INTERNATIONAL STANDARD

ISO 11267

Third edition
2023-08

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*

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

Reference number
ISO 11267:2023(E)

© ISO 2023

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

COPYRIGHT PROTECTED DOCUMENT

© ISO 2023

All rights reserved. Unless otherwise specified, or required in the context of its implementation, no part of this publication may be reproduced or utilized otherwise in any form or by any means, electronic or mechanical, including photocopying, or posting on the internet or an intranet, without prior written permission. Permission can be requested from either ISO at the address below or ISO's member body in the country of the requester.

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

Published in Switzerland

© ISO 2023 – All rights reserved

Contents

Page

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

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 document 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).

ISO draws attention to the possibility that the implementation of this document may involve the use of (a) patent(s). ISO takes no position concerning the evidence, validity or applicability of any claimed patent rights in respect thereof. As of the date of publication of this document, ISO had not received notice of (a) patent(s) which may be required to implement this document. However, implementers are cautioned that this may not represent the latest information, which may be obtained from the patent database available at www.iso.org/patents. ISO shall not be held responsible for identifying any or all such patent rights.

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

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

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

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

The main change is as follows:

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

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

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

Introduction

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

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

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

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

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

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

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

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

1 Scope

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

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

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

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 10390, *Soil, treated biowaste and sludge — Determination of pH*

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

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

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

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

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

3 Terms and definitions

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

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

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

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

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

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

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

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

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

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

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

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

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

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

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

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

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

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

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

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

EXAMPLE Euro soils, artificial soil, LUFA standard soil.

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

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

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

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

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

4 Principle

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

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

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

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

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

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

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

5 Reagents and material

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

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

5.2.1 Field-collected soil or waste

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

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

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

a) pH in accordance with ISO 10390,

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

c) water content in accordance with ISO 11465,

d) water-holding capacity according to Annex B,

e) cationic exchange capacity in accordance with ISO 11260,

f) organic carbon in accordance with ISO 10694,

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

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

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

a) If reference soils from uncontaminated areas near a contaminated site are available, they should be treated and characterized like the soils to be tested. If a toxic contamination or unusual soil properties cannot be ruled out, standard control soils should be preferred.

b) For testing the effects of substances mixed into soil, standard soils (e.g. artificial soil, LUFA) shall be used as test substrate. The properties of the field-collected standard soil shall be reported.

The substrate called artificial soil can be used as a standard soil and has the following composition:

	Percentage expressed on dry mass basis
— Sphagnum peat finely ground [a particle size of (2 ± 1) mm is acceptable] and with no visible plant remains	10 %

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

— Kaolinite clay containing not less than 30 % kaolinite 20 %

— Industrial quartz sand (dominant fine sand with more than 50 % of particle size 0,05 mm to 0,2 mm) 69 %

Approximately 0,3 % to 1,0 % calcium carbonate ($CaCO_3$, pulverized, analytical grade) are necessary to get a pH of 6,0 ± 0,5. Further guidance on how to proceed on the adjustment of pH of artificial soil is available in Annex C.

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

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

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

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

5.3 Food

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

5.4 Reference substance

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

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

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

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

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

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

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

6 Apparatus

Use laboratory equipment and the following apparatus:

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

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

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

6.4 Suitable accurate balances.

6.5 Apparatus capable of measuring pH.

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

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

6.8 Test environment.

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

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

7 Procedure

7.1 Experimental design

7.1.1 General

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

7.1.2 Range-finding test

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

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

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

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

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

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

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

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

7.1.3 Definitive test

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

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

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

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

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

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

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

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

7.2 Preparation of test mixture

7.2.1 Testing contaminated soil

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

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

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

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

A 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 are 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.

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. Choose the appropriate number of test concentrations above and below the LOEC/NOEC and use a spacing 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) moistened with deionized water to reach 40 % to 60 % of the total water holding capacity (determined according to Annex B).

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

Perform one control container for the range-finding test and six to eight control containers for the definitive test, depending on its experimental design (see 7.1.3).

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 exhauster as described in A.2. Before being transferred to the test containers, organisms are counted and checked for damage both to reduce control mortality and to avoid systematic trial errors.

An example for rearing and breeding the standard species *Folsomia candida* is provided in Annex A and for alternative test species in Annex E.

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 1 Other extraction methods (e.g. high-gradient extraction) can be used if they have proven to be effective.

NOTE 2 When Collembola species other than *F. candida* are used in the test, some modifications in the procedures of 7.3, 7.4 and 7.5 can be necessary (see Annex E).

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.

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

If dilution or concentration series are performed, indicate:

— in the ER*x*/EC*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;

— in the NOER/NOEC approach - the soil mixture ratio immediately below the LOER/LOEC 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 on reproductive output ($p < 0,05$).

9 Validity of the test

The results are considered to be valid if:

— the mortality of the adults in the control(s) does not exceed 20 % at the end of the test;

— the reproduction rate reaches a minimum of 100 juvenile springtails per control vessel; and

— the coefficient of variation of reproductive output in the control does not exceed 30 %.

See E.6.6 for Collembola species other than *F. candida.*

10 Statistical analysis

10.1 General

Most of test methods with sub-lethal end-points, for example, reproductive output, 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.

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 Reference [9]) or to contact a statistician.

10.2 Single-concentration tests

Quantitative single-concentration tests (e.g. effects on reproductive output) have different statistical methods. For sampling at several locations with field replication, an analysis of variance (ANOVA) is a first step if results are suitable. If the null hypothesis of no difference is rejected, the analysis proceeds 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 reproductive output after exposure to a sample of undiluted contaminated soil, compared to numbers of offspring exposed to a reference or standard soil. If there is 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 is suitable for statistical analysis.

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,

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

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, ER*x*/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:

— ER*x*/EC*x* approach.

The ER*x*/EC*x*-approach can only be used if a clear dose response relationship is found. Wherever possible, the R^2 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 ER50/EC50 values have smaller confidence limits compared with smaller effect concentrations (e.g. ER20/EC20), it is recommended to determine ER50/EC50 values.

— NOEC 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, for example, a one-way ANOVA, followed by a one-sided Dunnett test (α = 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, for example, the Mann & Whitney U-test 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 interpreted biologically.

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

11 Test report

The test report shall include the following information:

a) a reference to this document, i.e. 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 rate/concentration causing significant effects (LOER/LOEC), the highest rate/concentration causing no observed effects (NOER/NOEC), ER10/EC10 and ER50/EC50 for the inhibition of reproductive output 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.

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

Annex A
(informative)

Techniques for rearing and breeding *Folsomia candida*

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 Ix to 800 ix (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.

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

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.

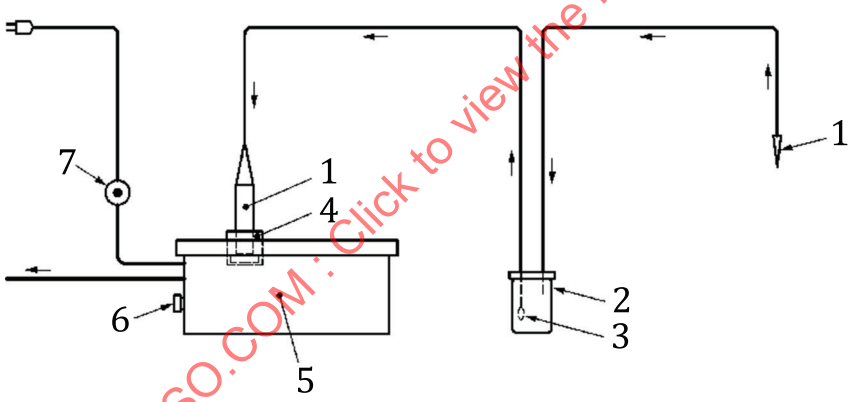
NOTE The egg clusters are easily removed if they are placed on small pieces of breeding substrate or cover glasses laid on the breeding substrate, and juvenile Collembola collected after a further 10 d incubation.

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 exhauster. An example is shown in Figure A.1.



Key

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

Figure A.1 — Low-suction exhauster 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.

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

Annex B
(normative)

Determination of water-holding capacity

B.1 General

The method described in this annex 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 ± 5) °C.

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

B.3 Procedure

Plug the bottom of the tube with a filter paper or a very fine gauze, 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 shall be satisfactory.

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

B.4 Calculation of water-holding capacity (C_{WH})

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

where

C_{WH} 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.

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

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 ± 0,5), an 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 ± 0,5 can be estimated.

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

Annex D
(informative)

Extraction and counting of Collembola

For extraction, two methods are available:

— A controlled temperature gradient extractor based on principles by MacFadyen can be used. Description is given in Reference [19].

— Assessment by flotation: For evaluating effects on reproductive output, the juvenile Collembola swimming on the surface of the watered substrate are counted. When reproductive output 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 can 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 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 with a culture-counter. Counting may also be performed using digital camera equipment and digital image processing techniques. Normal photographic equipment is adequate for this purpose (e.g. single-lens reflex camera, macro-lens or other device for close-up photography). Shutter speed shall be adjusted according to light intensity (e.g. flash or cold light source). 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 with dark ink. All techniques should be validated[19].

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.

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

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

Annex E
(informative)

Specific information of alternative Collembolan species other than *Folsomia candida*

E.1 General

When using alternative Collembolan species for the test according to this guideline, a number of prerequisites should be fulfilled.

— The species used should be unequivocally identified.

— The rationale for selection of the species should be given.

— The validity criteria of the test shall be met.

E.2 Geographic distribution and ecology

The geographic distribution and ecology of each of the alternative Collembolan species is presented in Table E.1.

Table E.1 — Geographic distribution and ecology of alternative Collembolan species

Species	Geographic distribution	Ecology
Folsomia fimetaria	Distributed worldwide; common in several soil types ranging from sandy to loamy soils and from mull to mor soils [28]. Preference for agricultural systems and other systems with high organic matter soils; occurs less frequently in meadows and soils of urban settlements [29].	Euedaphic, intermediate distribution within the 5 cm to 10 cm layer in soil [30]. Omnivorous feeding habit, including fungal hyphae, bacteria, protozoa and detritus in its food [28].
Onychiurus yodai	Asia [31].	Euedaphic species (permanent soil-dwellers). Can form bisexual populations [32]-[34].
Orthonychiurus folsomi	Common in soil environments in North America, with distribution in Europe, China and Australia.	Euedaphic detrivore, living primarily in the interstitial spaces of soil, or under stones or rotting wood on soil surface [35].
Proisotoma minuta	Worldwide distributed, exhibiting preference for agricultural systems and systems with high organic matter soils [31],[36].	Hemiedaphic species (intermediate vertical distribution).
Protaphorura fimata	Distributed through mild temperate to cold zones [31],[37].	Euedaphic species (permanent soil-dwellers [38],[39]). Can form bisexual populations[30],[32],[34]. Herbivorous [34] May feed on germinating lettuce seeds [41].
Sinella curviseta	Distributed in populations from North America to Europe, Southeast Asia and Japan [42].	Epedaphic species (upper vertical distribution).
Allonychiurus kimi	Distributed Korea, China, and Japan [43] common in paddy fields in Korea [44].	Euedaphic.
Yuukianura szeptyckii	Distributed Korea, China, and Japan [45]; common on a soil surface near a stream [46].	Hemiedaphic.

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

Table E.1 *(continued)*

Species	Geographic distribution	Ecology
Heteromurus nitidus	Widespread global distribution, usually found on the upper layers of forest and arable soils [47],[48].	*H. nitidus* predominantly lives in the litter layer (euedaphic species) a habitat where melanized and toxic fungi are most abundant [48],[49]. *H. nitidus*, can also be found in the soil surface layers[50], and is also considered a hemiedaphic species [51].
Onychiurus armatus	Worldwide distribution, common in temperate soils [52],[53].	Euedaphic fungivores species [52].
Orchesella cincta	Distributed in Europe and Asia, occupying a range of thermal habitats but mostly found in temperate regions. Lives in the litter layers, under bark or in moss on tree trunks [54],[55].	Epedaphic species with upper vertical distribution [54],[56].
Hypogastrura assimilis	Distributed worldwide; common in several soil types.	Epi- to hemiedaphic species, intermediate distribution within the 5 cm to 10 cm layer in soil; detritivores.
Mesophorura macrochaeta	Distributed worldwide; common in several soil types.	Euedaphic, typically endogeic; detrivores.

E.3 Morphology and reproduction mode

The morphology and reproduction mode for each of the alternative Collembolan species is presented in Table E.2.

Table E.2 — Morphology and mode of reproduction of the alternative Collembolan species

Species	Morphology	Reproduction mode
F. fimetaria	Elongated, eyeless and unpigmented (white); Sexual dimorphism in adults (at least 20 days after hatching): Females: clearly larger than males with a curvy body and a rather round head. Males: more stick-like body and triangle shaped head; males move generally faster than females [28].	Sexual; permanent presence of males required [28]
O. yodai	Unpigmented body, lack of eyes and furca. They present relatively slow movement and have defensive pores (pseudocelli) [56],[57]. No sexual dimorphism can be perceived.	Sexual
O. folsomi	Small (up to 1,9 mm length), elongated, pale white integument, with rounded abdomen, downward-pointing mouthparts and absence of anal spines; absence of ocelli, and lacking furca; conspicuous sexual dimorphism (age-synchronization allows for sexual differentiation); presence of females improve rate of hatching success [35]. Females: when age-synchronized, larger than males (~2 mm), with rounded abdomen. Males: when age-synchronized, more slender and smaller (half the size) than females.	Sexual; permanent presence of males required [35]

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

Table E.2 *(continued)*

Species	Morphology	Reproduction mode
P. minuta	Elongated greyish collembolans, with visible eyes and furca, and the size of adults is around 1,1 mm [58]. Sexual dimorphism is not very pronounced, yet some researchers reported a rounder abdomen of the females and shorter and slender size in males. Females are larger than males [35].	Sexual
P. fimata	Unpigmented body, with reduced compound eyes and furca[39]. They present relatively slow movement and have defensive pores (pseudocelli) [56],[57]. No sexual dimorphism can be perceived.	Sexual
S. curviseta	Long bodied (~2 mm [56]) brownish collembolans with long hair, antennae and furca. Sexual dimorphism is not very pronounced in adults, yet De Lima et al. 2021 reported that with some training is possible to distinguish males from females, since the later have a pronounced and rounder fourth part of the abdominal segment (personal communication from Maty P. Berg) [51],[59].	Sexual
A. kimi	Elongated, body length 2 mm to 3 mm, eyeless and unpigmented (white) [43],[44]. Sex ratio (% of females): 70,6 % (unpublished data).	Sexual (unpublished data)
Y. szeptyckii	Elongated, body length 1,5 mm to 1,7 mm, eyeless and pigmented (rose or orange colour) [45],[46]. Sex ratio (% of females): 88 % at 15 °C to 30 °C [46].	Sexual
H. nitidus	Adults up to 3,0 mm long[50],[56]. Brown pigmented body and long legs [50]. Sexual dimorphism is not very marked but according to De Lima et al.[51], females have a more pronounced and rounder fourth abdominal segment (personal communication from Maty P. Berg) [56].	Facultative parthenogenetic species [35],[51]
O. armatus	Small, Collembola rarely exceeding 2,5 mm in length. Unpigmented with a pale yellow or white tone, short legged with reduced furca lacking in jumping ability [53],[60].	Parthenogenetic species [61]
O. cincta	Adult size between 3 mm to 4,5 mm, yellow in colour with brownish/black dark pigments on abdomen with variable pattern, head usually dark in colour[56],[62]. Sexual reproduction by dissociated sperm transfer where Females collect spermatophores laid by males on soil [63],[64].	Sexual
H. assimilis	Elongated, pigmented brownish, and has eyespots; sexual dimorphism in adults. Females: twice the size of males, lighter colour. Males: more stick-like body, smaller; males move generally faster than females;	Sexual; permanent presence of males required
M. macrochaeta	Elongated, pigmented greyish; slower moving.	Sexual; permanent presence of males required

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

E.4 Life-history traits

The life-history traits of each of the alternative Collembolan species, including the age at maturation, duration of egg development and recommended age for exposure in tests are presented in Table E.3.

© ISO 2023 – All rights reserved

Table E.3 — Life-history traits of alternative Collembolan species

Species	Age at maturation	Duration of egg development	Instars subject to exposure
F. fimetaria	18 days [35]	9,5 days [29] (suggest 9 days to 10 days)	Adults aged 23 days to 26 days
O. yodai	No information available	No information available	Adults aged 20 days to 23 days
O. folsomi	28 days to 31 days	11 days to 14 days, temperature dependent	Adults aged 28 days to 31 days
P. minuta	~14 days	~6 days	Adults aged 10 days to 12 days [36] or 13 days to14 days [35]
P. fimata	No information available	No information available	Adults aged 20 days to 23 days [65]
S. curviseta	22 days to 26 days	5 days to 7 days	Adults aged 20 days to 23 days
A. kimi	42 days to 46 days at 20 °C [66],[67]	12 days to 14 days [68],[69]	Adults aged 42 days to 46 days
Y. szeptyckii	23 days at 25 °C [46]	12 days at 25 °C [46]	Adults aged 42 days [70]-[72]
H. nitidus	23 days to 25 days [51]	~8 days [51]	Adults aged 23 days and 25 days [51]
O. armatus	40 days [73]	12 days to 18 days [73],[74]	Adults aged 38 days to42 days
O. cincta	6 weeks to 7 weeks [75],[76]	6 days to 10 days [63],[75] Temperature dependant at 20 °C ~6 days [63]	35 days [77]
H. assimilis	23 days to 26 days	14 days to 16 days	23 days to 26 days
M. macrochaeta	23 days to 26 days	14 days to 16 days	23 days to 26 days

E.5 Culturing

E.5.1 Culture vessels and substrate

The proposed culturing vessels and substrate for each of the alternative Collembolan species are presented and described in Table E.4.

NOTE Some types of plastic can emit toxic organic compounds, which can influence the culturing and testing.

Table E.4 — Culture vessels and substrate for alternative Collembolan species

Species	Culture vessels	Culture substrate
F. fimetaria	Plastic containers (diameter 9 cm to11 cm) like Petri dishes	Plaster of Paris and activated charcoal (8:1, w:w) with a substrate layer ≥ 5 mm saturated with distilled water [28].
O. yodai	Plastic containers (diameter 11 cm) with perforated lid	Plaster of Paris and activated charcoal (11:1 or 8:1, w:w) with a substrate layer of ~1 cm saturated with distilled water.
O. folsomi	Plastic breeding boxes (1 L to 6 L capacity), rectangular, covered with solid or perforated lids; loading density 2 organisms to 3 organisms cm^{-2}	Plaster of Paris and activated charcoal (8:1, w:w) with a substrate layer depth of ~1 cm saturated with distilled water or a very thin layer of artificial soil layered on plaster of Paris and charcoal substrate. The presence of chards of plaster of Paris and charcoal substrate promote eggs laying[35]. Substrate should be renewed as needed (e.g. every 2 months).

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

Table E.4 *(continued)*

Species	Culture vessels	Culture substrate
P. minuta	Plastic containers the same as for *O. yodai*	Plaster of Paris and activated charcoal (11:1, 8:1 or 4:1, w:w) with a substrate layer of ~1 cm saturated with distilled water [35].
P. fimata	Plastic containers as for *O. yodai*	Culture substrate the same as for *O. yodai.*
S. curviseta	Plastic containers the same as for *O. yodai*	Culture substrate the same as for *O. yodai.*
A. kimi	Plastic Petri dish (9 cm diameter and 1,5 cm height) [66],[67]	Plaster of Paris and activated charcoal (4:1, w:w) with a substrate layer ≥ 0,5 cm saturated with distilled water [66],[67].
Y. szeptyckii	Glass jar (9 cm diameter and 9 cm height) [70]-[72]	Artificial OECD soil according to OECD guideline 207 [28],[46].
H. nitidus	Plastic containers (7 cm to 11 cm diameter and 3 cm to 4 cm height) (Haubert et al., 2011; Buse et al., 2013) or translucent plastic boxes of 125 ml[51]. with perforated lid	Plaster of Paris and activated charcoal (10:1, 9:1 or 2:1, w:w) with a substrate layer of ~2 cm saturated with distilled water[51],[78],[79]. Alternatively, moist quartz sand [80] or garden soil [50].
O. armatus	Petri dish (9 cm diameter) [61]	Plaster of Paris and activated charcoal (9:1, v:v) with a substrate layer of ~1 cm saturated with distilled water[61].
O. cincta	PVC boxes or plastic containers or Petri dishes (5 cm to 25 cm diameter), with a 1,5 cm to 2 cm layer of plaster of Paris [81]	Plaster of Paris and activated charcoal (10:1 or 9:1, w:w) with a substrate layer of 1,5 cm to 2 cm saturated with distilled water[51],[52],[61],[82]. Some twigs can be added to the culture box as long as these are from an unpolluted source.
H. assimilis	Plastic containers (diameter: 9 cm to 11 cm) like Petri dishes	Plaster of Paris and activated charcoal (8:1, w:w); minimum substrate layer of 5 mm saturated with distilled water [28]
M. macrochaeta	Plastic containers (diameter: 9 cm to 11 cm) like Petri dishes	Plaster of Paris and activated charcoal (8:1, w:w); minimum substrate layer of 5 mm saturated with distilled water [28].

E.5.2 Culturing conditions

The temperature and lighting conditions for culturing each of the alternative Collembolan species are presented in Table E.5.

The temperature used in the culturing conditions should be the same as in the test. This is important because, at least for some species and depending on the objective of the study, the test temperature can be 20 °C or 25 °C (exception, *Hetermurus nitidus*).

Table E.5 — Culturing conditions for alternative Collembolan species

Species	Temperature [°C]	Lighting conditions[a]
F. fimetaria	Mean 20 ± 2 [28]	16 h L:8 h D [28] or 12 h L:12 h D [35]; 400 lx to 800 lx.
O. yodai	20 or 25	Same conditions as for *F. fimetaria*.
O. folsomi	20 ± 3 [35]	Same conditions as for *F. fimetaria*.
P. minuta	20	16 h L: 8 h D.
P. fimata	20 or 25	Same conditions as for *F. fimetaria*.
S. curviseta	20 or 25 [83,84]; this species may reproduce faster at 25 °C	Same conditions as for *P. minuta*.
A. kimi	20 ± 1 [66],[67]	0 h L: 24 h D [66],[67].

[a] L = light; D = dark.

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

Table E.5 *(continued)*

Y. szeptyckii	25 ± 1 [70]-[72]	Same conditions as for *A. kimi*[70]-[72].
H. nitidus	Temperatures range from 15 to 22 [50],[79]; however, 15 is the most recommended to avoid the formation of spores [85]	0 h L: 24 h D [78],[79],[86] or 16 h L:8 h D [51].
O. armatus	12 or 19 [52],[61]	As for *A. kimi*[52].
O. cincta	20 ± 2 [87]	12 h L: 12 h D[82],[87]-[89]. or 16 h L: 8 h D [81].
H. assimilis	20 ± 2	Same conditions as for *F. fimetaria.*
M. macrochaeta	20 ± 2	Same conditions as for *F. fimetaria.*
[a] L = light; D = dark.		

E.5.3 Feeding and water replenishment

The procedures for feeding (including type of food) and water replenishment for culturing each of the alternative Collembolan species is provided in Table E.6.

Table E.6 — Feeding and water replenishment for alternative Collembolan species

Species	Feeding	Water replenishment
F. fimetaria	Granulated dry yeast; 2 mg to 15 mg divided into 2 or 3 piles; once or twice per week.	Loss should be replenished with distilled water, once or twice per week, depending on the culture's needs and culturing temperature.
O. yodai	Granulated dry yeast *ad libitum*. At least once a week, depending on the culture's needs. Uneaten food should be removed to prevent fungal growth.	Same procedure as for *F. fimetaria*.
O. folsomi	Granulated dry yeast; ~100 mg (for 15 cm × 23 cm × 8 cm breeding box) divided into two or three piles or sprinkled onto substrate surface; twice per week.	Moisture content sufficient to keep surface of substrate moist but with no standing water. Loss should be replenished with distilled or deionized water, with every feeding, depending on the culture's needs.
P. minuta	Granulated dry yeast ad libitum. At least once a week, depending on the culture's needs. Uneaten food should be removed to prevent fungal growth.	Same procedure as for *F. fimetaria*.
P. fimata	Same procedure as for *P. minuta*.	Same procedure as for *F. fimetaria*.
S. curviseta	Same procedure as for *P. minuta*.	Same procedure as for *F. fimetaria*.
A. kimi	Granulated dry yeast; ~100 mg; once or twice per week [44].	Same procedure as for *F. fimetaria* [44].
Y. szeptyckii	Granulated dry yeast; 10 mg; once or twice per week [71].	Same procedure as for *F. fimetaria* [71].
H. nitidus	Same procedure as for *P. minuta*. Alternatively, green algae (*Chlorella vulgaris*, *Chlorococcum infusorium* or *Desmococcus* spp.) can be provided [79],[80].	Same procedure as for *F. fimetaria* [78].
O. armatus	Hyphae of Mortierella isabelline or Verticillium bulbillosum [52],[61].	Same procedure as for *F. fimetaria* [61].
O. cincta	Green algae (*Desmococcus* spp., or *Pleorococcus* sp.) in suspension or algae collected from reference site growing on twigs [55],[81],[82],[88],[89].	Same procedure as for *F. fimetaria* [81].
H. assimilis	Same procedure as for *F. fimetaria*.	Same procedure as for *F. fimetaria*.
M. macrochaeta	Same procedure as for *F. fimetaria*.	Same procedure as for *F fimetaria*.

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

E.5.4 Culture maintenance and developmental rate

Procedures for producing synchronized Collembolan cultures for testing is provided for each of the alternative Collembolan species in Table E.7.

Table E.7 — Procedures for maintenance of age synchronized cultures of alternative Collembolan species

Species	Maintenance of age synchronized cultures
F. fimetaria	Collembolans are transferred into fresh containers for oviposition; after 10 days, freshly laid eggs are isolated in fragments of moistened substrate, using a paintbrush and a pipette, and placed in containers with fresh substrate. A few adult females (e.g. 6 to 8 of the biggest individuals) can be added to each hatching vessel, to improve the rate of hatching[35]. Eggs are incubated at similar conditions to those used for cultures. Three days after the emergence of the first juveniles, the fragments of substrate with unhatched eggs must be removed to guarantee the age synchronized individuals; organisms are fed, watered and aerated until test start when reaching an adult age of 23 days to 26 days.
O. yodai	Collembolans are frequently transferred into fresh containers, by tapping or using a manual exhauster, to induce oviposition. Freshly laid eggs are isolated in fragments of substrate, using a paintbrush and a pipette, and placed in containers with fresh substrate. Eggs are incubated at similar conditions to those used for cultures and two days after the emergence of the first juveniles, the fragments of substrate with unhatched eggs must be removed to guarantee the age synchronized individuals. Juveniles are fed, watered and aerated until test start when reaching an age of 20 days to 23 days.
O. folsomi	Monitor existing cultures for large egg clutches; 7 days after the first egg clutches appear in new culture vessels or large egg clutches appear in existing cultures, transfer (using moistened paintbrush) egg clusters to hatching vessels (i.e., Petri dish ~10 cm diam. and ≥ 1 cm high) containing fresh plaster of Paris and charcoal substrate; a few adult females (e.g. 6 to 8 of the largest individuals) can be added to each hatching vessel, to improve the rate of hatching; incubate as per culture conditions; monitor daily for hatching ; remove unhatched eggs 72 h after appearance of juveniles; organisms are fed, watered and aerated until test start when reaching an adult age of 28 days to 31 days
P. minuta	Same procedure as for *O. yodai*, except that juveniles are fed, watered and aerated until the test start, when they have reached the age of 10 days to 12 days or 13 days to 14 days.
P. fimata	Same procedure as for *O. yodai*.
S. curviseta	Same procedure as for *O. yodai*.
A. kimi	Adult collembolans are transferred into fresh containers for oviposition; after 3 days, freshly laid eggs are isolated in fragments of moistened substrate, using a paintbrush and a pipete, and placed in containers with fresh substrate. Eggs are incubated at similar conditions to those used for cultures. Fourteen days after the emergence of the first juveniles, the fragments of substrate with unhatched eggs must be removed to guarantee the age synchronized individuals. Organisms are fed, watered and aerated until test start when reaching an age of 42 days to 46 days [66]-[68].
Y. szeptyckii	Adult collembolans are transferred into compressed OECD artificial soil substrate for oviposition[46]. After 1 week, freshly laid eggs are isolated in a fresh soil substrate using a fine hair brush[71]. Eggs are incubated at similar conditions to those used for cultures. Eggs are hatched after 10 days, and the hatched organisms must be watered and aerated until test start when reaching an age of 6 weeks [46],[71].
H. nitidus	Collembolans are frequently transferred into fresh containers, by tapping or using a manual exhauster, to induce oviposition. Alternatively, to ensure a high number of juveniles for the tests and/or for enlarging cultures, 30 to 40 males and the same number or a bit more of females may be transferred into fresh containers. After 2 days to 3 days, adults start laying eggs[51], that should be isolated in fragments of substrate and placed in containers with fresh substrate. Eggs are incubated at similar conditions to those used for cultures for around 8 days[51], when they start emerging. Three days after the emergence of the first juveniles, the fragments of substrate with unhatched eggs must be removed to guarantee age synchronized individuals. Juveniles are fed, watered and aerated until test start when they have reached the age of 23 days to 25 days.

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

Table E.7 *(continued)*

Species	Maintenance of age synchronized cultures
O. armatus	Synchronisation is difficult. For testing, Collembola are collected from the culture population and, in Petri-dishes, individuals of similar size are selected using digital image processing (DIP) [74].
O. cincta	Twigs are refreshed in the culture boxes daily before a testing period. During this process Collembola are separated from eggs attached to twigs from the culture box. Collected eggs on twigs are left for 7 days until hatching, after, unhatched eggs on twigs are removed[82]. Juveniles are fed, watered and aerated until test start when they have reached the age of 35 days. Alternatively, for testing, individuals of similar size range (2 mm to 3 mm) can be selected and distributed to test treatments randomly [89].
H. assimilis	Collembolans are transferred into fresh containers, by tapping or using a manual exhauster, for oviposition. After 10 days, freshly laid eggs are isolated in fragments of moistened substrate, using a paintbrush and a pipette, and placed in containers with fresh substrate. A few adult females (e.g. 6 to 8 of the biggest individuals) can be added to each hatching vessel, to improve the rate of hatching [35]; Eggs are incubated at similar conditions to those used for cultures. Three days after the emergence of the first juveniles, the fragments of substrate with unhatched eggs must be removed to guarantee the age synchronized individuals. Organisms are fed, watered and aerated until test start when reaching an age of 23 days to 26 days.
M. macrochaeta	Same procedure as for *H. assimilis*.

E.6 Test procedure

E.6.1 Test individuals

The number of individuals (total number or number of each sex for sexual reproducing species) and appropriate age for testing is provided for each of the alternative Collembolan species in Table E.8.

Table E.8 — Number of individuals and appropriate age for testing alternative Collembolan species

Species	Number & Sex	Age
F. fimetaria	10 females and 10 males	Synchronized individuals between 23 days and 26 days [28],[35].
O. yodai	20 individuals	Synchronized individuals between 20 days and 23 days (unpublished data).
O. folsomi	10 females and 5 males	Synchronized individuals between 28 days and 31 days [35].
P. minuta	5 females and 5 males	Synchronized individuals between 10 days and 12 days or 13 days and 14 days [35].
P. fimata	20 individuals	Synchronized individuals between 20 days and 23 days (unpublished data).
S. curviseta	10 females and 10 males [51]	Synchronized individuals between 20 days and 23 days [83].
A. kimi	10 or 20 individuals	Synchronized individuals between 42 days and 46 days [66],[67].
Y. szeptyckii	10 or 20 individuals	Synchronized individuals with 6 weeks[70]-[72].
H. nitidus	10 females and 10 males	Synchronized individuals between 20 days and 25 days [51].
O. armatus	10 individuals [74]	Individuals of similar size between 4 weeks to 10 weeks old.
O. cincta	20 individuals [89]	Synchronized individuals at 35 days[77],[82]. Alternatively, if synchronization is not possible, individuals with the same size range (2 mm to 3 mm) [89].
H. assimilis	10 females and 10 males	Synchronized individuals with a minimum age between 16 days and 19 days to make it possible to distinguish between female and male individuals.

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

Table E.8 *(continued)*

Species	Number & Sex	Age
M. macrochaeta	10 females and 10 males	Synchronized individuals between 23 days and 26 days.

E.6.2 Test vessels and test substrate

A suggestion of appropriate dimensions of test vessels and testing substrate for each alternative Collembolan species is provided in Table E.9.

Table E.9 — Test vessel dimensions and testing substrate for alternative Collembolan species

Species	Test vessels	Test substrate
F. fimetaria	Test containers made of glass (or other chemically inert material) of 125 ml volume (4,5 cm diameter × 10,5 cm height).	Artificial soil or natural soil as described for *F. candida*.
O. yodai	Same containers as for *F. fimetaria.*	Artificial soil or natural soil as described for *F. candida*. Natural soils must be sieved at 2 mm or 4 mm mesh.
O. folsomi	Test containers made of glass (or other chemically inert material) of 100 ml to 125 ml volume (5 cm to 8 cm diameter); loosely covered to allow air exchange.	Artificial soil or natural soil as described for *F. candida*.
P. minuta	Same containers as for *F. fimetaria*.	Artificial soil or natural soil as described for *F. candida*.
P. fimata	Same containers as for *F. fimetaria*.	Artificial soil or natural soil as described for *F. candida*. Natural soils must be sieved at 2 mm or 4 mm mesh.
S. curviseta	Same containers as for *F. fimetaria*.	Artificial soil or natural soil as described for *F. candida*).
A. kimi	Test containers made of glass (or other chemically inert material) of 160 ml volume (5,5 cm diameter × 6,0 cm height) [66],[67].	Artificial soil or natural soil as described for *F. candida*.
Y. szeptyckii	Test containers made of glass (or other chemically inert material; 7 cm diameter ×7,5 cm height).	Artificial soil or natural soil as described for *F. candida*.
H nitidus	Same containers as for *F. fimetaria*.	Artificial soil or natural soil as described for *F. candida*.
O. armatus	Same containers as for *F. fimetaria*.	Artificial soil or natural soil as described for *F. candida*.
O. cincta	Same containers as for *F. fimetaria*.	Artificial soil or natural soil as described for *F. candida*.
H. assimilis	Same containers as for *F. fimetaria*.	Artificial soil or natural soil as described for *F. candida*.
M. macrochaeta	Same containers as for *F. fimetaria*.	Artificial soil or natural soil as described for *F. candida*.

E.6.3 Test conditions

The test conditions required (i.e. temperature, light conditions and soil moisture) for each Collembolan alternative species are presented in Table E.10.

Table E.10 — Test conditions required in laboratory tests when using alternative Collembolan species

Species	Temperature [°C]	Lighting conditions[a]	Moisture [%C_{WH}]
F. fimetaria	Mean 20 ± 2 [28],[35]	16 h L:8 h D [28]. or 12 h L:12 h D [35]; 400 lx to 800 lx	> 50
O. yodai	20 or 25	As for *F. fimetaria*	> 40

[a] L = light; D = dark.

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

Table E.10 *(continued)*

Species	Temperature [°C]	Lighting conditions[a]	Moisture [%C_{WH}]
O. folsomi	mean 20 ± 3 [35]	As for *F. fimetaria*	> 50
P. minuta	20 ± 3 or 25 ± 2 (or others depending on the objective of the study)	16 h L: 8 h D	40-50
P. fimata	20 or 25	As for *F. fimetaria*	> 40
S. curviseta	20 or 25 [83]	As for *P. minuta*	40 to 75
A. kimi	20 ± 1 [66],[67]	0 h L: 24 h D [66],[67]	40 to 60 [35],[66],[67]
Y. szeptyckii	25 ± 1 [70]-[72]	As for *A. kimi* [70]-[72]	60 [46]
H. nitidus	15 or 22	16 h L: 8 h D or 12 h L: 12 h D	Same conditions as for *A. kimi*
O. armatus	12 or 19	0 h L: 24 h D or 12 h L: 12 h D	Same conditions as for *A. kimi*
O. cincta	20 ± 2	As for *H. nitidus*	Same conditions as for *A. kimi*
H. assimilis	20 ± 2 [28],[35]	As for *F. fimetaria*	40 to 60 [28]
M. macrochaeta	20 ± 2 [28],[35]	As for *F. fimetaria*	40 to 60 [28]
[a] L = light; D = dark.			

E.6.4 Maintenance during the test

The procedures for feeding (including type of food) and water replenishment for the duration of the test exposure and for each of the alternative Collembolan species are provided in table E.11.

Table E.11 — Feeding and water replenishment during the test exposure for alternative Collembolan species

Species	Feeding	Water replenishment
F. fimetaria	2 mg to 10 mg granulated dry yeast added to each container at the beginning of the test and after 14 days [28,35].	At least once a week replicates must be aerated and test vessels are weighted to monitor soil moisture. Weight loss > 2 % is replenished by the addition of de-ionised water [28].
O. yodai	2 mg to 5 mg of granulated dry yeast, both at the beginning and 14th day of test (more food can be added if necessary) with a small droplet of water on it.	Same procedure as for *F. fimetaria*.
O. folsomi	~5 mg granulated dry yeast added to each test vessel on days 0, 7, 14 and 21 [35].	Same procedure as for *F. fimetaria*.
P. minuta	~2 mg of granulated dry yeast on days 0 and 14 when using a test period of 28 days (more food can be added if necessary) with a small droplet of water on it. Alternatively, ~2 mg of granulated dry yeast on days 0, 7 and 14 when using a test period of 21 days with a small droplet of water on it [35].	Same procedure as for *F. fimetaria*.
P. fimata	As for *O. yodai*.	Same procedure as for *F. fimetaria*.
S. curviseta	~2 mg of granulated dry yeast, both at the beginning and 14th day of test (more food can be added if necessary) with a small droplet of water on it.	Same procedure as for *F. fimetaria*.
A. kimi	~30 mg of granulated dry yeast, both at the beginning and 14th day of test (more food can be added if necessary) with a small droplet of water on it [68].	Same procedure as for *F. fimetaria* [28],[66],[67].
Y. szeptyckii	~10 mg of granulated dry yeast, both at the beginning and 14th day of test (more food can be added if necessary) with a small droplet of water on it [70]-[72].	At least once a week replicates must be aerated for 15 min and test vessels are weighted to monitor soil moisture. Weight loss > 2 % is replenished by the addition of de-ionised water [72].

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

Table E.11 *(continued)*

Species	Feeding	Water replenishment
H. nitidus	~1 mg of granulated dry yeast, once a week if needed [51].	Same procedure as for *F. fimetaria*.
O. armatus	~4 mg of granulated dry yeast, once a week if needed [74].	Same procedure as for *F. fimetaria*.
O. cincta	Thick suspension of green algae (e.g. *Pleurococcus* spp.) provided on filter papers. Filter papers replaced with new filter papers containing fresh food once a week.	Same procedure as for *F. fimetaria*.
H. assimilis	As for *F. fimetaria*.	Same procedure as for *F. fimetaria*.
M. macrochaeta	As for *F. fimetaria*.	Same procedure as for *F. fimetaria*.

E.6.5 Test termination

The total test exposure duration and the extraction method for determining adult and juvenile counts is provided for each alternative species in Table E.12.

Table E.12 — Total test exposure duration and extraction method for alternative Collembolan species

Species	Test duration [days]	Extraction method
F. fimetaria	21	Heat extraction or flotation similar to the methods described for *F. candida* given in Annex D.
O. yodai	28	Same procedure as for *F. fimetaria*.
O. folsomi	28	Same procedure as for *F. fimetaria*.
P. minuta	21 or 28	Same procedure as for *F. fimetaria*.
P. fimata	28	Same procedure as for *F. fimetaria*.
S. curviseta	21 or 28	Same procedure as for *F. fimetaria*.
A. kimi	28 [66],[67]	Same procedure as for *F. fimetaria*.
Y. szeptyckii	28	Same procedure as for *F. fimetaria*.
H. nitidus	21	Same procedure as for *F. fimetaria*.
O. armatus	28 [74]	Same procedure as for *F. fimetaria*.
O. cincta	49 to 85 [82],[90]	Same procedure as for *F. fimetaria*.
H. assimilis	28	Heat extraction as described for *F. candida* in Annex D.
M. macrochaeta	28	Same procedure as for *H. assimilis*.

E.6.6 Validity criteria

The validity criteria that should be satisfied in the untreated controls for a test result to be considered valid at the end of the test are provided for each alternative Collembolan species in Table E.13.

Table E.13 — Validity criteria for alternative Collembolan species

Species	Adult mortality [%] per control vessel	Number of juveniles produced per control vessel	Coefficient of variation of juveniles produced in control vessels [%]
F. fimetaria	Mean ≤ 20 [28] or mean ≤ 30 [35]	Mean ≥ 100 [28],[35]	≤ 30 [28]
O. yodai	Mean ≤ 20	Mean ≥ 100	≤ 30
O. folsomi	Mean ≤ 30 [35]	Mean ≥ 100 [35]	≤ 30

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

Table E.13 *(continued)*

Species	Adult mortality [%] per control vessel	Number of juveniles produced per control vessel	Coefficient of variation of juveniles produced in control vessels [%]
P. minuta	Mean ≤ 20; or alternatively, mean ≤ 30 for artificial soil and mean ≤ 40 for natural soil [35]	Mean ≥ 100	≤ 30
P. fimata	Mean ≤ 20	Mean ≥ 100	≤ 30
S. curviseta	Mean ≤ 20	Mean ≥ 100	≤ 40
A. kimi	Mean ≤ 20 [28],[67]	Mean ≥ 100 [28],[67]	≤ 30 [28],[67]
Y. szeptyckii	Mean ≤ 30 [70]-[72]	Mean ≥ 60 [72]	≤ 30 [28]
H. nitidus	Mean ≤ 20	Mean ≥ 100	e.g. ≤ 30 (no data available)
O. armatus	Mean ≤ 20	Mean ≥ 100	e.g. ≤ 30 (no data available)
O. cincta	Mean ≤ 20	Mean ≥ 100	e.g. ≤ 30 (no data available)
H. assimilis	Mean ≤ 20 [91]	Mean ≥ 100 [91]	≤ 30
M. macrochaeta	Mean ≤ 20 [91]	Mean ≥ 50 [91]	≤ 30

E.6.7 Sensitivity and suitability

In Table E.14., toxicity data of the alternative Collembolan species to different chemical substances are presented, taken from publicly available literature. These data aim to provide evidence of the sensitivity of the alternative Collembolan species to chemical substances.

Table E.14 — Sensitivity of alternative Collembolan species to chemical contaminants

Species	Toxicity endpoints [mg/kg sdw] [a]	Additional comments
F. fimetaria	Boric acid: LC50 = 560; EC50 (repro) = 107 [29]; EC50 (repro) = 100 [28]	
O. yodai	Cadmium: EC50(repro)= 154,7 [33]	
Orthonychiurus folsomi	Boric acid: 35-d LC50 = 1 476 in artificial soil; 35-d EC50 = 503 in artificial soil [35]	
P. minuta	Cadmium: EC50 (repro)= 125 [92] Copper: EC50 (repro)= 696 natural soil [92] Zinc: EC50 (repro)= 283 [92] Metsulfuron-methyl (as c.f.): EC50 (repro) > 10 [93] Metsulfuron-methyl (as c.f.) + mineral oil (as c.f.): EC50 (repro) = 0,003 [93] Mercury: LC50 = 6,87 to 7,16 (depending on test soil and temperature) [36] Mercury: EC50 (repro) = 4,43 to 4,58 (depending on test soil and temperature) [36] Copper: EC50 (repro) = 157,5; LC50 = 1 180 [94] Phenol: EC50 (repro) = 186 [94] Arsenic: EC50 (repro) = 4,4; LC50= 36,7 [94] Nonylphenol: EC50 (repro) = 3,31 [94]	

[a] sdw = soil dry weight.

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved

Table E.14 *(continued)*

Species	Toxicity endpoints [mg/kg sdw] [a]	Additional comments
P. fimata	2-phenylethyl isothiocyanate: EC50(repro) = 11,2 nmol/g sdw; LC50 = 15,2 nmol/g sdw [95] α-cypermethrin: EC50 (abund) =14,7 - 15,9 mg/kg (varies with community composition) [96]	
S. curviseta	Pyrimethanil (a.s.): EC50 (repro) (at 20 °C or 26 °C, varies with % C_{WH}) = 60 to 80 [97] Lambda-cyhalothrin (a.s.): EC50(repro) (at 20 °C or 26 °C, varies with % C_{WH}) = 10 to 20 [83] Copper: EC50 (repro) = 442 [98] Zinc: EC50(repro) = 2 760 [98] Lead: EC50(repro) = 3 212 [98] Boric acid: LC50 = 279; EC50 = 95; EC20 = 83; EC10 = 77; NOEC: 31,5 (all values from Reference [51])	
A. kimi	Cadmium: 28-d LC50=90,1 mg/kg[68], EC50(repro) = 60,0 mg/kg [68] Mercury: 28-d LC50 = 2,6 mg/kg[68], EC50(repro) = 0,23 mg/kg [68] Lead: 28-d LC50 = 1 299 mg/kg[68], EC50(repro) = 428 mg/kg [68] Copper: EC50(repro) = 277 mg/kg [99] Manganese: EC50(repro) = 326 mg/kg [99] Nickel: EC50(repro) = 52,6 mg/kg [99] in the OECD artificial soil	
Y. szeptyckii	Tebufenozide: 28-d LC50 > 700 mg/kg, EC50(repro) = 43,8 mg/kg [70] Teflubenzuron: 28-d LC50 > 0,90 mg/kg, EC50(repro) = 0,07 mg/kg [71] Fenoxycarb: 28-d LC50 = 955,2 mg/kg, EC50(repro) = 0,2 mg/kg [72]	Higher sensitive to fenoxycarb toxicity than *F. candida*. The EC50(repro) was 62 times smaller than that of *F. candida*.
H. nitidus	Imidacloprid [51]: LC50 = 1,50 (1,0 to 2,0) EC50 (repro) = 0,50 (0,30 to 0,60) EC20 (repro) = 0,30 (0,10 to 0,50) EC10 (repro) = 0,20 (0 to 0,40) NOEC = 0,37 Thiacloprid [51]. LC50 = > 10 EC50 (repro) = 1,5 (0,20 to 2,5) EC20 (repro) = 0,70 (0 to 1,5) EC10 (repro) = 0,45 (0 to 1,0)	
O. armatus	Betanal [100] LOEC (mortality): 1,2 LOEC (avoidance): 1,2	

[a] sdw = soil dry weight.

STANDARDSISO.COM : Click to view the full PDF of ISO 11267:2023

© ISO 2023 – All rights reserved