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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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Contents

Page

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).

$$x = \left(\frac{n_{\text{c}} - n_{\text{t}}}{N} \right) \times 100 \qquad (1)$$

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.

8.4 EC$_x$ calculation

The desired EC$_x$ shall be obtained through Probit regressions, taking into account the percentage of surviving springtails found in a control soil along the gradient of dilutions of a contaminated test soil or a gradient of concentrations of a test substance.

If the control soil and test soil differ in the main properties and in the presence of contaminants, statistical calculations to assess effects of contaminants only are not appropriate, since soil properties can mask an effect of the contaminants. In this case, for the assessment of the habitat function, the application of a fixed threshold value, instead of a statistically significant difference between the number of springtails in the control and the test soil, is recommended. Test soils with less than 30 % of the total number of surviving springtails (when more than 70 % of surviving springtails are found in the control) are classified as having limited habitat function.

9 Test report

The test report shall provide 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, age, 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 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 (LOEC, NOEC and/or EC$_x$) including 95 %-confidence limits, method of calculation, plot of the exposure concentration-response or dilution-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)

Techniques for rearing and breeding of Collembola

A.1 General

The following procedures are appropriate. They are cited in accordance with ISO 11267:1999, Annex A.

A.2 Conditions for rearing and breeding

A.2.1 Breeding substrate

Plaster of Paris (plaster for stucco, pH 6,4) and activated charcoal (pulverized chemically activated charcoal, pH 6 to 7), are mixed in a mass ratio of 8:1, but higher ratios, (9:1 or 10:1) may also be used. Depending on the type of plaster, 60 g to 100 g of water are added to 100 g of the mixture. This provides a highly moist substrate, while the charcoal adsorbs waste gases and excretion products. The dark background facilitates observation.

The presence of water on the saturated substrate surface is essential for breeding springtails, and the pH can readily be determined by using pH indicator paper placed on this wet substrate surface.

A.2.2 Breeding containers

Commercial plastic containers with a volume of about 400 ml should be used. Fill the containers to a depth of about 1 cm with the breeding substrate, and add deionized water to almost saturation. The moisture content can be maintained automatically by using an absorbent wick, implanted in the substrate and running to a water bath below the container, or by supplying distilled water with a pipette until the substrate is saturated but there is no water standing on the substrate surface.

Close the breeding containers tightly using suitable covers, and aerate periodically (e.g. in combination with feeding) by lifting the cover for a short period of time.

The covers may also be perforated with a needle for aeration.

CAUTION — Care should be taken that predacious mites do not penetrate the containers.

A.2.3 Climatic conditions

For keeping and breeding the springtails, a climatic chamber with a controlled temperature of 20 °C to 22 °C and 70 % to 80 % relative humidity with constant lighting at 400 lx to 800 lx (or light:dark cycle 16 h:8 h) is most suitable.

A.2.4 Food

For breeding and for the test, use granulated dry yeast as the food supply. Adding food to the breeding containers once or twice a week is necessary, but to avoid spoilage by fungi, food should be applied in small amounts at frequent intervals.

A.2.5 Transfer

After about eight weeks, transfer the springtails to fresh breeding containers by tapping or blowing. The transfer to fresh containers usually induces oviposition.

A.2.6 Test organisms of a standard age

To obtain 10 d to 12 d old juvenile springtails for the test, transfer egg clusters from breeding containers to a freshly prepared breeding substrate, using a fine spatula or a hair brush. After 48 h remove the egg clusters and feed the instars hatched from the eggs. Juveniles that are 10 d to 12 d old can be collected after another 10 d incubation.

NOTE The egg clusters are easily removed if they are placed on small pieces of breeding substrate or cover glasses laid on the breeding substrate.

Alternatively, 10 d to 12 d old juvenile springtails can be obtained by placing a number of adult springtails in small containers with plaster of Paris in the base, and allowing them to lay eggs over a two-day period. After this time remove the adults. Twelve days after the first juveniles have emerged from the eggs, they can be used for the test. To ensure successful synchronization, it is advisable to check the containers for egg production before removing the adults. In some cases, the adults do not start laying eggs immediately, and only a few eggs are produced in the first two days. If this is the case, then keep the adults in the containers for an additional day or more.

For both methods, avoid overcrowding in the containers, as this may lead to a reduced growth. As a consequence, the 10 d to 12 d old animals used for the test may be too small and may show differences in sensitivity when compared to sub-adults.

A.3 Transfer of springtails to the test containers

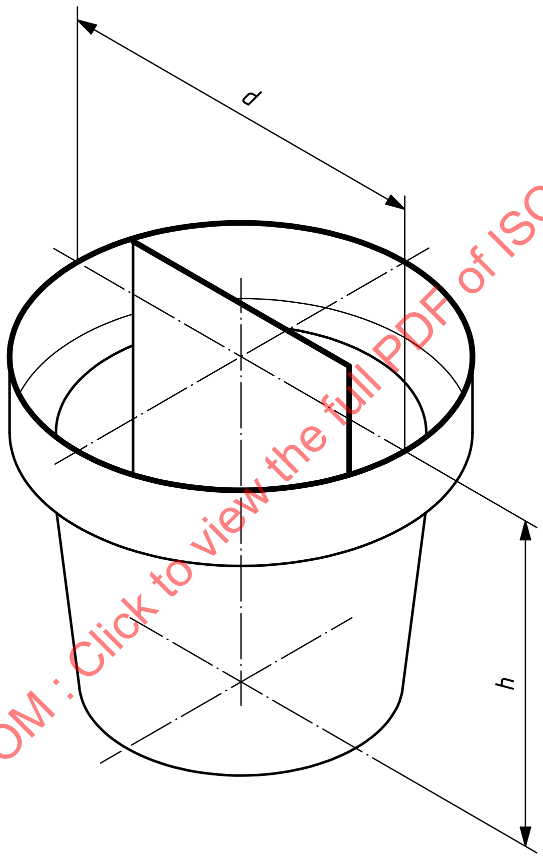
The springtails are easily transferred from the breeding substrate to the test substrate by an exhaustor. The springtails are sucked individually through a pipette tip to a small covered container. Care shall be taken to ensure that the suction of the pump is low, thus avoiding damage to the springtails. After removing the cover, springtails provided for one test vessel, and showing no signs of physical damage, are transferred onto the substrate surface of that test vessel.

Alternatively, a manual exhaustor can be hand-made from a small glass tube and a piece of flexible plastic tube. For this purpose a Pasteur pipette can be used, from which the small end has been removed and the narrow aperture is smoothed in a flame to remove the cutting edges. The other end of the tube is covered with a piece of very fine mesh gauze, and a piece of flexible plastic tube is fixed to it. With such an exhaustor, the juveniles can be sucked up by mouth and transferred to the test containers.

Annex B
(informative)

Test chambers

Round containers (plastic, glass or stainless steel) of 200 ml to 300 ml capacity with a cross-sectional area of about 40 cm^2 to 60 cm^2.



Key

d diameter

h height

Figure B.1 — Example of a test container for an avoidance test with springtails

Annex C
(normative)

Testing of chemicals in the avoidance test

C.1 General

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

C.2 Test substrate

If the test is used for substance testing, prepare artificial soil in accordance with ISO 11268-2 or use a sandy soil with the characteristics specified in ISO 11269-2 (carbon content: $C_{org} \leq 1,5$ %, sand with 50 % to 75 % of particle size 0,063 mm to 2 mm, particles less than 0,02 mm < 20 %; pH of 5 to 7,5). The sandy soil should be sieved to ≤ 2 mm. Adjust the water content according to 5.2.

C.3 Control soil

The same test substrate should be used as the control and test soil (see 5.3). For the control soil, however, no test substance is added. Adjust the water content according to 5.2.

C.4 Procedure

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

C.4.2 Water-soluble test substances. Immediately before starting the test, an emulsion or dispersion of the test substance in deionized 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.

C.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.

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.

C.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 premoistened artificial soil and with the amount of deionized water in order to get the final moisture required before introducing it into the test vessels.

C.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.

