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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*)*

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Foreword

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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).

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

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).

