



« Soil quality – Biological methods »

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**ISO/CD 11267 – Inhibition of reproduction of Collembola
(Folsomia candida) by soil pollutants**

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

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

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Foreword

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

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ISO 11267 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

This second/third/... edition cancels and replaces the first/second/... edition (), [clause(s) / subclause(s) / table(s) / figure(s) / annex(es)] of which [has / have] been technically revised.

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 and ISO 17616). ISO 15799 includes a list and short characterisation of recommended and standardized test systems and ISO 17616 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 relevant ecologically 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 may be the most important soil invertebrates besides enchytraeids with respect to that function, since earthworms are typically absent [23]. Additionally, collembolans represent arthropod species with a different route and a different rate of exposure compared to earthworms [3] and enchytraeids [8]. Various species were used in bioassays of which four species were used most commonly, *Folsomia candida*, *Folsomia fimetaria*, *Onychiurus armatus*, and *Orchesella cincta* [24]. 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 collembolans [14]. The method prepared by EC includes three species, *Orthonychiurus folsomi*, *Folsomia candida*, and *Folsomia fimetaria*. As standardized test systems using springtails as indicator organisms for the habitat function of soil, another two methods exist. One is designed for assessing the effects of chemicals on the reproductive output of the collembolans, *Folsomia fimetaria* L. and *Folsomia candida* Willem in soil [23], [25], and the other method being described here, focusses on testing contaminated soil. Optionally the method can be used for testing chemicals added to standard soils (e.g. artificial soil) for their sublethal hazard potential to collembolans.

This International Standard describes a method that is based on the determination of sublethal effects of contaminated soils to adult springtails of the species *Folsomia candida* (Willem). The species is distributed world wide. It plays a similar ecological role as *Folsomia fimetaria* [14], [23]. *Folsomia candida* reproduces partheogenetically and is an easy accessible species as it is commercially available and easy to culture. *Folsomia candida* is considered to be a representative of soil arthropods and collembolans in particular. Background information on the ecology of springtails and their use in ecotoxicological testing is available [26].

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

1 Scope

This International Standard specifies one of the methods for evaluating the habitat function of soils and determining effects of soil contaminants and chemicals to the reproduction of *Folsomia candida* (Willem) by dermal and alimentary uptake. This chronic test is applicable to soils and soil materials of unknown quality e. g. from contaminated sites, amended soils, soils after remediation, agricultural or other sites under concern and waste materials.

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

Information is provided how to use this method for testing chemicals. 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 0,013 3 Pa at 25 °C. The stability of the test substance cannot be assured over the test period. No allowance is made in the test method described for possible degradation of the test substance over the course of the experiment.

WARNING — Contaminated soils may contain unknown mixtures of toxic, mutagenic, or otherwise harmful chemicals or infectious micro-organisms. Occupational health risks may arise from dust or evaporated chemicals during handling and incubation. Precautions should be taken to avoid skin contact.

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 10381-6, *Soil quality — Sampling — Part 6: Guidance on the collection, handling and storage of soil under aerobic conditions for the assessment of microbial processes, biomass and diversity in the laboratory*

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

ISO 10390:2005, *Soil quality — Determination of pH*

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

ISO/DIS 11268-2:2011, *Soil quality — Effects of pollutants on earthworms — Part 2: Determination of effects on reproduction to Eisenia fetida/Eisenia andrei*

ISO 11274, *Soil quality — Determination of the water retention characteristic — Laboratory methods*

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

ISO 11461, *Soil quality — Determination of soil water content as a volume fraction using coring sleeves — Gravimetric method*

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

ISO 15176:2002, *Soil quality — Characterization of excavated soil and other soil materials intended for re-use*

3 Terms and definitions

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

3.1

contaminant

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

[ISO 15176:2002].

3.2

ER_x (effective rate) or EC_x (effective concentration)

x % effect rate or concentration of the test soil or test substance at which reproduction is reduced by x % compared to the control

3.3

lowest observed effect rate (LOER) or effect concentration (LOEC)

lowest tested percentage of a test soil in a reference or a standard control soil or concentration of a substance at which a statistically significant effect is observed

NOTE The LOEC is expressed as percentage of test soil dry mass per test mixture dry mass. All test mixtures above the LOEC have a harmful effect equal or greater than observed at the LOEC. If this condition cannot be satisfied, an explanation should be given for how the LOEC and NOEC (3.4) have been selected.

3.4

NOER (No Observed Effective Rate) or NOEC (No Observed Effect Concentration)

test soil percentage immediately below the LOER/LOEC or, highest tested concentration of a test substance which when compared to the control has no statistically significant lethal or other effect such as reduced reproduction or mass alteration ($p < 0,05$)

NOTE The NOEC is expressed as percentage of test soil dry mass per test mixture dry mass.

3.5

reference soil

uncontaminated site-specific soil (e. g. collected in the vicinity of a contaminated site) with similar properties (nutrient concentrations, pH, organic carbon content and texture) as the test soil

3.6

standard soil

field collected soil or artificial soil whose main properties (e. g. pH, texture, organic matter content) are within a known range

EXAMPLE Euro-Soils [20], artificial soil [22], LUFA Standard Soil [21].

NOTE The properties of standard soils can differ from the test soil.

3.7**control soil**

reference or standard soil used as a control and as medium for preparing dilution series with test soils or a reference substance, that allows the presence of earthworms

3.8**test mixture**

mixture of contaminated soil or the test substance with control soil (3.9)

NOTE Test mixtures are given in per cent contaminated soil based on soil dry mass.

3.9**test mixture ratio**

ratio between the test soil and the control soil in a test mixture

NOTE Different ratios may be applied in a dilution series to establish a dose-response relationship.

4 Principle

The effects on reproduction of 10- to 12-day old springtails (*Folsomia candida*) exposed to the test soil are compared to those observed to samples exposed to a control soil. If appropriate, effects based on exposure to a dilution range of contaminated soil or a range of concentrations of a test substance are determined. Test mixtures are prepared at the start of the test and are not renewed within the test period.

The springtails are incubated until offspring (F_1) emerge from eggs laid by mature adults, and the number of offspring is determined. Normally offspring emerge within 28 days in control experiments. The results obtained from the tests are compared with a control soil or, if appropriate, are used to determine the dilutions or concentrations which cause no effects on mortality and reproduction (NOER/NOEC) and the dilution (concentration) resulting in x % reduction of juveniles hatched from eggs compared to the control (ERx/ECx, 28 d) respectively.

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

- a preliminary 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;
- the definitive test to determine sublethal effects of (dilutions of) contaminated soil or the concentration of a chemical 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).

NOTE The use of a reference soil is an essential requirement to demonstrate the present status of the test population, and to avoid misinterpretation of results.

5 Reagents**5.1 Biological material**

In this test, 10- to 12-day old juvenile springtails of the species *Folsomia candida* (Willem) are used (see A.1 for details on synchronization of breeding).

5.2 Test substrate may consist of field-collected soil or control soil amended by the test substance.

5.2.1 Field-collected soils, soil or waste materials

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.

Test soils shall be sieved by 4 mm mesh and thoroughly mixed. If necessary, soil may be air dried without heating before sieving. Storage of test soils should be as short as possible. Store the soil in accordance with ISO 10381-6 using containers that minimise losses of soil contaminants by volatilisation and sorption to the container walls. If soils or soil 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 D,
- e) cationic exchange capacity in accordance with ISO 11260,
- f) organic carbon in accordance with ISO 10694,

NOTE It is important to measure the water holding capacity of all mixtures used in the test.

5.2.2 Control soil, either a) reference soil (3.5) or b) standard soil (3.7) that allows the presence of earthworms.

- g) If reference soils from uncontaminated areas near a contaminated site are available, they should be treated and characterised like the test soils. If a toxic contamination or unusual soil properties cannot be ruled out, standard control soils should be preferred.
- h) For testing the effects of substances mixed into soil or making dilutions of the test soil, standard soils 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 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 (CaCO₃, pulverised, analytical grade) are necessary to get a pH of 6,0 ± 0,5.

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

NOTE 2 It has been demonstrated that *Folsomia candida* can comply with the validity criteria even on reproduction 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 comply with the validity criteria unless the peat contents lowered more than specified above [??].

Prepare the artificial soil at least three days prior to start 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 while mixing is continued. 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 pH and the maximum water holding capacity, the dry artificial soil is pre-moistened one or two days before starting the test by adding deionised water to obtain half of the required final water content of 40 % to 60 % of the maximum water holding capacity.

The total water holding capacity is determined according to Annex D, the pH is determined according to ISO 10390.

NOTE 3 Allowance should be made for any water that is used for introducing the test substance into the soil.

5.3 Reference substance

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

The agricultural chemical Betosip (a.i. 157 g/l Phenmedipham) and boric acid have been tested in a ring test, and are recommended as reference substances.

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

NOTE 1 Betosip: Effects on reproduction ($\alpha = 0,05$) were observed at concentrations of between 100 mg and 200 mg of the product per kilogram dry mass of the substrate.

NOTE 2 Boric acid: Effects on reproduction were observed at concentrations (IC_{50}) of 147 mg boric acid/kg dry weight of artificial soil and 169 mg boric acid/kg clay-loam soil dry weight) [9], [25].

6 Apparatus

Standard laboratory equipment, and:

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

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 substrate (5.2).

6.4 Precision balance with an accuracy of at least 1 mg.

6.5 Apparatus capable of measuring pH and water content of the substrate.

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

6.7 Test environment

6.7.1 Enclosure, capable of being controlled to a temperature of $20\text{ °C} \pm 2\text{ °C}$.

6.7.2 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 test soil can be tested at a single concentration (typically 100 %) or evaluated for toxicity in a multi-concentration test whereby a series of dilutions are 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). Depending on the knowledge of relevant response levels a preliminary test may precede the definitive test. Each definitive test consists of a series of soil mixtures (treatments). Each treatment is replicated at least four times.

7.1.2 Preliminary test

A preliminary test to find the range of mixture ratio affecting earthworms is optional, e.g. 0 %, 1 %, 5 %, 25 %, 50 %, 75 %, 100 %, or of the test substance, e.g. 0 mg/kg, 1 mg/kg, 10 mg/kg, 100 mg/kg and 1 000 mg/kg (the concentrations being expressed in milligrams of test substance per kilogram of dried control soil (5.2.2) and a control using ten worms per container). The preliminary test is conducted without replication.

When no effects are observed, even at 100 % contaminated soil or at concentrations of 1 000 mg test substance/kg 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 substrate. To ensure easy migration of springtails, the substrate in the test container should not be compressed.

Use 10 specimens of 10- to 12-day old springtails per mixture ratio or concentration and per container. Prepare the test containers as indicated in 7.2.1. Place the test containers in the test enclosure (6.7.1) with the light source (6.7.2).

At the beginning of the test, add about 2 mg of granulated dry yeast 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 days, count the live springtails in each container, and determine the percentage mortality for each test substance concentration. Also observe surviving springtails and record any symptoms. Due to the rapid degradation of dead springtails, missing springtails 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 reproduction could 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 test soil are characterised by comparison of the biological effects found in the test soil(s) with those found in the control soil (3.7) (single-concentration tests). If a reference soil (3.5) to be used as control is not available or not appropriate due to toxicity or atypical physicochemical characteristics, effects are compared to a standard soil instead. If a reference soil is available to be used as control soil, it is recommended to include additionally a standard soil exhibiting a typical known response, and to use these results to judge the validity and acceptability of the test [24]. Results found for the standard soil assist in distinguishing contaminant effects from non-contaminant effects caused by soil physicochemical properties of the test soil and/or the control soil.

If for characterisation 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 approach, at least five concentrations in a geometric series should be used. Four replicates for each concentration plus eight controls are recommended.
- For the 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, 6 to 8 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 EC_x evaluation.

A limit test can be sufficient if in the preliminary test no toxic effect was observed. In the limit test only the test soil without any dilution and the control (or the test soil vs. the control soil) shall be tested with at least four replicates each.

To facilitate checking of the pH and humidity of the test substrate, 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 substrate. To ensure easy migration of springtails, the substrate in the test container should not be compressed.

7.2 Preparation of test mixture

7.2.1 Testing contaminated soil

Mix the test soil with the reference soil or the standard soil thoroughly (either manually or by using a hand mixer) according to the selected dilution range. Check the homogeneity of the mixture visually. The total mass of the test soil and the reference soil or the standard soil shall be 30 g (wet mass) in each test container (6.1). Wet the test mixture with deionised water to reach an appropriate water content of usually 40 % to 60 % of the total water holding capacity determined according to Annex C. In some cases, e. g. 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).

Proceed simultaneously with at least four replicates per concentration and the control(s).

WARNING — Contaminated soils may contain unknown mixtures of toxic, mutagenic, or otherwise harmful chemicals or infectious micro-organisms. Precautions should be taken to avoid skin contact. Occupational health risks may arise from dust or evaporated chemicals during handling and incubation.

7.2.2 Testing substances added to the test substrate

Standard soil (5.2.2) is used as test substrate. For each test container (6.1), the mass of the substrate used shall be 30 g (wet mass). Add substances to the test substrate and mix thoroughly.

For the introduction of test substances use either method a), b) or c), as appropriate:

a) Water-soluble substance

- immediately before starting the test, dissolve the quantity of the test substance in the water required for the replicates of one concentration in water (or that portion of it necessary to wet the soil substrate) in order to meet the requirements of 5.2.2. Mix it thoroughly with the soil substrate before introducing it into a test container.

b) Substances insoluble in water but soluble in organic solvents

- Dissolve the quantity of test substance required to obtain the desired concentration in a volatile solvent (such as acetone or hexane) mix it with a portion of the quartz sand required. After evaporating the solvent by placing the container under a fume hood, add the remainder of the standard soil and the water and mix it thoroughly before introducing it into the test containers.

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

WARNING — Take appropriate precautions when dealing with solvent vapour to avoid danger from inhalation or explosion, and to avoid damage to extraction equipment, pumps etc.

c) Substances insoluble in water or organic solvents

- For a substance insoluble in a volatile solvent, prepare a mixture of 10 g of finely ground industrial quartz sand (see 5.2.2) and the quantity of the test substance required to obtain the desired concentration. Add that mixture to the remainder of the standard soil and the water and mix thoroughly before introducing it into a test container.

Mix the test substance into the standard soil before the earthworms are added.

Base the concentrations selected to provide the LOEC/NOEC on the results of the preliminary test. Space the concentrations by a factor not exceeding 2. Substances mixed into the substrate do not need to be tested at concentrations higher than 1 000 mg/kg mass of test substrate. Proceed simultaneously with at least four replicates per concentration and the control(s).

Determine the pH for each test substrate (one container per concentration) according to ISO 10390 at the beginning and end of the test.

7.2.3 Preparation of control container

The control container contains the control soil (5.2.2) wetted with deionised water to reach 40 % to 60 % of the total water holding capacity (determined according to Annex C).

Perform one control container for the preliminary test and at least four control containers for the definitive test.

Prepare the control containers in the same way as the test containers. If the preparation of the test requires the use of a solvent (see 7.2.2), use an additional control prepared with solvent but without the test substance. Cover the containers as indicated in 6.1.

7.3 Introduction of the test organisms

Ten juvenile springtails (10 to 12 days old) are placed in each test container.

Springtails are tapped or sucked from the breeding containers to transfer them to the test containers. This can easily be done using an exhaustor as described in clause A.2. Before they are transferred to the test containers, organisms are counted and checked for damage both to reduce control mortality and to avoid systematic trial errors.

7.4 Test conditions and measurements

At the beginning of the test and after a period of 14 days, add about 2 mg of granulated dry yeast 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.

Determine the water content and the pH (in the presence of 1 mol/l KCl) of the artificial soil at the beginning and end of the test. When acidic or basic substances are tested, do not adjust the pH.

After two weeks, check the water content by reweighing the additional test containers, and compensate for water loss if it exceeds 2 % of the initial water content.

7.5 Determination of surviving springtails

Determine the number of springtails present four weeks after introducing the parental springtails onto the test and control substrates. Pour the test substrate into a 500 ml to 600 ml container and add water. After gentle stirring of the suspension with a spatula, springtails drift to the water surface. Count adults and juveniles, if present, by a suitable procedure (see Annex B) and report the numbers.

NOTE Other extraction methods (e.g. high-gradient extraction) may be used if they have proved to be effective.

8 Calculation and expression of results

8.1 Calculation

For each dilution or concentration, determine the percent mortality and number of offspring produced after a period of four weeks.

8.2 Expression of results

A graphical presentation of the mean values of the endpoints including standard deviation of the measured values against the test soil(s), control soil(s) or the selected series of soil mixture ratios should be prepared. This comparison or curve gives an impression of the quality of effects and their magnitudes. Express the mixture ratio as based on soil dry mass.

If dilution or concentration series were performed indicate:

- in the EC_x-approach the % soil mixture based on dry mass or in milligrams per kilogram of dried soil substrate, the median percent dilution of contaminated soil or median concentration of the test substance, which reduces the number of juvenile worms to 50 % (EC₅₀) compared to the control within the test period or
- in the NOEC-approach the soil mixture ratio immediately below the LOEC or highest tested concentration of a test substance which when compared to the control has no statistically significant lethal or other effect such as mass alteration and reduction of reproduction ($p < 0,05$).

9 Validity of the test

The results are considered to be valid if:

- The mortality of the adults in the control(s) should not exceed 20 % at the end of the test.
- The reproduction rate should reach a minimum of 100 instars per control vessel.
- The coefficient of variation of reproduction in the control should not exceed 30 %.

10 Statistical analysis

10.1 General

Most of test methods with sub-lethal endpoints, e. g. growth, reproduction, involve quantitative effects, e. g. measuring the weight of the organisms or counting juvenile worms. Quantal effects may also be measured in the same test, such as mortality after four weeks exposure.

NOTE Guidance given here for statistical evaluation of test results aims to make the investigator aware of problems that can arise in consequence of a test design selected. Computer programs do not necessarily guard against violations of rules that can cause erroneous analyses. It is strongly recommended to look for more information in specific guidance documents (e. g. as provided by [13]) or to contact a statistician.

10.2 Single-concentration tests

Quantitative single-concentration tests (e. g. effects on reproduction or the biomass development) have different statistical methods. For sampling at several locations with field replication, ANOVA would be a first step if results were suitable. If the null hypothesis of no difference was rejected, analysis would proceed to one of several multiple-comparison tests [13].

An example of a single-concentration test for quantitative effects can be counting juvenile worms as endpoint of effects on reproduction or measuring the average biomass of earthworms after exposure to a sample of undiluted contaminated soil, compared to numbers of juvenile worms or biomass of earthworms exposed to a reference or standard soil. If there was only one sample tested, and one control material, without any replicates, results can be not compared by any statistical test. In a quantitative test with replication for the test soil (material) and for the control soil, a standard t-test would be suitable for statistical analysis.

Analysis of variance (ANOVA) involving multiple comparisons of endpoint data derived for undiluted test soils including field replicates of field-collected soil from more than one sampling location is commonly used for statistical interpretation of the significance of quantitative findings (e. g. biomass) from soil toxicity tests. This is a hypothesis-testing approach, and is subject to appreciable weaknesses [13]. The parametric analyses (e. g. ANOVA and multiple comparisons) for such data assume that the data are normally distributed, that the treatments are independent, and that the variance is homogenous among the different treatments. These assumptions shall be tested. If the data satisfy these assumptions, analysis may proceed. If not, data may be transformed and tested again. As parametric tests are reasonably robust in the face of moderate deviations from normality and equality of variance, parametric analysis should proceed, even if moderate nonconformity continues after transformation, [13].

10.3 Multi-concentration tests

10.3.1 Preliminary test

If a clear dose-response is obvious, EC_x-values can be estimated by using regression techniques like logistic regression function or probit analysis. In other cases the effect range should be determined by expert knowledge.

10.3.2 Definitive test

A point estimate (ER_x/EC_x-approach) is recommended as the best quantitative endpoint. This is usually a specific degree of reduction in performance compared to the control. Linear and non-linear regression methods are widely applied for statistical analysis. Operators should be aware of being able to understand the judgements in selecting appropriate mathematical models.

Hypothesis testing (NOEC-approach) is commonly used to identify dilutions (concentrations) with significant effects compared to the control. As this method has many flaws it is not recommended for future use,

Therefore, in cases where various dilutions (concentrations) of each sample of field-collected soil with negative control soil are tested, data preferably are analysed by the EC_x-approach, or if required by legislation, by the NOEC-approach:

— ER_x/EC_x (effect concentration)-approach

The ER_x/EC_x-approach can only be used if a clear dose response relationship is found. Wherever possible, the R² should be 0,7 or higher and the test mixtures used encompass 20 % to 80 % effects. If these requirements are not fulfilled, expert knowledge is necessary for the interpretation of the test results.

To compute an ER_x/EC_x-value, the treatment means are used for regression analysis after an appropriate dose-response function has been found (e. g. probit or logistic function). A desired ER_x/EC_x is obtained by inserting a value corresponding to x% of the control mean into the equation found by regression analysis. Since EC₅₀ values have smaller confidence limits compared with smaller effect concentrations (e. g. ER/EC₂₀), it is recommended to determine ER/EC₅₀ values.

— NOEC (No-observed-effect-concentration)-approach

First of all, a statistical analysis of the homogeneity of the variances shall be made, e. g. by using Cochran's test. With homogeneous data, an appropriate statistical analysis, e. g. a „One-Way Analysis of Variance (ANOVA)“, followed by a one-sided Dunnett test ($\alpha = 0,05$), should be performed. If the homogeneity requirement is not fulfilled, it is recommended to evaluate if an appropriate transformation of the data can solve the problem. Otherwise non-parametric methods, e. g. the U-test by Mann & Whitney or the Bonferroni-U-Test can be used.

If a limit test has been performed and the pre-requisites (normality, homogeneity) of parametric test procedures are fulfilled, the Student-t-test, otherwise the Mann-Whitney-U-test procedure should be used.

In any case the results of the statistical evaluation shall be biologically interpreted.

NOTE Guidance given here for statistical evaluation of test results aims to make the investigator aware of problems that can arise in consequence of a test design selected. Computer programs do not necessarily guard against violations of rules that can cause erroneous analyses. It is strongly recommended to look for more information in specific guidance documents (e. g. as provided by [13]) or to contact a statistician in any case, a special design is used.

11 Test report

The test report shall include the following information:

- a) a reference to ISO 11267;
- b) the results, expressed as in 8.2;
- c) detailed description of the test substance and information on physical and chemical properties if helpful for the interpretation of the test result;
- d) complete description of the biological material employed (species, age, breeding conditions, supplier);
- e) method of preparation of the test substrate together with an indication of the auxiliary substances used for a low-/non-water-soluble substance;
- f) results obtained with the reference substance, if performed;
- g) detailed conditions of the test environment;
- h) a table giving the percent mortality of adults at each concentration and in the control(s);
- i) number of dead or missing adults and number of offspring per test container at the end of the test;

- j) depending on the statistical approach selected, list the lowest concentration causing significant effects (LOEC), the highest concentration causing no observed effects (NOEC), EC₁₀ and EC₅₀ for the inhibition of reproduction and the method used for calculation (optional);
- k) description of any pathological or other symptoms, or distinct changes in behaviour observed in the test organisms per test container;
- l) water content and pH of artificial soil at the start and at the end of the test for the control and for each concentration;
- m) any operating details not specified in this International Standard, as well as any factors that may have affected the results.

Annex A (informative)

Techniques for rearing and breeding of Collembola

A.1 Conditions for rearing and breeding

A.1.1 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 to 11:1) may also be used. Depending on the type of plaster, 60 g to 100 g 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 indicators placed on this wet substrate surface.

A.1.2 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 time.

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

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

A.1.3 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.1.4 Food

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

A.1.5 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.1.6 Test organisms of a standard age

To obtain 10- to 12-day old juvenile springtails for the test, transfer egg clusters from breeding containers to a freshly prepared breeding substrate, using a fine spatula or hair brush. After 48 h, remove the egg clusters and feed instars hatched from the eggs.

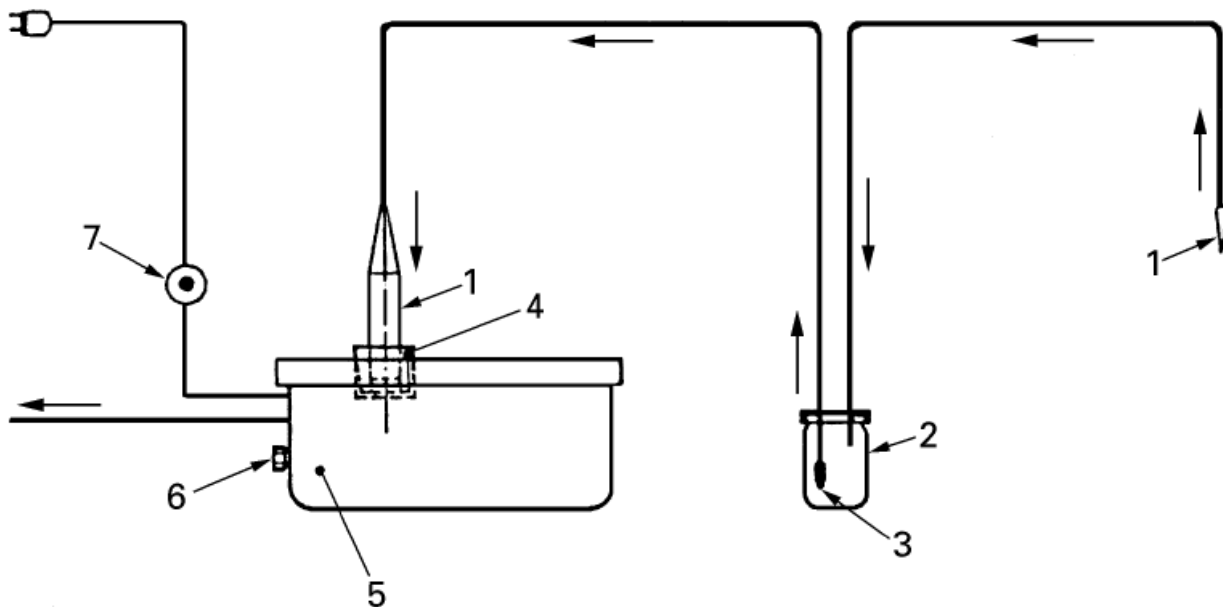
NOTE The egg clusters are easily removed if they are placed on small pieces of breeding substrate or cover glasses laid on the breeding substrate, and juvenile springtails collected after a further 10 days incubation.

Alternatively, 10- to 12-day old juvenile springtails may 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 few eggs will be produced in two days. If this is observed to be 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 reduced growth. As a consequence, the 10- to 12-day old animals used for the test may be too small and not yet able to produce a sufficient number of eggs to meet the requirements of the test.

A.2 Transfer of springtails to the test containers

The springtails are easily transferred from the breeding substrate to the test substrate by an exhaustor. An example is shown in Figure A.1.



Key

- | | |
|--|----------------------------|
| 1 Eppendorf-pipette tip | 4 rubber stopper |
| 2 cylinder (10 ml volume) with rolled flange and plastic cover for control of sucked springtails | 5 commercial aquarium pump |
| 3 gauze | 6 regulator |
| | 7 foot switch |

Figure A.1 — Low-suction exhaustor apparatus for transfer of springtails

The springtails are sucked individually through a pipette tip to a small covered container to control damage of springtails. Care shall be taken to ensure that the suction of the pump is low to avoid damage to the springtails. After removing the cover, springtails provided for one test container are transferred onto the substrate surface of the test container.

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 annealed in a flame to smooth the cut 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.

Warning: If tests are performed regularly, sucking up the juveniles by mouth should be avoided as technicians may run the risk of becoming allergic to materials (e.g. fungal spores, faeces, exoskeleton particles etc.) inhaled.

Annex B **(informative)**

Techniques for counting juvenile springtails

For evaluating effects on reproduction, the juvenile springtails swimming on the surface of the watered substrate are counted. When reproduction is high, the use of technical devices to facilitate counting is recommended.

If the swimming juveniles are distributed evenly over the water surface, a counting grid may be used and a sample for counting taken at random. Aggregations of instars on the water surface can be a problem and can be prevented by adding a drop of sewing-machine oil.

A culture-counter may also be used to count the animals on a projected picture (slide) of the water surface. Normal photographic equipment is adequate for this purpose (e. g. single-lens reflex camera, macro-lens or other device for close-up photography). Film speed has to be adjusted according to light intensity (e. g. flash or cold light source) and the desired shutter speed. If a cold light source is used, the minimum film speed value is 400 ASA. A useful relation between a sufficient projection format on a screen and the beaker volume is met with volumes of 50 ml to 100 ml (lens used: Zeiss S-Planar 1:2,8 f = 60 mm, angular field 39°, min. focus 0,24 m). To improve the contrast between white springtails and surrounding water surface, water may be coloured dark with ink.

Overall, the average error of counting should not exceed 10 %.

NOTE To avoid errors in determining mortality of the parental springtails, the number of live adults floating on the water surface should be counted by using a binocular microscope.

Annex C (informative)

Determination of water-holding capacity of artificial soil

C.1 General

The following method has been found to be appropriate for the purposes of determination of water-holding capacity (WHC) of artificial soil and may be used as an alternative to ISO 11274.

C.2 Apparatus

C.2.1 Glass tube, approximately 20 mm to 50 mm diameter and at least 100 mm in length.

C.2.2 Water bath, at room temperature.

C.2.3 Filter paper.

C.2.4 Drying oven, set to 105 °C ± 5 °C.

C.2.5 Balance, capable of weighing with an accuracy of ± 0,1 g.

C.3 Procedure

Plug the bottom of the tube with a filter paper, and after filling with the artificial soil substrate to a depth of 5 cm to 7 cm, place the tube on a rack in a water bath. Gradually submerge the tube until the water level is above the top of the soil. Leave the substrate sample in the water for about 3 h.

As not all water absorbed by the substrate capillary can be retained, the tube containing the sample should be placed for a period of 2 h on very wet finely ground quartz sand for draining. The same quartz sand as is used for the soil substrate is satisfactory.

Weigh the sample, dry it to constant mass at 105 °C and reweigh it.

C.4 Calculation of water-holding capacity (WHC)

$$\text{WHC} = \frac{S - T - D}{D} \times 100$$

where

WHC is the water-holding capacity, expressed as a percent of dry mass;

S is the mass of water-saturated substrate + mass of tube + mass of filter paper;

T is the tare (mass of tube + mass of filter paper);

D is the dry mass of substrate.

Annex D
(informative)

Guidance on adjustment of pH of artificial soil

To estimate how much CaCO_3 is needed to obtain the desired pH ($6,0 \pm 0,5$), artificial soil is prepared by mixing peat, sand, kaolin and water as described in 5.2.1. Small portions are taken and mixed with different amounts of CaCO_3 e.g. corresponding with concentrations of 0,2 %, 0,4 %, 0,6 %, 0,8 % and 1,0 % dry mass. From these portions, the pH is determined as described in ISO 10390 and the results are plotted as a graph of pH versus the amount of CaCO_3 . From this graph, the amount of CaCO_3 necessary to obtain a pH of $6,0 \pm 0,5$ can be estimated.

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