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Soil quality — Inhibition of reproduction of Collembola (*Folsomia candida*) by soil contaminants

*Qualité du sol — Inhibition de la reproduction de Collembola (*Folsomia candida*) par des contaminants du sol*

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Foreword

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This document was prepared by Technical Committee ISO/TC 190, *Soil quality,* Subcommittee SC 4, *Biological characterization*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 444, *Environmental characterization of solid matrices,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This third edition cancels and replaces the second edition (ISO 11267:2014), which has been technically revised.

The main change is as follows:

— addition of an annex to provide specific information when using alternative Collembola species for reproduction test.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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 References [2] and [4]). Reference [2] includes a list and short characterization of recommended and standardized test systems and Reference [4] gives guidance on the choice and evaluation of the bioassays. Aquatic test systems with soil eluate are applied to obtain information about the fraction of contaminants potentially reaching the groundwater by the water path (retention function of soils), whereas terrestrial test systems are used to assess the habitat function of soils.

Soil-dwelling Collembola are ecologically relevant species for ecotoxicological testing. Springtails are prey animals for a variety of endogeic and epigeic invertebrates and they contribute to decomposition processes in soils. In acidic soils they are probably the most important soil invertebrates besides enchytraeids with respect to that function, since earthworms are typically absent.[19] Additionally, Collembola represent arthropod species with a different route and a different rate of exposure compared to earthworms[1] and enchytraeids.[3] Various species were used in bioassays of which four species were used most commonly, *Folsomia candida* Willem, *Folsomia fimetaria* L., *Onychiurus armatus*, and *Orchesella cincta*.[20] Numerous soil toxicity tests supported by Environment Canada (EC) resulted in the development and standardization of a biological test method for determining the lethal and sublethal toxicity of samples of contaminated soil to Collembola.[10] The method prepared by EC includes four species, *Orthonychiurus folsomi*, *Proisotoma minuta*, *F. candida*, and *F. fimetaria*. As standardized test systems using Collembola as indicator organisms for the habitat function of soil, another two methods exist. One is designed for assessing the effects of substances on the reproductive output of the Collembola, *F. fimetaria* and *F. candida* in soil[19],[21], and the other method described here, focuses on testing contaminated soil. Optionally the method can be used for testing substances added to standard soils (e.g. artificial soil) for their sublethal hazard potential to Collembola.

This document describes a method that is based on the determination of sublethal effects of contaminated soils to adult Collembola of the species *Folsomia candida* Willem. The species is distributed worldwide. It plays a similar ecological role to *F. fimetari*[10],[19]. *F. candida* reproduces parthenogenetically and is an easily accessible species as it is commercially available and easy to culture. *F. candida* is considered to be a representative of soil arthropods and Collembola in particular. Background information on the ecology of springtails and their use in ecotoxicological testing is available in Reference [22].

Distinct Collembolan species inhabit various ecological niches at different soil depths and in different soil types across the globe. Although considered a surrogate species and therefore frequently used in ecotoxicological reproduction tests, *F. candida* is not common in most natural soils.[28] Furthermore, species specific morphological adaptations can influence exposure and toxic effects of chemicals on organisms.[102] Thus, the use of a variety of Collembolan species representing different morphological adaptations can be advantageous to obtain a broad spectrum of sensitivities for this group. Therefore, other species like *F. fimetaria* (euedaphic, distributed worldwide and found in agricultural soils[28]), *Onychirus yodai* (an euedaphic Asian species,[31] *Proisotoma minuta* (hemiedaphic, distributed worldwide and inhabiting agricultural soils [31],[36]), *Protaphorura fimata* (euedaphic, occuring through mild temperate to cold zones [31],[37]), and *Sinella curviseta* (epedaphic, distributed from North America to Europe, Southeast Asia and Japan [42]) were added as potential alternative test species (Annex E). These species have been used as ecotoxicological test species before, but available testing experience is limited.

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

NOTE The stability of the test substance cannot be ensured over the test period. No provision is made in the test method for monitoring the persistence of the substance under test.

Soil quality — Inhibition of reproduction of Collembola (*Folsomia candida*) by soil contaminants

1 Scope

This document specifies one of the methods for evaluating the habitat function of soils and determining effects of soil contaminants and substances on the reproduction of *Folsomia candida* Willem by dermal and alimentary uptake. This document also provides information on how to use this method for testing substances under temperate conditions.

The chronic test described is applicable to soils and soil materials of unknown quality, e.g. from contaminated sites, amended soils, soils after remediation, industrial, agricultural or other sites of concern and waste materials.

The method is not applicable to volatile substances, i.e. substances for which H (Henry's constant) or the air/water partition coefficient is greater than 1, or for which the vapour pressure exceeds 300 Pa at 25 °C.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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, treated biowaste and sludge – Determination of pH*

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

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

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

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

ISO 18400-206, *Soil quality — Sampling — Part 206: Collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory*

3 Terms and definitions

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

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

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

3.2
EC*x*
effect concentration for *x* % effect
concentration (mass fraction) of a test sample or a test substance that causes *x* % of an effect on a given end-point within a given exposure period when compared with a control

EXAMPLE An EC50 is a concentration estimated to cause an effect on a test end-point in 50 % of an exposed population over a defined exposure period.

Note 1 to entry: The EC*x* is expressed as a percentage of soil to be tested (dry mass) per soil mixture (dry mass). When substances are tested, the EC*x* is expressed as mass of the test substance per dry mass of soil in milligrams per kilogram.

3.3
ER*x*
effect rate for *x* % effect
rate of a contaminated soil that causes *x* % of an effect on a given end-point within a given exposure period when compared with a control

3.4
limit test
single concentration test consisting of at least four replicates each, the soil to be tested without any dilution or the highest concentration of test substance mixed into the *control soil* (3.11) and the control

3.5
LOEC
lowest observed effect concentration
lowest test substance concentration that has a statistically significant effect ($p < 0,05$) when compared with the control

Note 1 to entry: In this test, the LOEC is expressed as a mass of test substance per dry mass of the soil to be tested. All test concentrations above the LOEC should usually show an effect that is statistically different from the control.

3.6
LOER
lowest observed effect rate
lowest rate of a contaminated soil tested in a *control soil* (3.11) that has a statistically significant effect ($p < 0,05$) when compared with the control

3.7
NOEC
no observed effect concentration
highest test substance concentration immediately below the *LOEC* (3.5) at which no statistically significant effect is observed when compared to the control

Note 1 to entry: In this test, the concentration corresponding to the NOEC has no statistically significant effect ($p < 0,05$) within a given exposure period when compared with the control.

3.8
NOER
no observed effect rate
highest rate of a contaminated soil to be tested immediately below the *LOER* (3.6) at which no statistically significant effect is observed when compared to the control

3.9
reference soil
uncontaminated soil with comparable pedological properties (nutrient concentrations, pH, organic carbon content and texture) to the soil being studied

3.10
standard soil
field-collected soil or artificial soil whose main properties (pH, texture, organic matter content) are within a known range

EXAMPLE Euro soils, artificial soil, LUFA standard soil.

Note 1 to entry: The properties of standard soils can differ from the soil to be tested.

3.11
control soil
reference soil (3.9) or *standard soil* (3.10) used as a control and as a medium for preparing dilution series with soils to be tested or a reference substance, which fulfils the validity criteria

Note 1 to entry: In the case of natural soil, it is advisable to demonstrate its suitability for a test and for achieving the test validity criteria before using the soil in a definitive test.

3.12
test mixture
mixture of contaminated soil or the test substance (e.g. chemical, biosolid, waste) with *control soil* (3.11)

3.13
test mixture ratio
ratio between the soil to be tested and the *control soil* (3.11) in a *test mixture* (3.12)

4 Principle

The effects on reproductive output of 10 d to 12 d old Collembola (*F. candida*) exposed to the soil to be tested are compared to those observed in a control soil. If appropriate, effects based on exposure to a test mixture of contaminated soil and control soil or a range of concentrations of a test substance mixed into control soil are determined. Test mixtures are prepared at the start of the test and are not renewed within the test period.

The Collembola are incubated until offspring (F_1) emerge from eggs laid by mature adults, and the number of offspring is determined. Usually offspring emerge within 28 d in control experiments. The results obtained from the tests are compared with a control or, if appropriate, are used to determine the concentrations which cause no effects on mortality and reproductive output (NOER/NOEC) and the concentration resulting in *x* % reduction of juveniles hatched from eggs compared to the control (ER*x*/EC*x*, 28 d) respectively.

If testing a concentration series, all test dilutions/concentrations above the LOER/LOEC have a harmful effect equal to or greater than that observed at the LOER/LOEC. Where there is no prior knowledge of the concentration of the soil to be tested or the test substance likely to have an effect, then it is useful to conduct the test in two steps.

— An acute toxicity test (range-finding test) is carried out, to give an indication of the effect dilution/concentration, and the dilution/concentration giving no mortality (NOER/NOEC). Dilutions/concentrations to be used in the definitive test can then be selected.

— A definitive test on the reproductive output determines sublethal effects of (dilutions of) the contaminated soil or the concentration of a substance which, when evenly mixed into the standard soil, causes no significant effects on numbers of offspring hatched from eggs compared with the control (NOER/NOEC), and the lowest concentration causing effects (LOER/LOEC).

A reference soil shall be used to demonstrate the appropriate status of the test population, and to avoid misinterpretation of results.

5 Reagents and material

5.1 Biological material, in this test, 10 d to 12 d old juvenile springtails of the species *Folsomia candida* Willem are used (see A.1 for details on synchronization of breeding).

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

5.2.1 Field-collected soil or waste

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

The field-collected soils used in the test shall be passed through a sieve of 4 mm square mesh to remove coarse fragments and thoroughly mixed. If necessary, soil may be air-dried without heating before sieving. Storage of soil to be tested should be as short as possible. The soil shall be stored in accordance with ISO 18400-206 using containers that minimize losses of soil contaminants by volatilization and sorption to the container walls. If soils or test mixtures have been stored, they should be mixed a second time immediately before use. Soil pH should not be corrected as it can influence bioavailability of soil contaminants.

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

a) pH in accordance with ISO 10390,

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

c) water content in accordance with ISO 11465,

d) water-holding capacity according to Annex B,

e) cationic exchange capacity in accordance with ISO 11260,

f) organic carbon in accordance with ISO 10694,

g) percentage of material (mineral and organic) removed by the 4 mm sieve.

The water holding capacity of all mixtures used in the test should also be measured.

5.2.2 Control soil, either a reference soil or a standard soil that allows the presence of Collembola. Control soil and soil used for dilution shall not differ in one test (either a reference soil or a standard soil).

a) If reference soils from uncontaminated areas near a contaminated site are available, they should be treated and characterized like the soils to be tested. 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, standard soils (e.g. artificial soil, LUFA) shall be used as 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 [a particle size of (2 ± 1) mm is acceptable] 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. Further guidance on how to proceed on the adjustment of pH of artificial soil is available in Annex C.

NOTE 1 Taking the properties of highly non-polar (log Kow > 2) or ionizing substances into account, 5 % of peat has proven to be sufficient for maintaining the desired structure of the artificial soil.

NOTE 2 It has been demonstrated that *F. candida* can conform to the validity criteria even on reproductive output when tested in field soils with lower organic carbon content (e.g. 2,7 %), and there is experience that this can be achieved in artificial soil with 5 % peat. Therefore, it is not necessary, before using such a soil in a definitive test, to demonstrate the suitability of the artificial soil for allowing the test to conform to the validity criteria unless the peat content is lower than that specified above.

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

The total water-holding capacity shall be determined according to Annex B; the pH shall be determined according to ISO 10390.

5.3 Food

A sufficient amount, for example, 2 mg to 10 mg, of granulated dried baker's yeast, commercially available for household use, is added to each container as a suitable food source, at the beginning of the test and after about two weeks.

5.4 Reference substance

To ensure the quality of the test system, tests should be performed regularly (once or twice a year) with a reference substance.

Boric acid and the plant protection product Betosip[1)] (a.i. 157 g/l phenmedipham) have been tested in an interlaboratory test and are recommended as reference substances.

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

NOTE 1 Boric acid: Effects on reproductive output (i.e. EC50) are observed at concentrations of 147 mg boric acid per kilogram of artificial soil (dry mass), 111 mg boric acid per kilogram of artificial soil with 5 % peat, and 169 mg boric acid per kilogram of clay loam soil *for F. candida*.[5],[21] Taking into consideration these data and due to the variability of organism sensitivity, an EC50 value between 50 mg and 175 mg boric acid/kg dry mass of artificial soil is acceptable based on current laboratory experience and in previous studies [103],[104].

NOTE 2 Betosip: Effects on reproductive output (α = 0,05) are observed at concentrations between 100 mg and 200 mg of the product per kilogram of the substrate (dry mass).

1) Betosip is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

6 Apparatus

Use laboratory equipment and the following apparatus:

6.1 Test containers made of glass or other chemically inert material of about 100 ml capacity and with a diameter of about 5 cm, with lids (e.g. plastic, glass discs or parafilm, able to be closed tightly).

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

6.3 Large scale laboratory mixer for the preparation of the test mixture (5.2).

6.4 Suitable accurate balances.

6.5 Apparatus capable of measuring pH.

6.6 Apparatus to determine water-holding capacity of the substrate (see B.2).

6.7 Exhauster for transfer of springtails (see A.2).

6.8 Test environment.

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

6.8.2 Light source, capable of delivering a constant light intensity of 400 lx to 800 lx at the substrate surface at a controlled light: dark cycle of between 12 h:12 h and 16 h:8 h.

7 Procedure

7.1 Experimental design

7.1.1 General

A sample of field-collected soil can be tested at a single concentration (typically 100 %) or evaluated for toxicity in a multi-concentration test whereby a series of concentrations (dilutions) is prepared by mixing measured quantities with a control soil (5.2.2). When testing substances, a series of concentrations is prepared by mixing quantities of the test substance with a standard soil (e.g. artificial soil). The concentrations being expressed in milligrams of test substance per kilogram of dried control soil (5.2.2). Depending on the knowledge of relevant response levels a range-finding test may precede the definitive test. Each definitive test consists of a series of soil mixtures (treatments).

7.1.2 Range-finding test

A test to find the range of contaminated soil mixture ratios (e.g. 0 %, 1 %, 5 %, 25 %, 50 %, 75 %, 100 %) or concentrations of the test substance (e.g. 0 mg/kg, 1 mg/kg, 10 mg/kg, 100 mg/kg, 1 000 mg/kg) affecting Collembola is optional. The range-finding test is conducted without replication.

When no effects are observed, even at 100 % contaminated soil or at concentrations of 1 000 mg test substance per kilogram of standard soil (dry mass), the definitive test can be designed as a limit test.

Each test container (replicate) is filled with 30 g wet mass of the test sample. To ensure easy migration of springtails, the substrate in the test container should not be compressed.

Use 10 specimens of 10 d to 12 d old Collembola per container. Prepare the test containers as indicated in 7.2.1. Place the test containers in the test enclosure (6.8.1) with the light source (6.8.2).

At the beginning of the test, add about 2 mg of granulated dry yeast (5.3) to each test container, and cover the containers tightly (e.g. using plastic, glass discs or parafilm). Open the test containers briefly twice a week to allow aeration.

After 14 d, count the live Collembola in each container, and determine the percentage mortality for each test substance concentration. Also, observe surviving Collembola and record any symptoms. Due to the rapid degradation of dead Collembola, missing Collembola are assumed to have died during the test period.

NOTE To obtain additional information for the determination of the concentration range for the final test, the test period can be extended to four weeks to allow qualitative determination of effects at concentrations at which effects on reproductive output can be expected.

7.1.3 Definitive test

The design of the definitive test depends on the test objectives. Typically, the habitat properties of samples of a field-collected soil are characterized by comparison of the biological effects found in the soil to be tested with those found in a reference soil, or if not available or not appropriate due to toxicity or atypical physicochemical characteristics, in a standard soil. Results for the standard soil assist in distinguishing contaminant effects from non-contaminant effects caused by soil physicochemical properties. Regardless of whether a reference soil or standard soil is used for the statistical comparisons, the results from standard soil shall be used to judge the validity and acceptability of the test[20].

If for characterization purposes a test design including dilution series is required, three designs are possible (the concentrations shall be spaced by a factor not exceeding 2).

— For the NOEC/NOER approach, at least five concentrations in a geometric series should be used. Four replicates for each concentration plus eight controls are recommended.

— For the ER*x*/EC*x* approach, 12 concentrations should be used. Two replicates for each concentration plus six controls are recommended. The spacing factor can be variable; smaller at low concentrations, larger at high concentrations.

— For the mixed approach, six concentrations to eight concentrations in a geometric series should be used. Four replicates for each concentration plus eight controls are recommended. This mixed approach allows a NOEC as well as an ER*x*/EC*x* evaluation.

A limit test can be sufficient if no toxic effect is observed in the range-finding test.

To facilitate checking of the pH and humidity of the test sample, use of additional containers for each concentration and for the control is recommended.

Each test container (replicate) is filled with 30 g wet mass of the test sample. To ensure easy migration of Collembola, the substrate in the test container should not be compressed.

7.2 Preparation of test mixture

7.2.1 Testing contaminated soil

According to the selected dilution range, the soil to be tested is mixed with the reference soil or the standard soil thoroughly (either manually or by using a hand mixer). The homogeneity of the mixture is checked visually. The total mass of the soil to be tested and the reference soil or the standard soil shall be 30 g (wet mass) in each test container (6.1). The test mixture shall be wetted with deionized water to reach 40 % to 60 % of the total water holding capacity determined according to Annex B. In some cases, for example, when testing waste materials, higher percentages are required. A rough check of the soil moisture content can be obtained by gently squeezing the soil in the hand; if the moisture content is correct, small drops of water should appear between the fingers.

Determine the pH for each test mixture (one container per concentration) according to ISO 10390 at the beginning and end of the test (when acid or basic substances are tested, do not adjust the pH).