
**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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Contents

Page

Foreword	iv
Introduction	v
1 Scope	1
2 Normative references	1
3 Terms and definitions	1
4 Principle	3
5 Reagents and material	4
6 Apparatus	6
7 Procedure	6
7.1 Experimental design	6
7.2 Preparation of test mixture	7
7.3 Addition of the biological material	9
7.4 Test conditions and measurements	9
7.5 Determination of surviving Collembola	9
8 Calculation and expression of results	9
8.1 Calculation	9
8.2 Expression of results	9
9 Validity of the test	10
10 Statistical analysis	10
10.1 General	10
10.2 Single-concentration tests	10
10.3 Multi-concentration tests	11
11 Test report	11
Annex A (informative) Techniques for rearing and breeding of Collembola	13
Annex B (informative) Determination of water-holding capacity	15
Annex C (informative) Guidance on adjustment of pH of artificial soil	16
Annex D (informative) Extraction and counting of Collembola	17
Bibliography	18

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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information

The committee responsible for this document is ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

This second edition cancels and replaces the first edition (ISO 11267:1999), which has 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 [2] and [4]). Reference [2] includes a list and short characterization of recommended and standardized test systems and [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 may be 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*, *Folsomia fimetaria*, *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 three species, *Orthonychiurus folsomi*, *Folsomia candida*, and *Folsomia 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, *Folsomia fimetaria* L. and *Folsomia candida* Willem 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 International Standard 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 *Folsomia fimetaria*. [10],[19] *Folsomia candida* reproduces parthenogenetically and is an easily accessible species as it is commercially available and easy to culture. *Folsomia 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.[22]

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

1 Scope

This International Standard 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 chronic test 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.

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).

This International Standard provides information on how to use this method for testing substances under temperate conditions.

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.

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.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. 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 microbiological processes, biomass and diversity in the laboratory*

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

ISO 10390, *Soil quality — Determination of pH*

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*

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
- 3.2**
EC_x
effect concentration for x % effect
concentration (mass fraction) of 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
rate of a soil to be tested that causes an 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 and the control
- 3.5**
LOEC
lowest observed effect concentration
lowest test substance concentration that has a statistically significant effect (probability $p < 0,05$)
- 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 soil to be tested in a control soil at which a statistically significant effect is observed
- 3.7**
NOEC
no observed effect concentration
highest test substance concentration immediately below the LOEC at which no effect is observed
- Note 1 to entry: In this test, the concentration corresponding to the NOEC has no statistically significant effect (probability $p < 0,05$) within a given exposure period when compared with the control.
- 3.8**
NOER
no observed effect rate
lowest rate of a soil to be tested immediately below the LOER which, when compared to the control, has no statistically significant effect (probability $p < 0,05$) within a given exposure period
- 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 or standard soil 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

3.13**test mixture ratio**

ratio between the soil to be tested and the control soil in a test mixture

4 Principle

The effects on reproduction of 10 d to 12 d old Collembola (*Folsomia 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 reproduction (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;
- the definitive test on reproduction to determine sublethal effects of (dilutions of) 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).

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 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 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, composted 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 10381-6 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 removed by the 4 mm sieve

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 ([3.9](#)) or b) standard soil ([3.10](#)) that allows the presence of Collembola. Control soil and soil used for dilution shall not differ in one test (either a) or b)).

- 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 $6,0 \pm 0,5$.

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 is lower than 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 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 is determined according to [Annex C](#), the pH is determined according to ISO 10390.

5.3 Food

A sufficient amount, e.g. 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 Betosip¹⁾ (a.i. 157 g/l phenmedipham) have been tested in a ring 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 reproduction were observed at concentrations (EC50) of 147 mg boric acid/kg dry mass of artificial soil and 169 mg boric acid/kg clay-loam soil dry mass).^{[5],[21]}

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.

NOTE 2 Betosip: Effects on reproduction ($\alpha = 0,05$) were observed at concentrations between 100 mg and 200 mg of the product per kilogram dry mass of the substrate.

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 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 $(20 \pm 2) ^\circ\text{C}$.

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 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 mixture ratio affecting *Collembola* 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 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/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 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 ([6.7.1](#)) with the light source ([6.7.2](#)).

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 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 soil are characterized by comparison of the biological effects found in the soil to be tested(s) 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 ECx 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 ECx evaluation.

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

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, 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 can contain unknown mixtures of toxic, mutagenic, or otherwise harmful substances or infectious microorganisms. Occupational health risks can arise from dust or evaporated substances as well as via dermal contact during handling and incubation.

7.2.2 Testing substances added to the test substrate

Standard soil (5.2.2) is used to prepare the test sample. For each test container (6.1), the mass of the substrate used shall be 30 g (wet mass). Substances are added to the test substrate and mixed 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 or a portion of it required to wet the soil samples for the replicates of one concentration in order to meet the requirements of 5.2.2, and mix it thoroughly with the soil before introducing it into the test containers.

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 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 soil and the water and mix thoroughly before introducing it into a test container.

Base the concentrations selected to provide the LOEC/NOEC on the results of the range-finding 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 sample.

Proceed simultaneously with all replicates per concentration and the control(s) required according to the selected approach.

Determine the pH for each test mixture (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 deionized water to reach 40 % to 60 % of the total water holding capacity (determined according to Annex B).

Perform one control container for the range-finding 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 Addition of the biological material

Ten juvenile Collembola (10 d to 12 d old) are placed in each test container.

Collembola 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 [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 d, 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.

Determine the water content and the pH of the soil at the beginning and end of the test for one vessel of each test conditions. 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 Collembola

Determine the number of Collembola present four weeks after introducing the parental Collembola onto the test and control substrates. Pour the test sample into a 500 ml to 600 ml container and add water. After gentle stirring of the suspension with a spatula, Collembola drift to the water surface. Count adults and juveniles, if present, by a suitable procedure (see [Annex D](#)) 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 end-points including standard deviation of the measured values against the soil(s) to be tested, control soil(s) or the selected series of test 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/ ER_x -approach the % soil to be tested 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 springtails to 50 % (EC₅₀) compared to the control within the test period; or

- in the NOEC/NOER-approach the soil mixture ratio immediately below the LOEC/LOER or highest tested concentration/rate of a test substance which when compared to the control has no statistically significant lethal or other effect such as 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 juvenile springtails per control vessel, and
- 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 end-points, e.g. reproduction, involve quantitative effects, e.g. counting juvenile springtails. 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 [9]) or to contact a statistician.

10.2 Single-concentration tests

Quantitative single-concentration tests (e.g. effects on reproduction) 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.[9]

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

Analysis of variance (ANOVA) involving multiple comparisons of end-point data derived for undiluted soil to be tested 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 from soil toxicity tests. This is a hypothesis-testing approach, and is subject to appreciable weaknesses.[9] 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.[9] If the original or transformed data do not satisfy either test for distribution of data, then analysis by nonparametric methods shall be carried out.

10.3 Multi-concentration tests

10.3.1 Range-finding 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 end-point. This is usually a specific degree of reduction in performance compared to the control. Linear and nonlinear 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 are preferably analysed by the EC_x-approach, or if required by legislation, by the NOEC-approach:

— EC_x/EC_x (effect concentration)-approach

The EC_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 EC_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 EC_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 unequal-variance *t*-test (Welch *t*-test) or a nonparametric test, such as the Mann-Whitney-U-test may be used.

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

11 Test report

The test report shall include the following information:

- a) a reference to this International Standards: 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 sample 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, pH and CEC of the soil to be tested and the control soil at the start and at the end of the test 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 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 Collembola, 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 water 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 Collembola, 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 the 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 Collembola 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 d to 12 d old juvenile Collembola 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.

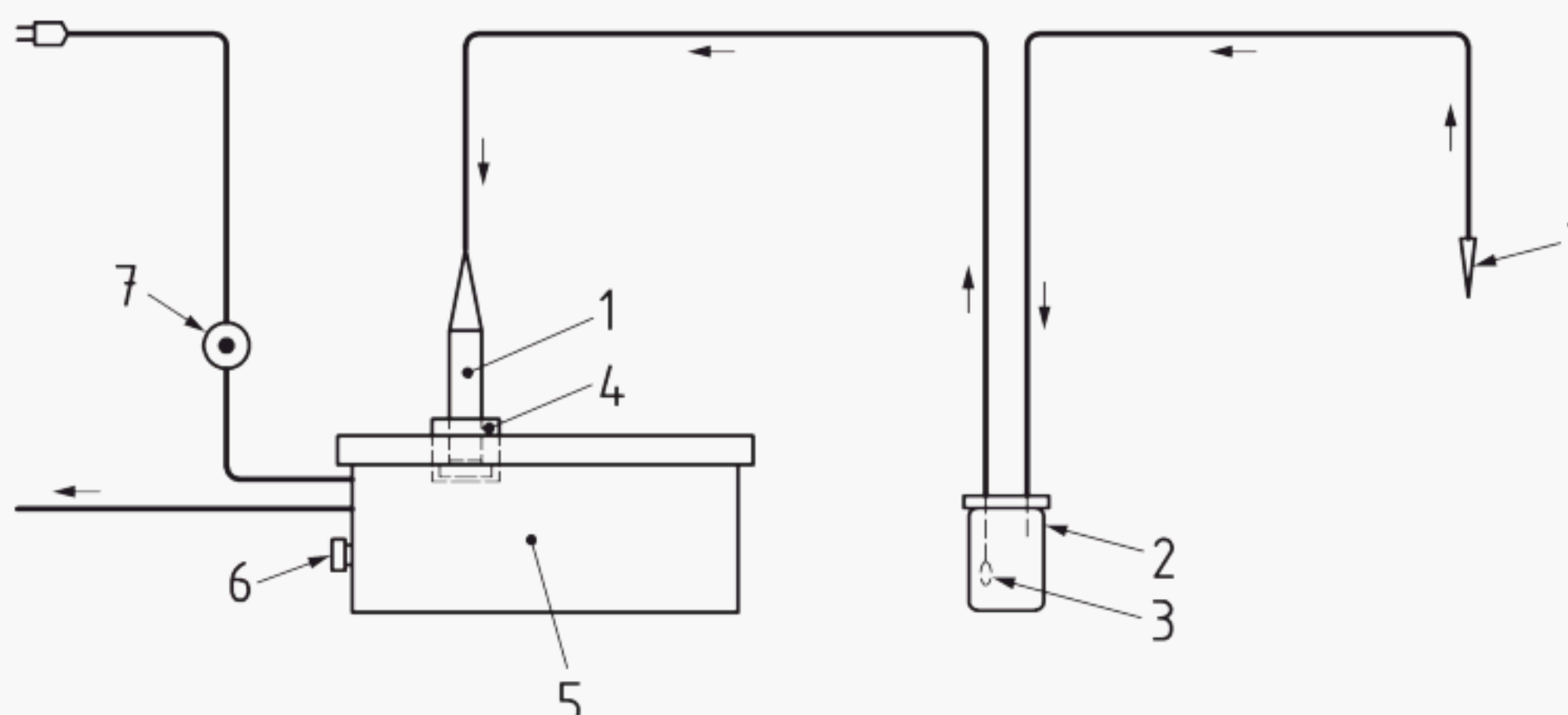
NOTE The egg clusters are easily removed if they are placed on small pieces of breeding substrate or cover glasses laid on the breeding substrate, and juvenile Collembola collected after a further 10 d incubation.

Alternatively, 10 d to 12 d old juvenile Collembola can be obtained by placing a number of adult Collembola 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 are 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 d to 12 d 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 Collembola to the test containers

The Collembola are easily transferred from the breeding substrate to the test sample 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 Collembola | 5 commercial aquarium pump |
| 3 gauze | 6 regulator |
| | 7 foot switch |

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

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

Annex B (informative)

Determination of water-holding capacity

B.1 General

The following method has been found to be appropriate for laboratory samples of soils to be tested and standard soils.

B.2 Apparatus

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

B.2.2 Water bath, at room temperature.

B.2.3 Filter paper.

B.2.4 Drying oven, set to $(105 \pm 5) ^\circ\text{C}$.

B.2.5 Balance, capable of weighing with an accuracy of $\pm 0,1$ g.

B.3 Procedure

Plug the bottom of the tube with a filter paper, and after filling with the control or test sample 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, but below the upper edge of the tube. 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 those used for the soil substrate is satisfactory.

Weigh the sample, dry it to constant mass at $105 ^\circ\text{C}$ and reweigh it.

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

$$WHC = \frac{m_S - m_T - m_D}{m_D} \times 100 \quad (\text{B.1})$$

where

WHC is the water-holding capacity, in percentage of dry mass, %;

m_S is the mass of the water-saturated substrate plus the mass of the tube plus the mass of the filter paper;

m_T is the tare (mass of tube plus mass of filter paper);

m_D is the dry mass of substrate.

Annex C (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 [5.2.2](#). 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.

Annex D (informative)

Extraction and counting of Collembola

For extraction two methods are available:

- A controlled temperature gradient extractor based on principles by MacFayden can be used. Description is given in [\[19\]](#)
- Assessment by flotation: For evaluating effects on reproduction, the juvenile Collembola 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.

Counting: Counts of numbers can be carried out by eye or under light microscope using a grid placed over the flotation vessel or by photographing the surface of each vessel and later counting the Collembola on enlarged print or projected slides with a culture-counter. 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 shall 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. To improve the contrast between white Collembola and surrounding water surface, water can be coloured dark with ink. Counts may also be performed using digital camera equipment and digital image processing techniques. All techniques should be validated. [\[19\]](#)

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

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

Bibliography

- [1] ISO 11268-2, *Soil quality — Effects of pollutants on earthworms — Part 2: Determination of effects on reproduction of Eisenia fetida/Eisenia andrei*
- [2] ISO 15799, *Soil quality — Guidance on the ecotoxicological characterization of soils and soil materials*
- [3] ISO 16387, *Soil quality — Effects of pollutants on Enchytraeidae (Enchytraeus sp.) — Determination of effects on reproduction*
- [4] ISO 17616, *Soil quality — Guidance on the choice and evaluation of bioassays for ecotoxicological characterization of soils and soil materials*
- [5] BECKER-VAN SLOOTEN K., CAMPICHE S., TARRADELLAS J. 2003): Research in Support of Environment Canada's Toxicity Test Method with *Folsomia fimetaria* for Assessment of Contaminated Soils, Report Prepared by Laboratory of Environmental Chemistry and Ecotoxicology /CECOTOX), Ecole polytechnique fédérale de Lausanne (EPFL) ENAC-ISTE (Lausanne, Switzerland) for Biological Methods division, Environmental Technology Centre, Environment Canada, ON
- [6] DUNNETT C.W. A multiple comparison procedure for comparing several treatments with a control. *Amer. Statist. Ass. J.* 1955, **50** pp. 1096–1121
- [7] DUNNETT C.W. New tables for multiple comparisons with a control. *Biometrics.* 1964, **20** pp. 482–491
- [8] EG (1988): Teil C, Toxizität für Regenwürmer: Prüfung in künstlichem Boden. *Amtsblatt der Europäischen Gemeinschaften*, Nr. L 133/3, Teil C
- [9] EC (ENVIRONMENT CANADA). 2005): "Guidance Document on Statistical Methods for Environmental Toxicity Tests" Method Development and Applications Section, Environment Canada, Ottawa, ON, Report EPS 1/RM/46, 241 p
- [10] EC (ENVIRONMENT CANADA). 2007): Biological Test method: Test for Measuring Survival and Reproduction of Springtails Exposed to Contaminant in Soil. Environment Canada, Ottawa, ON, Report EPS 1/RM/47, 176 p
- [11] FINNEY D.Y. *Probit Analysis*. Cambridge University Press, Cambridge, UK, 1971
- [12] FINNEY D.Y. *Statistical Methods in Biological Assay*. Charles Griffin & Company Ltd, London, UK, 1978
- [13] HAMILTON H.A., RUSSO R.C., THURSTON R.V. 1978): Trimmed Spearman-Kärber method for estimating median lethal concentrations in toxicity bioassays. *Environ. Sci. Technol.* 11, pp. 714–719. [Correction]. *Environ. Sci. Technol.* 1977, **12** p. 417
- [14] HOLM S. A simple sequentially rejective multiple test procedure. *Scand. J. Stat.* 1979, **6** pp. 65–70
- [15] Jancke (1989): *Zool. Beiträge, neue Folge*, 32, pp. 261–299
- [16] KUHNT G. Muntau, H. EURO-Soils: Identification, Collection, Treatment, Characterization. JRC Ispra, European Commission
- [17] SPEYER LUFA Obere Langgasse 40, 67346 Speyer, stdboden@lufa-speyer.de
- [18] OECD (1984): Earthworms, Acute Toxicity Tests, *Guideline for testing of chemicals*, No. 207
- [19] OECD (2009): Collembolan Reproduction Test in Soil, *Guideline for testing of chemicals*, No. 232

- [20] RIEPERT F., & KULA C. *Development of laboratory methods for testing effects of chemicals and pesticides on Collembola and earthworms. Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft*, 320. Berlin-Dahlem, 1996
- [21] SCOTT-FORDSMAND J.J., & KROGH P.H. *Background report on Pre-Validation of an OECD Springtail Test Guideline, report prepared by NERI*. Silkeborg, Denmark, 2005
- [22] SPAHR H.J. The importance of Collembola for soil biology and their suitability as test organism for ecotoxicology. *Anz. Schädlingskde., Pflanzenschutz. Umweltschutz*. 1981, **54** pp. 27–29
- [23] STÄMPFLI C., BECKER-VAN SLOOTEN K., CAMPICHE S., TARRADELLAS J. (2005): Research in Support of Environment Canada's Toxicity Test Method with *Folsomia fimetaria* for Assessment of Contaminated Soils, Report Prepared by Laboratory of Environmental Chemistry and Ecotoxicology /CECOTOX), Ecole polytechnique fédérale de Lausanne (EPFL) ENAC-ISTE (Lausanne, Switzerland) for Biological Methods division, Environmental technology Centre, Environment Canada, ON
- [24] Ulber (1983) Einfluss von *Onychiurus fimatus* Gisin (Collembola, Onychiuridae) und *Folsomia fimetaria* L. (Collembola, Isotomidae) auf *Pythium ultimum* Trow., einen Erreger des Wurzelbrandes der Zuckerrübe. In New trend in soil Biology (Lebrun Ph, André HM, De Medts A, Grégoire-Wibo, Wauthy G (Eds), Proceedings of the VI. international colloquium on soil zoology, Louvain-la-neuve Belgium), 30 August-2 September 1982, I Dieu-Brichart, Ottignies-Louvain-la-Neuve, pp. 261-268
- [25] WOLF-ROSKOSCH. (1983): *Chemikaliengesetz*, Heft 3, Texte 27/38, Umweltbundesamt, pp. 83–109
- [26] WILLIAMS D.A. A test for differences between treatment means when several dose levels are compared with a zero dose control. *Biometrics*. 1971, **27** pp. 103–117
- [27] WILLIAMS D.A. The comparison of several dose levels with a zero dose control. *Biometrics*. 1972, **28** pp. 519–531

