worms at the end of the test in the test soil is compared to the mean of the control soil treatment using a one-tailed Student t-test or another appropriate statistic for pairwise comparisons.

For statistical analysis of ecotoxicity data, see Reference [16].

Annex F
(normative)

Determination of water-holding capacity

This method is in accordance with ISO 11268-2:1998, Annex C.

Take a defined quantity (e.g. 5 g) of the test soil substrate with a suitable device (auger tube, etc.) to achieve saturation with water. Close the bottom of the tube with filter paper, and after filling, place the tube with substrate on a rack in a water bath. The water level should first be beneath the upper lid of the tube and later above this lid. Leave the soil substrate sample in the water for about 3 h. As not all water absorbed by the soil substrate capillary can be retained, the sample should be placed for a period of 2 h on very wet finely ground quartz sand for draining in a closed vessel. Weigh the sample, dry it to constant mass at 105 °C and re-weigh it. The water holding capacity (WHC), expressed as a percentage of dry mass, is calculated according to Formula (F.1):

$$\left(\frac{m_S - m_T - m_D}{m_D}\right) \times 100 \qquad \text{(F.1)}$$

where

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 substrate.

Annex G
(informative)

Comparison of the results obtained in the two section chamber and six section chamber system

G.1 Comparison of different soil test options

Based on the threshold value of 20 %, the assessment of the results determined in the two section chamber system and in the six section chamber system indicates that they correspond to each other in 76 % of the experiments. The same is true for the assessment on the basis of statistical significance. For this evaluation only avoidance, not attraction (only observed for the six chamber system) was regarded. Furthermore the different statistical levels were not considered. For further information see References [8], [11] and [17].

Table G.1 lists characteristics of the applied soils. Results obtained in the tests are presented in Table G.2.

Table G.1 — Characteristics of the applied soils

Characteristic	Sandy soil	Loamy soil
Particle size, d, distribution		
$d < 2$ µm	3,6	31,5
2 µm $\leqslant d < 63$ µm	25,6	46,8
63 µm $\leqslant d < 2\,000$ µm	70,8	21,7
Organic carbon content, $w_{C,org}$, %	1,03	3,3
Total nitrogen content, $w_{N,tot}$, %	0,09	0,36
pH ($CaCl_2$)	5,5	5,4
Water-holding capacity, WHC_{max}, g/kg dry mass	269	653

Table G.2 — Results obtained in the tests

Results			Worms in the contaminated soil		Correspondence of the results	
Soil	Contaminant	Concentration [mg/kg]	2-chamber system (five replicates)	6-chamber system (two replicates)	According to 20 % value	According to statistical significance
Sandy	PCP	2,5	4,5 ± 0,6 [c]	3,0 ± 1,4	d	d
		10	2,0 ± 1,4 [c]	2,0 ± 0,0 [b]	d	d
		40	0,5 ± 0,7	0 ± 0,0 [c]	d	d
Loamy	PCP	2,5	5,6 ± 1,3	7,0 ± 0,0	d	d
		10	4,8 ± 1,3 [c]	8,8 ± 1,1 [b]	d	d
		40	0,8 ± 0,6 [c]	3,8 ± 0,4	—	—
Sandy	TBT	0,3	4,8 ± 2,0	4,0 ± 0,0	d	d
		1,25	3,6 ± 1,4 [a]	1,0 ± 0,0 [c]	—	d
		5,0	1 ± 1,0 [c]	1,5 ± 0,7 [b]	d	d
Loamy	TBT	1,25	3,3 ± 1,0 [a]	5,0 ± 1,4	d	—
		5,0	1,7 ± 1,5 [c]	4,5 ± 4,9	—	—
		20	0,8 ± 1,1 [c]	0,0 ± 0,0 [c]	d	d
Sandy	TNT	32	0,2 ± 0,4 [c]	0,5 ± 0,7 [c]	d	d
Loamy	TNT	8,0	6,5 ± 1,5 [a]	9,0 ± 0,0 [c]	d	d
		16	4,9 ± 2,1	5,5 ± 0,7	d	d
		32	1,2 ± 0,9 [c]	8,5 ± 2,1 [b]	—	d
		64	0,2 ± 0,9 [c]	6,0 ± 1,4	—	—
Sandy	Cu	50	0,0 ± 0,0 [c]	0,5 ± 0,7 [c]	d	d
Loamy	Cd	10	5,7 ± 0,8 [c]	6,0 ± 1,4	d	d
		40	4,8 ± 2,8	5,8 ± 1,8	d	d
		160	2,8 ± 1,1	6,3 ± 2,5	d	—

[a] Significantly different $\alpha = 0{,}05$.

[b] Significantly different $\alpha = 0{,}01$.

[c] Significantly different $\alpha = 0{,}001$.

[a] to [c]: Assessment concerning the statistical difference of the number of worms detected in the contaminated soil and the number of worms in the control soil (χ^2-test).

[d] The assessment of the results determined in the two test systems correspond to each other.

G.2 Tests with boric acid: Comparison between the six chamber and the two chamber test vessel

In March 2003, a series of tests was performed to evaluate the avoidance response of *Eisenia andrei* when exposed to concentrations of boric acid in a field-collected reference soil (Alberta black chernozem soil). The tests were performed in parallel to compare the observed avoidance response between the two types of test vessels (i.e. six chamber versus two chamber design).

As observed with the calculated EC_{50}s (Table G.4), *Eisenia andrei* was able to detect and avoid lower concentrations of boric acid when exposed to the contaminated soil in the six chamber design, relative to the two chamber design.

Table G.3 provides a detailed summary of the test results for the six chamber and two chamber test vessels.

Table G.3 — Total number of *Eisenia andrei* observed in each treatment (e.g. control vs. treated soil) and test concentration after 48 h of exposure to boric acid in Alberta black chernozem soil using six chamber and two chamber test vessels

Boric acid mg/kg soil, dry basis	Six chamber test vessel			Two chamber test vessel		
	Worms in control soil	Worms in treated soil	Avoidance %	Worms in control soil	Worms in treated soil	Avoidance %
125	16	14	7	19	11	27
250	13	16	−10	16	14	7
500	20	10	33	20	10	33
750	25	5	67	22	8	47
1 000	26	4	73	24	6	60
NOTE Negative values were analysed as 0 % avoidance. The results are presented on a per treatment basis (n = 30).						

Table G.4 — Calculated EC_{50} and corresponding 95 % confidence limits for the avoidance response test using the six chamber and two chamber test vessel design (*Eisenia andrei* was exposed to boric acid for 48 h in Alberta black chernozem soil)

Statistical method	Six chamber test vessel			Two chamber test vessel		
	EC_{50}	95 % LCL	95 % UCL	EC_{50}	95 % LCL	95 % UCL
Spearman-Kärber	617 [a]	513	741	794 [b]	537	1 202
[a] The analysis required 27 % trimming.						
[b] The analysis required 40 % trimming.						
LCL lower confidence limit						
UCL upper confidence limit						