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

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

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

Partie 2: Essai avec des collemboles (Folsomia candida)

Reference number
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Foreword

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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 17512-2 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*)*

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

Introduction

The use of the avoidance behaviour of soil invertebrates as an indicator of unfavourable conditions allows a preliminary assessment of contaminated soils in a short period of time, with a high degree of sensitivity. Being rapid, cost-effective and ecologically relevant, the avoidance tests with earthworms were proposed to complement conventional chemical analysis. Supporting the results obtained in the chronic tests, the avoidance bioassays can be used as a first screening tool in the assessment of the habitat function of soils. Considering the fact that the avoidance response of soil invertebrates differs between species due to their distinct sensitivity to contaminants and modes of exposure, it is recommended to standardize a second rapid cost-effective and ecologically relevant avoidance bioassay.

Springtails have shown a distinct sensitivity towards several contaminants when compared with earthworms, complementing the information obtained in the avoidance tests with earthworms [1][2]. Until now, the species *Folsomia candida* has been the most commonly used collembolan test species due to a great facility to keep laboratory cultures and due to their high locomotor ability [3]. *Folsomia candida* is considered to be a hemi-edaphic species, meaning that it lives mainly in the soil. Furthermore, this species is already used in ISO 11267.

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

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

1 Scope

This part of ISO 17512 specifies a rapid screening method for evaluating the habitat function of soils based on the avoidance behaviour of springtails.

The test is a rapid method that reflects the bioavailability of contaminants in natural soils and substances spiked into soils to *Folsomia candida*. In both cases, it is possible to establish a dose-response-relationship. The avoidance behaviour of the springtails is the measurement endpoint of the test. This test is not intended to replace the Collembola reproduction 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 10390, *Soil quality — Determination of pH*

ISO 11267:1999, *Soil quality — Inhibition of reproduction of Collembola (Folsomia candida) by soil pollutants*

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 contaminated soils on the emergence and early 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 soils 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

[ISO 17512-1:2008]

3.2
test soil
either a natural or an artificial clean soil that is spiked with the test substance or a contaminated natural soil (a site soil)

3.3
control soil
natural or artificial uncontaminated soil

See 5.3.

3.4
limited habitat function
habitat function is limited if, on average, > 70 % of springtails are found in the control soil (indication of an impact on behaviour) after they were allowed to choose between the control soil and tested soil

3.5
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]

NOTE In this part of ISO 17512-2, an EC_{50} means the concentration of a test substance, or dilution of a contaminated test soil, that is estimated to cause an avoidance response of 50 %.

EXAMPLE An avoidance of 50 % occurs when the number of springtails in the test soil is 50 % of the number that should be there in the case of no avoidance: no avoidance (control soil = 10 individuals; test soil = 10 individuals); 50 % avoidance (control soil = 15 individuals; test soil = 5 individuals).

3.6
lowest observed effect concentration
LOEC
lowest tested concentration of a test substance, or dilution of a contaminated test soil, that is observed to cause a statistically significant avoidance response ($p \leq 0{,}05$)

NOTE 1 In the case of a test substance, the concentration is expressed as mass of the test substance per dry mass of test substrate; in the case of a contaminated test soil, the concentration is expressed as the percentage dilution of the test soil.

NOTE 2 All tested concentrations/dilutions above the LOEC should have a harmful effect equal to or greater than those observed at the LOEC. When this condition is not observed, a full explanation should be given for how the LOEC (and hence the NOEC) has been selected.

3.7
no observed effect concentration
NOEC
test concentration/dilution tested immediately below the LOEC, which causes a not statistically significant avoidance response ($p > 0{,}05$)

4 Principle

Springtails (*Folsomia candida*) are exposed at the same time to the control soil and the test soil. These soils are filled into the two sections of the same vessel. After an incubation period of two days, the number of springtails is determined in each section of the vessels.

5 Reagents and materials

5.1 Biological material, only springtails of the species *Folsomia candida* (Willem) (see A.2.6) coming from synchronized cultures should be used.

NOTE Typically, in this test springtails can be used when they are 10 d to 12 d old (or alternatively adult, e.g. 20 d to 22 d old).

5.2 Test substrate, in the case of a natural soil, the substrate to be tested should be sieved (2 mm) and moisture-adjusted to about 40 % to 60 % of the maximum water-holding capacity. If standing water or free water appears when the soil is compressed before achieving the desired percentage of maximum WHC, a lower percentage might be used. 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 ≤ 2 mm with an acceptable expenditure of work. The holes of the sieves might 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 ≤ 5 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 D.

If testing a substance, a different procedure should be followed (see Annex C).

5.3 Control soil, two choices are possible (see also ISO 15799). Either a) a reference soil or b) a standard soil that allows the presence of springtails.

a) If reference soils from uncontaminated areas near a contaminated site are available, they should be treated and characterized like the test soils. 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 soil, standard soils (e.g. LUFA 2.2) shall be used as the test substrate. 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 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 size 0,05 mm to 0,2 mm)	69 %

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

Natural soil should be sieved and the water content should be adjusted according to 5.2.

5.4 Reference substance, having Phenmedipham as the only active ingredient.

6 Apparatus

Usual laboratory equipment and the following.

6.1 Containers (see Annex B).

Cylindrical containers of capacity 200 ml to 300 ml with a cross-sectional area of about 50 cm^2, such that a depth of 3 cm to 4 cm of 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 springtails from escaping (e.g. by using a tape to fix the cover). To avoid lateral effects of light, test vessels should be made of opaque material, otherwise they should be wrapped with aluminium foil.

NOTE Due to the short test period and the proportionally large volume of soil in the vessels (considering the small amount of soil needed by the springtails), a reduction of the chemical concentration in the soil resulting from sorption to the vessel walls is negligible. Therefore, plastic vessels can be used, although, when available, the use of inert material (e.g. glass or stainless steel) is preferred.

6.2 Divider, made of plastic or thin sheets of metal. It shall divide the test containers vertically into two identical sections.

6.3 Equipment for measuring the water content of a substrate (in accordance with ISO 11465).

6.4 Apparatus, for measuring the pH of the substrate.

6.5 Exhaustor, to transfer the springtails (see A.3).

6.6 Test environment.

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

6.6.2 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.

7 Procedure

7.1 Appropriate concentration/dilution range

The avoidance test is designed to detect sublethal effects. Therefore, the test is invalid if more than 20 % of the total number of springtails are dead or missing at the end of the test (see 7.4). In order to avoid mortality, a range-finding test should be performed in those cases when testing a substance or a dilution gradient of contaminated natural soil.

7.2 Testing of soils

At the beginning of the test, the containers (6.1) are divided into two equal sections by means of a vertically introduced divider (6.2). Vessels are filled with sieved soil up to a height of 3 cm to 4 cm (±30 g wet mass per side; the soil is freshly moistened and should not be pressed). One half of the vessel is filled with test soil (section A), the other half is filled with control soil (section B). Then the divider is removed and 20 springtails are placed on top of the line separating the two soils on each test vessel. The containers are covered according to 6.1 and placed into the environmental chamber or in the enclosure (6.6.1).

No feeding of the animals is required during the test.

The test runs with five replicates. To facilitate checking of the pH and humidity of the test substrates at the end of the test period, the use of an additional container without animals for each tested combination is recommended. 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 divider. It shall be inserted before the test units are moved from the environmental chamber. One section of the test container is emptied (alternately the control and the treated soil sections to avoid systematic errors) into a small vessel followed by the removal of the divider. Once separated, both soils are flooded with water and, after the addition of a few drops of ink and gentle stirring with a spatula, the animals floating on the water surface are counted. Missing springtails are considered as dead organisms.

NOTE Although some studies had shown that *Folsomia candida* can have an avoidance reaction before 48 h of exposure for some substances [4][5][6], the use of 48 h as the test period for avoidance tests with springtails is still recommended.

7.3 Reference substance

Substances having Phenmedipham as the only active ingredient (5.4) are recommended as reference toxicants for this test to be used in a positive control treatment. Springtails can detect and avoid sublethal concentrations that adversely affect reproduction [4][7]. A significant avoidance behaviour response (see Clause 8) should be obtained in a range of 5 mg to 20 mg reference toxicant (5.4) per kilogram of soil measured on the dry-mass basis when artificial soil is used [2].

Alternatively, boric acid can be used as a reference substance. A significant avoidance-behaviour response (see Clause 8) should be obtained in a range of 700 mg to 2 800 mg of boric acid per kilogram of soil measured on a dry-mass basis when artificial soil is used [10].

WARNING — When handling these chemicals, appropriate precautions should be taken to avoid ingestion or skin contact.

7.4 Validity criteria

The test is invalid if the number of dead or missing springtails is > 20 % per treatment.

To validate the test set, check the homogeneity of distribution of the springtails. For this purpose, test vessels with the control soil on both sections shall be prepared and an even distribution of the springtails among both sections should be observed. On average, the number of springtails in one section should be within the range of 40 % to 60 % of the total number of animals present.

8 Calculation and expression of results

8.1 General

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. 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.

8.2 Calculation of the percentage of avoidance

For calculation of the percentage effect per concentration of the tested substance or per soil dilution (in the case of contaminated natural soil), the number of springtails in the test soil is compared with the number of springtails in the control soil in accordance with Equation (1).

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

where

x is the avoidance, expressed as a percentage;

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

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

N is the total number of springtails (usually 20 per vessel, or that in all replicates).

A neutral response (0 % avoidance) is considered when an equal number of individuals is found in both soils. Negative responses (when the springtails prefer the test soil) are considered as 0 % of avoidance.

8.3 NOEC/LOEC calculation

For a single concentration or a single dilution test, the statistical significance of the avoidance response can be evaluated using the Fisher's exact test or another statistic appropriate for comparing observed with predicted distributions [9]. With this test, comparisons between the observed and a theoretical distribution (assuming a "no avoidance" situation) of the organisms in the test soil are performed. What is tested is an avoidance response towards the test soil, and the null hypothesis assumes a situation of no avoidance. Results showing a statistically significant ($p < 0{,}05$) lower number of surviving springtails in the test soil, relative to the expected number in case of no avoidance, indicate an avoidance response towards the test soil. LOEC and NOEC values can be evaluated along a gradient of dilutions of a contaminated test soil, or a gradient of concentrations of a test substance can be determined by analysing the significance of each single Fisher's exact test.