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Soil quality — Test for measuring the inhibition of reproduction in oribatid mites (*Oppia nitens*) exposed to contaminants in soil

Qualité du sol — Essai de détermination de l'inhibition de la reproduction chez les acariens oribates (Oppia nitens) *exposés aux contaminants dans le sol*

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Foreword

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

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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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*.

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

Introduction

Ecotoxicological test systems are applied to obtain information about the effects of contaminants in soil and are proposed to complement conventional chemical analysis (see ISO 15799[1] and ISO 17616[2]). ISO 15799 includes a list and short characterization of recommended and standardized test systems and ISO 17616 gives guidance on the choice and evaluation of the bioassays. Aquatic test systems with soil eluate are applied to obtain information about the fraction of contaminants potentially reaching the groundwater by the water path (retention function of soils), whereas terrestrial test systems are used to assess the habitat function of soils with regards to supporting soil biota and interactions within.

Mites (Acari) are a world-wide and diverse group of arthropods belonging to a sub-class of Arachnida with over 40 000 species recorded, divided into two super-orders (Acariformes and Parasitiformes). Due to their relative small size (a few µm to a few cm), they occupy specific ecological niches on plants as well as in soils[5].

In recent years, there has been an increase in the development of biological test methods for assessing contaminated soil, which has historically lagged behind that for other media (e.g., water and sediment). Ecotoxicology tests for soil are challenged, among other things, by the complexity of soil systems (e.g., lack of homogeneity) and the variety of exposure routes (e.g., via alimentary uptake, exposure to pore water or soil vapours, or direct contact with soil particles). A recently developed method (ISO 21285[3]) assesses the effects of contaminated soil on the reproduction of the predatory mite (*Hypoaspis aculeifer*), mainly through alimentary uptake. Oribatid mites represent a different but essential ecological niche than *H. aculeifer* within soil, contributing to carbon mineralization and soil formation, as well as nitrogen and phosphorous release through grazing activities. Oribatid mites are among the most diverse and abundant micro-arthropod species within soil, however, their slower metabolism and development, coupled with low fecundity, long life span, and limited dispersal capacity increase the potential for susceptibility and sensitivity to short- and long-term disturbances[6]. The use of oribatid mites in the context of soil ecotoxicology testing has been thoroughly reviewed[7][8][9][10][11]. Recent research using *Oppia nitens* for soil testing has demonstrated applicability and relative sensitivity of the species for the assessment of contaminated soils from both agronomic regions, and those from the boreal and taiga ecozones[6][12][13][14][15]. Research has also demonstrated its sensitivity to metals[16][17][18][19], pesticides[20][21], and organic compounds[16][17][22]. *Oppia nitens* is an oribatid mite, inhabiting the upper organic layer of soil, and is a member of the largest oribatid family (Oppiidae) with approximately 1 000 species in 129 genera widely distributed throughout Holarctic and Antarctic regions[23]. They are sexually reproducing, polyphagous fungivores that can be easily reared in the laboratory in soil or on plaster of Paris, and on a diet of Baker's yeast[10].

This method outlines procedures for conducting 28-day tests for determining the effects of contaminated soils on the survival and reproduction of the oribatid mite, *Oppia nitens*. Optionally, the method can be used for testing chemicals added to standard soils (e.g., artificial soil) for their lethal and sublethal hazard potential to oribatid mites. The performance of this method has been assessed in an international inter-laboratory investigation[15], as summarized in Annex E. Mites represent communities that cannot be omitted from environmental hazard assessment. This species is considered to be representative of non-predatory soil mites.

Soil quality — Test for measuring the inhibition of reproduction in oribatid mites (*Oppia nitens*) exposed to contaminants in soil

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

1 Scope

This document specifies one of the methods for evaluating the habitat function of soils and determining effects of soil contaminants and individual chemical substances on the reproduction of the oribatid mite *Oppia nitens* by dermal and alimentary uptake. This chronic (28-day) test is applicable to soils and soil materials of unknown quality (e.g., contaminated sites, amended soils, soils after remediation, agricultural or other sites under concern and waste materials). This method is not intended to replace the earthworm or Collembola tests since it represents another taxonomic group (= mites; i.e., arachnids), nor the predatory mite test since this species represents a different trophic level and ecological niche.

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

Information is provided on how to use this method for testing substances under temperate conditions.

This document is not applicable to substances for which the air/soil partition coefficient is greater than 1, or to substances with vapour pressure exceeding 300 Pa at 25 °C.

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

2 Normative references

The following documents 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 quality — 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 11265, *Soil quality — Determination of the specific electrical conductivity*

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 terminological databases for use in standardization at the following addresses:

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

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

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

3.2
effect concentration
EC*x*
concentration (mass fraction) of a test sample or 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
effect rate
ER*x*
rate of a soil to be tested that causes an *x* % of an effect on a given end-point within a given exposure period when compared with a control

3.4
lethal concentration
LC*x*
concentration (mass fraction) of a test sample or test substance that causes *x* % mortality within a given exposure period when compared with a control

EXAMPLE An LC50 is a concentration estimated to cause mortality in 50 % of an exposed population over a defined exposure period.

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

3.5
limit test
single concentration test consisting of at least five replicates each, the *test soil* (3.14) without any dilution or the highest concentration of test substance mixed into the control soil and the control

3.6
lowest observed effect concentration
LOEC
lowest tested concentration (mass fraction) of a test sample or test substance that has a statistically significant effect (probability $p < 0,05$)

Note 1 to entry: In this test, the LOEC is expressed as a percentage of soil to be tested (dry mass) per soil mixture (dry mass) or 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.

3.7
lowest observed effect rate
LOER
lowest rate of a soil to be tested in a control soil at which a statistically significant effect is observed

3.8
no observed effect concentration
NOEC
highest tested concentration (mass fraction) of a test sample or test substance immediately below the LOEC at which no effect is observed

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

3.9
no observed effect rate
NOER
lowest rate of a soil to be tested immediately below the LOER which, when compared to the control, has no statistically significant effect (probability $p < 0,05$) within a given exposure period

3.10
reproduction
mean number of offspring per test vessel after a 28-day incubation under the specified test conditions

3.11
reference soil
uncontaminated soil with comparable pedological properties (nutrient concentrations, pH, organic carbon content and texture) to the *test soil* (3.14)

3.12
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 those of the test soil.

3.13
control soil
reference or *standard soil* (3.12) used as a control and as a medium for preparing dilution series with test soils/samples 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.

3.14
test soil
sample of field-collected soil or chemical-spiked soil to be evaluated for toxicity to mites

3.15
test mixture
mixture of contaminated soil or the test substance (e.g. chemical, biosolid, waste) with *control soil* (3.13)

Note 1 to entry: Test mixtures are given in percent of contaminated soil based on soil dry mass.

3.16
test mixture ratio
ratio of test soil to control soil in a *test mixture* (3.15)

Note 1 to entry: Different ratios may be applied in a dilution series to establish a dose-response relationship.

4 Principle

The effects on reproduction of adult, laboratory-cultured mites, *Oppia nitens,* exposed to the test soil are compared to those observed for organisms in 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 during the test period.

The test is started with 15 adult mites from age-synchronized cultures (aged 8 to 10 d after ecdysis (i.e., moult) to adult form) per test vessel. The test is performed in 30 mL glass vessels with a wet-weight equivalent to a volume of ~20 mL of soil, and a minimum of five replicates are prepared for each treatment. The test runs for 28 d at (20 ± 2) °C by which time offspring (F_1) have emerged from eggs laid by the adults and the number of offspring produced is determined. Survival of the adults is also determined at the end of the test. The results obtained from the tests are compared with a control or, where a serial dilution design is used, to determine the concentration resulting in *x* % reduction of juveniles produced compared to the control (EC*x*, 28 d), depending on the experimental design. An estimate of the test concentration resulting in *x* % mortality (LC*x*, 28 d) is an optional test endpoint. If a multi-concentration hypothesis test design is used, the reproductive output of the mites exposed to the test mixtures is compared to that of the controls in order to determine the concentration which causes no effects on mortality and reproduction (NOEC).

In cases where there is no prior knowledge of the dilution/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:

— a range-finding test is carried out to give an indication of the effect dilution/concentration, and the dilution/concentration resulting in no mortality. Dilutions/concentrations to be used in the definitive test can then be selected; and

— the definitive test to determine lethal and sub-lethal effects of (dilutions of) contaminated soil or the concentration of a substance which, when evenly mixed into the standard soil, results in: 1) *x* % inhibition of reproduction, EC*x* (e.g., EC10, EC20, or EC50), or 2) causes no significant effects on numbers of offspring hatched from eggs compared with the control for estimation of the NOEC and LOEC.

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

5 Reagents and material

5.1 Biological material

In this test, adult mites, *Oppia nitens* C.L. Koch 1836, aged 8 to 10 d (i.e., 8 to 10-d post-ecdysis to adult form), established from newly emerged adults collected over a 1- to 3-d period, are required to start the test. The mites shall be selected from an age-synchronised culture. A method for culturing *Oppia nitens* and for obtaining age-synchronised test organisms is provided in Annex A.

Adult *Oppia nitens* are obtained from laboratory cultures maintained under conditions of temperature, photoperiod, and food similar to those in the test. Species identification should be confirmed by qualified personnel experienced in identifying soil mites using the distinguishing taxonomic features, described in taxonomic keys[26], or using DNA-based taxonomic identification (i.e., barcoding) as outlined in ISO 21286[4]. All mites used in a test shall be derived from the same population and source. Sources of animals to be used to establish cultures include government or private laboratories that are culturing *Oppia nitens* for soil toxicity tests, or commercial biological suppliers[24].

5.2 Test mixture can consist of field-collected soil or control soil mixed with the test soil or spiked with 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 works, plant-derived compost, or manure) under consideration for possible land disposal.

The field-collected soils used in this test shall be passed through a sieve of 4 to 10 mm square mesh to remove coarse fragments and thoroughly mixed. If necessary, soil may be air-dried without heating before sieving. Storage of the test soil 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.

NOTE A 4-mm mesh sieve is appropriate for use with any mineral-based soil with relatively low organic matter (e.g., agricultural soil), however for forest soils or wetland soils with higher organic matter content, sieves with larger mesh sizes (e.g., 6 to 8 mm for forest soils and 8 to 10 mm for wetland soils) could be required.

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) electrical conductivity in accordance with ISO 11265

g) organic carbon in accordance with ISO 10694;

h) percentage of material removed by the sieve.

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

5.2.2 Control soil, either a) reference soil (3.11) or b) standard soil (3.12) that allows the presence of oribatid mites. Control soil and soil used for dilution shall not differ in one test (either a) or b)).

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

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

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

	Percentage expressed on dry mass basis
— Sphagnum peat finely ground (a particle size of (2 ± 1) mm is acceptable) and with no visible plant remains	10 %
— Kaolinite clay containing not less than 30 % kaolinite	20 %
— Industrial quartz sand (dominant fine sand with more than 50 % of particle size 0,05 mm to 0,2 mm)	70 %

NOTE It has been demonstrated that *Oppia nitens* can conform with the validity criteria (survival and reproduction) when tested in field soils with lower organic matter content (e.g., 2,6 %)[24], and experience shows that the validity criteria can be achieved in artificial soil with 10 % peat. It is therefore not necessary, before using such a soil in a definitive test, to demonstrate the suitability of the artificial soil in complying with the validity criteria, unless the peat content is lowered more than specified above[24].

Prepare the artificial soil at least three days prior to starting the test, by mixing the dry constituents listed above thoroughly in a large-scale laboratory mixer. Guidance on the adjustment of pH of the artificial soil is provided in Annex C; the optimal pH range for the *O. nitens* test is 7,0 ± 0,5 pH units[24]. A portion of the deionized water required is added while mixing is continued. 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 the 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 three days to equilibrate acidity. To determine pH and the maximum water holding capacity, the dry artificial soil is pre-moistened one or two days before starting the test by adding deionized water to obtain approximately half of the required final water content of 50 % to 70 % of the maximum water holding capacity.

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

5.3 Food

As a suitable food source, a sufficient amount, e.g., 0,5 mg to 1 mg, of granulated dried baker's yeast, commercially available for household use, is added to each container at the beginning of the test (Day 0) and every 7 days up to and including Day 21.

5.4 Reference substance

The EC*x* of a reference substance shall be determined to provide assurance that the laboratory test conditions are adequate and to verify that the response of the test organisms did not change over time. The reference substance can be tested in parallel to the determination of the toxicity of each test sample at one concentration, which shall be demonstrated beforehand in a dose response study to result in an effect of about 50 %. In this case, the number of replicates should be the same as that in the controls. Alternatively, the reference substance is tested 1 to 2 times a year in a dose-response test. Depending on the design chosen, the number of concentrations and replicates and the spacing factor differ (see 7.1.3), but a response of 10 % to 90 % effect should be achieved (spacing factor of 1,8). Boric acid is a suitable reference substance that has been shown to affect reproduction[14][15][19].

The EC50 for boric acid based on the number of juveniles should fall in the range between 290 mg/kg (dry mass) soil and 410 mg/kg (dry mass) soil in artificial soil, and in the range between 80 mg/kg (dry mass) soil and 120 mg/kg (dry mass) soil in field-collected soil including standard LUFA 2.2[15].

5.4.1 Boric acid (CAS 10043-35-3), H_3BO_3 (99 %).

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

6 Apparatus

Use laboratory equipment and the following apparatus.

6.1 Test containers made of glass (e.g., glass shell vials) or other chemically inert material of a capacity of about 30 mL (about 2,6 cm inner diameter), should be used. Test containers shall be covered to prevent mites from escaping but shall allow gas exchange and light penetration (e.g. perforated transparent cover or removable lid that allows for aeration on a weekly basis).

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 temperature, pH, and water content of the test substrate.

6.6 Apparatus for heat extraction of mites, as described in Annex D.

6.7 Test environment

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

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

7 Procedure

7.1 Experimental design

7.1.1 General

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

7.1.2 Range-finding test (preliminary test)

A preliminary test to find the range of mixture ratio (e.g. 0 %, 1 %, 5 %, 25 %, 50 %, 75 %, 100 % soil), or of the test substance (e.g., 0 mg/kg, 1 mg/kg, 10 mg/kg, 100 mg/kg and 1 000 mg/kg; the concentrations being expressed in milligrams of test substance per kilogram of dried control soil), affecting *O. nitens* is optional. The range-finding test is conducted with reduced replication (e.g., 3 replicates), relative to the definitive test. The duration of the range-finding test is 28 d, after which mortality of the adult mites and the number of juveniles is determined. Based on the results of the range finding test, the EC50 for inhibition of reproduction should be estimated. The concentration/dilution range in the definitive test should preferably be chosen so that it includes concentrations that span a wide range, including a low concentration that evokes no adverse effects (similar to the negative control treatment) and a high concentration that results in “complete” or severe effects. To keep the wide range of concentrations and also obtain the important mid-range effects, it might be necessary to use additional treatments in order to split the selected range more finely.

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

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[24].

If for characterization purposes a test design including serial dilution series is required, three designs are possible:

1) for the EC*x* approach, a minimum of 7 concentrations plus the control treatment(s) shall be used, and more (i.e., ≥10 plus controls) are recommended to improve the likelihood of bracketing each endpoint sought[25]. The spacing factor can be variable; smaller at lower concentrations, larger at high concentrations. Five replicates for each treatment plus the controls are recommended;

NOTE If a range-finding test is conducted prior to definitive testing, fewer concentrations (e.g., 6 or 5) can be possible in the definitive test, since more information on the effect concentration/dilution range will be available.

2) for the NOEC hypothesis approach, at least five concentrations or test mixtures in a geometric series should be used. Five replicates for each treatment plus eight controls are recommended; or

3) for the mixed approach, 6 to 8 concentrations or test mixtures in a geometric series should be used. Five replicates for each treatment plus eight controls are recommended. This mixed approach allows a NOER/NOEC as well as an ER*x*/EC*x* evaluation.

Regardless of the test design chosen, the test concentrations shall be spaced by a factor not exceeding 2.

A limit test can be sufficient if, in the range-finding test, no toxic effect was observed. In the limit test, only the highest concentration tested in the range-finding test (i.e., 1,000 mg/kg) or test soil without any dilution (100 %) and the control shall be tested with a minimum of five replicates for each treatment.

To facilitate checking of the physicochemical analyses at the beginning and end of the test, use of additional containers for each concentration and for the control is recommended.

Each test container (replicate) is filled with the wet-weight equivalent to measured volume of about 20 mL at optimal soil moisture content (see 7.2.1). To ensure easy migration of mites, the substrate in the test container should be smoothed but not compressed.

7.2 Preparation of test mixture

7.2.1 Testing contaminated soil

According to the selected dilution range, the test soil 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 the wet weight equivalent to a volume of about 20 mL in each test container (6.1). The test mixture shall be wetted with deionised water to reach 40 % to 60 % of the total water holding capacity, or that which results in a crumbly texture (i.e. 2 to 3 mm clumps) that is optimal for testing, determined according to Annex B. In some cases, e.g. when testing forest soils, wetland soils, or waste materials, higher or lower percentages might be required. A rough check of the soil moisture content can be obtained by gently squeezing the soil by hand, if the moisture content is optimal, small drops of water should appear between the fingers.

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

Proceed simultaneously with the replicate requirement per concentration according to the selected test design (7.1.3).

For soils collected as distinct horizons (e.g., boreal or taiga soils), each horizon shall be tested separately in independent definitive tests[24].

WARNING — Contaminated soils can contain unknown mixtures of toxic, mutagenic, or otherwise harmful substances or infectious micro-organisms. Occupational health risks can arise from dust or evaporated substances as well as via dermal contact during handling and incubation.

7.2.2 Testing substances added to the test substrate

Standard soil is used to prepare the test sample. For each test container (6.1), the mass of the substrate used shall be the wet-weight equivalent to a volume of about 20 ml. Substances are added to the test substrate and mixed thoroughly.

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

a) water-soluble substance

— immediately before starting the test, dissolve the quantity of the test substance in the water or a portion of it required to wet the soil samples for the replicates of one concentration in order to meet the requirements of 5.2.2, and mix it thoroughly with the soil before introducing it into the test containers;

b) substances insoluble in water but soluble in organic solvents

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

NOTE Ultrasonic dispersion, organic solvents, emulsifiers or dispersants can be used to disperse substances with low aqueous solubility. When such auxiliary substances are used, all test concentrations and any additional controls will 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) or standard field soil and the quantity of the test substance required to obtain the desired concentration. Add that mixture to the remainder of the soil (i.e. artificial or standard field soil, respectively), add the water and mix thoroughly before introducing it into test containers.

Base the concentrations selected to provide the EC*x* or the NOEC/LOEC on the results of the range-finding test. Space the concentrations by a factor not exceeding 2.

Substances mixed into a reference or control soil do not need to be tested at concentrations higher than 1 000 mg/kg mass of reference or standard soil.

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 containers

The control container contains the control soil wetted with deionised water to reach 40 % to 60 % of the total water holding capacity, or that which results in a crumbly texture (i.e., 2 to 3 mm clumps) that is optimal for testing (determined according to Annex B).

Perform three control containers for the range-finding test and at least five control containers for the definitive test.

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

7.3 Addition of the mites

Fifteen age-synchronized adult mites (see Annex A) are placed in each control and treatment vessel. Test organisms should be added the day after preparation of the test and control soils (i.e. after dilution of a test soil or application of the test item, and preparation of replicate vessels).

Mites are gently transferred from the age-synchronized cultures to a piece of folded cardboard, a small glass container, or a weigh boat (previously washed and dried to remove a waxy film that coats the weigh boats) using a fine paintbrush[24]. Before they are transferred to the test containers, organisms are counted and checked for colouration or damage both to reduce control mortality and to avoid systematic trial errors.

Vessels are covered as indicated in 6.1.

7.4 Test conditions and measurements

On the day of addition of test organisms to the soil (Day 0) and every 7 d up to and including Day 21, 0,5 mg to 1 mg of granulated yeast (see 5.3) is distributed uniformly over the surface of the moist test soil. If food consumption is low in a given vessel, reduce feeding to ensure that the same amount of yeast is available to organisms in each vessel. Lids are removed from each test vessel briefly at least once per week or more frequently (≥2 times per week), as necessary, to allow aeration. Observations and records should be made at this time regarding appearance of soil (growth of bacteria or fungi), feeding activity, and presence and quantity of uneaten food.

Temperature should be recorded at least daily and adjusted, if necessary. The pH (7.2.1 and 7.2.2) and water content (according to ISO 11465) of soil representing each treatment shall be measured and recorded at the beginning and end of the test. Additionally, it is recommended that conductivity (see ISO 11265) be measured at the beginning and end of the test in instances where the test soil is anticipated to have high salt content.

The water content of the soil substrate in the test vessels is maintained throughout the test by examining the apparent “wetness” (see 7.2.1) of each vessel during weekly observations. Soil that appears too dry should be moistened with a test water dropper, being careful not to over-hydrate the soil. Alternatively, the water content of the soil substrate can be maintained throughout the test by weighing and if needed re-watering the test vessels periodically (e.g., once per week). Losses are replenished as necessary with deionised water. The moisture content during the test should not differ by more than 10 % from the start value. When acidic or basic substances are tested, do not adjust the pH.

7.5 Determination of adult survival and reproductive output

On Day 28, the live mites (adults and progeny) are extracted from the soil via heat/light extraction (see Annex D). The juveniles (i.e. larvae, protonymphs, deutonymphs, and tritonymphs) and adults are counted separately. All organisms, alive or dead are counted and recorded. Any adult mites not found at this time are to be recorded as dead, assuming that such mites have died and decomposed prior to the assessment. The parameters (e.g., bulb wattage, height above soil, etc.) of the soil organism heat extraction method shall be optimized to allow live organisms to move efficiently through the soil to a collection vessel without becoming desiccated. The temperature gradient should also be optimized to create an effective temperature and moisture gradient to enable the movement of the organisms and should be performed between 48 h and 120 h (see Annex D). The heat extraction efficiency should be validated once or twice a year in controls with known numbers of adults and juveniles. Efficiency should be ≥90 % on average combined for all developmental stages (see Annex D). Adult and juvenile counts are not adjusted for efficiency.

Any observed differences between the behaviour and the morphology of the mites in the control and the treated vessels should be recorded.

Adults can be easily distinguished from juveniles by their significantly greater size and darker colouration (see Annex A.3). A mite is considered dead if there is complete cessation of movement of any body part. Dead *O. nitens* tend to have their legs curl underneath them and can, but generally do not, change colour. Care should be taken to distinguish dead individuals from molted carapaces; the latter are translucent and collapsed.

The extraction efficiency can be demonstrated by counting the number of adults and juveniles present in the negative control treatment. The extraction efficiency can be assessed by counting the number of individuals remaining within the heat-extracted soil, after the heat extraction is complete, relative to the total amount of mites. The heat-extracted soil can be checked by traditional floatation methodology. If the criterion is consistently met, then the heat extracted soil may be disposed of accordingly. Otherwise, all other treatments shall be processed in a similar manner.

8 Calculation and expression of results

8.1 Calculation

For each dilution or concentration, determine the percent adult mortality and number of offspring produced after a period of 28 days.

8.2 Expression of results

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

If dilution or concentration series were performed:

— for the EC*x* approach, indicate the % soil mixture 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 resulted in reduced the number of juvenile mites to 50 % (EC50) compared to the control within the test period[25];

— for the NOEC approach, indicate the soil mixture ratio immediately below the 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 reproduction ($p < 0,05$).

9 Validity of the test

The results are considered to be valid, if:

— the mean percent mortality of the adults in the control(s) is ≤30 % at the end of the test;

— the mean number of live juvenile mites in control vessels at the end of the test is ≥30;

— the coefficient of variation of reproduction in the control should not exceed 40 %.

NOTE Validity criteria for this method are based on testing of 13 soil types and horizons (agricultural and forest soil)[24] and the results of international inter-laboratory testing (see Annex E and [15]).

10 Statistical analysis

10.1 General

Although this method primarily measures sub-lethal endpoints (e.g., reproduction involve quantitative effects such as counting juvenile mites, quantal effects (e.g., survival of adults) may also be measured at the end of the test (Day 28).

NOTE Guidance given here for statistical evaluation of test results aims to make the investigator aware of problems that can arise in consequence of a test design selected. Computer programs do not necessarily guard against violations of rules that can cause erroneous analyses. It is strongly recommended to look for more information in specific guidance documents (e.g., as provided by [25]) or to contact a statistician.

10.2 Single-concentration tests

Quantitative single-concentration tests (e.g. effects on reproduction) have different statistical methods. For sampling at several locations with field replication, ANOVA can be a first step if results are suitable. If the null hypothesis of no difference was rejected, analysis can proceed to one of several multiple-comparison tests[25].

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

Analysis of variance (ANOVA) involving multiple comparisons of end point data derived for undiluted soil to be tested including field replicates of field-collected soil from more than one sampling location, is commonly used for statistical interpretation of the significance of quantitative findings (e.g., reproduction) from soil toxicity tests. This is a hypothesis-testing approach, and is subject to appreciable weaknesses[25]. The parametric analyses (e.g., ANOVA and multiple comparisons) for such data assume that the data are normally distributed, that the treatments are independent, and that the variance is homogenous among the different treatments. These assumptions shall be tested. If the data satisfy these assumptions, analysis may proceed. If not, data may be transformed and tested again. As parametric tests are reasonably robust in the face of moderate deviations from normality and equality of variance, parametric analysis should proceed, even if moderate nonconformity continues after transformation[25]. Data which fail to satisfy either test might be transformed to meet the requirements. If the original or transformed data do not satisfy either test for distribution of data, then analysis by nonparametric methods shall be carried out.

10.3 Multi-concentration tests

10.3.1 Range-finding test

If a clear dose-response is obvious, EC*x*-values can be estimated using regression techniques like logistic regression function and LC*x* values using probit analysis. In other cases, the effect range could be determined by expert knowledge.

10.3.2 Definitive test

The quantal mortality data for a specific period of exposure (i.e., 28 d) can be used to calculate (data permitting), the appropriate point estimate (LC*x*), together with 95 % confidence limits. The optimization of the calculation of the LC*x* is based on the number of partial effects observed[25]. Several methods are available for statistically estimating a test endpoint from quantal mortality data. For further guidance see[25].

A point estimate (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 non-linear regression methods are widely applied for statistical analysis. Operators should be aware of being able to understand the judgements in selecting appropriate mathematical models.

Hypothesis testing (NOEC-approach) is commonly used to identify dilutions (concentrations) with significant effects compared to the control. As this method has many flaws, it is not recommended for use[25]. 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. However, if legislation requires NOEC/LOEC estimates, then these values can be calculated in addition to the point estimate endpoints.

— EC*x* (effect concentration)-approach

The EC*x*-approach can only be used if a clear dose response relationship is found. Wherever possible, the R^2 (where R is the regression coefficient) should be 0,7 or higher and the test mixtures used encompass 20 % to 80 % effects. If these requirements are not fulfilled, expert knowledge is necessary for the interpretation of the test results.

To compute an EC*x*-value, the treatment means are used for regression analysis after an appropriate dose-response function has been found (e.g. linear and non-linear regression). A desired EC*x* is obtained by inserting a value corresponding to *x* % of the control mean into the equation found by regression analysis. Since EC50 values have smaller confidence limits compared with smaller effect concentrations (e.g., EC20 or EC10), it is recommended that EC50 values be determined.

For the NOEC approach, statistical analyses shall be made to assess the normality of data (e.g., using Kolmogorov-Smirnov test) and the homogeneity of the variances (e.g., using Cochran's test). With normal and homogeneous data, an appropriate statistical analysis (e.g., "One-Way Analysis of Variance (ANOVA)") followed by a one-sided Dunnett test (α = 0,05), should be performed. If the normality and/or homogeneity requirements are not fulfilled, it is recommended to evaluate if an appropriate transformation of the data can solve the problem. Otherwise non-parametric methods (e.g., the U-test by Mann & Whitney or the Bonferroni-U-Test) can be used.

If a limit test has been performed and the pre-requisites (normality, homogeneity) of parametric test procedures are fulfilled, the Student-*t*-test, otherwise the unequal-variance *t*-test (e.g., Welch *t*-test) or a nonparametric test, such as the Mann-Whitney-U-test, may be used.

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

11 Test report

The test report shall include the following information:

a) a reference to this document, i.e. ISO 23266:2020;

b) the results, expressed as in 8.2;

c) a detailed description of the test substance and information on physical and chemical properties if helpful for the interpretation of the test result;

d) the origin of the field soil used as a control and dilution soil (if appropriate);

e) the complete description of the biological material employed (species, age, breeding conditions, supplier);

f) the method of preparation of the test sample together with an indication of the auxiliary substances used for a low-/non-water-soluble substance;

g) the identity of the reference substance and the results obtained when used;

h) detailed conditions of the test environment;

i) a table giving the percent mortality of adults at each concentration and in the control(s);

j) number of dead or missing adults and number of offspring per test container at the end of the test;

k) depending on the statistical approach selected, list the EC10, EC20 and/or EC50 for the inhibition of reproduction, the LC50 (if calculated) for adult mortality, and the methods used for calculation;

l) in additional to k), it is optional to list the lowest concentration causing significant effects (LOEC) and the highest concentration causing no observed effects (NOEC);

m) description of any pathological or other symptoms, or distinct changes in behaviour observed in the test organisms per test container;

n) water content, pH and conductivity of the soil to be tested and the control soil at the start and at the end of the test for each concentration;

o) any operating details not specified in this document, as well as any factors that may have affected the results.

Annex A
(informative)

Techniques for rearing and breeding of *Oppia nitens*

A.1 Condition for rearing and breeding

A.1.1 General biology[24]

Oppia nitens C.L. Koch 1836 is a member of the largest oribatid family, Brachypylina. Adult *O. nitens* are approximately 510 µm in length and 290 µm in breadth while larvae are approximately 200 µm in length and 105 µm in width, and tritonymphs approximately 372 µm in length and 195 µm in width[27]. During juvenile stages, the mites are white/translucent in colour but transition through golden hues to a rich chestnut-brown within a week of reaching adulthood. Development time (14 to 46 d) appears to be dependent on environmental conditions[10][28][29][30]. There is conflicting information regarding reproductive mode (i.e., sexual or parthenogenetic); however, observations of spermatophores under laboratory conditions[29] are suggestive of sexual reproduction. There is no documented sexual dimorphism exhibited within the species (i.e. males do not differ in size from females), and sexual dimorphism has not been observed to date. *Oppia nitens* eggs are oval, whitish with a smooth surface, with sizes ranging from 90 µm to 150 µm[27] and eggs hatch within about one week of oviposition[10].

Diagnostic features of *O. nitens* have historically included the number and type of setae but more recently, emphasis is being placed on genetic barcoding. Sequencing of the 5' region of mitochrondrial Cytochrome Oxidase Subunit 1 from several *O. nitens* specimens are available for comparison on the International Barcode of Life data portal: http://www.boldsystems.org.

Oppia nitens has been documented as a polyphagous fungivore but does show some selective feeding preferences[31][32]. This species also has a strong preference for ground leaf litter mixed with dried mushrooms, with a moderate preference for leaf litter or mushrooms alone, and very little preference for granulated yeast. However, *O. nitens* has been successfully reared on granulated yeast alone[10].

Like most oribatid mites, growth and maturity of *O. nitens* is characterized by six post-embryonic developmental stages (an inactive prelarva, and an active larva, protonymph, deutonymph, tritonymph, and adult) whereby growth is accomplished with shedding of the exoskeleton. Some aspects of development are unique in that the legs of subsequent instars are formed within the body, rather than within the hull of previous instars' legs[33]. The moult (ecdysis) involves a pre-ecdysial (development of integument) resting stage, characterized by immobility for a prolonged period of time, and depending on the species, can occupy up to one third of a mite's total life span[34]. The shedding of exoskeleton stops with the final developmental stage, as the exoskeleton within the adult form becomes hardened and sclerotized. Melanization is also typical, manifesting in a medium to dark brown colouration.

A.1.2 Breeding substrate

In the laboratory, *O. nitens* cultures demonstrate higher survival and reproductive rates when maintained on a soil substrate (i.e., sandy soil with high leaf litter/organic matter content)[24]. When organisms are needed for testing, they can be extracted from the soil cultures with heat and then transferred to a plaster of Paris substrate for observation and handling. Plaster of Paris (plaster for stucco, pH 6,4) and activated charcoal (pulverized analytical-grade activated charcoal 375 µm mesh, pH 6 to 7), are mixed in a mass ratio of 8:1. To prepare the substrate 120 g of plaster of Paris and 15 g of charcoal are mixed with 130 mL of water. The amount of water needed can vary depending on the type of plaster used. This provides a highly moist substrate, while the charcoal adsorbs waste gases and excretion products. The dark background facilitates observation. The plaster of Paris substrate shall be kept at or near saturation to prevent the substrate from drying out and shrinking away for the edges of the vessel. The substrate should be rinsed with water and lightly blotted with paper towel before organisms are added. The pH of the substrate can be verified by placing pH paper on the wet substrate surface.

A.1.3 Breeding containers

Plastic trays or breeding boxes with a volume of about 1 l are suitable for culturing *O. nitens*. Each culture vessel is filled with the plaster of Paris breeding substrate to a depth of about 1 cm. Pie-shaped pieces of filter paper coated with plaster of Paris or plaster of Paris caps (made by filling aluminium weigh boats with plaster of Paris and allowing them to harden) should be placed on the surface of the substrate to provide hiding places for the mites and to promote egg production. Culture vessels shall be sealed with solid or perforated lids, and the vessels' sides and or lids shall be transparent or translucent to enable light to contact the surface of the culturing substrate. Culture vessels should be re-hydrated with water once or twice per week to maintain the humidity. This is accomplished by adding several drops of water with a pipette or by gently spraying the sides of the vessel until the water just begins to remain on the surface. The culture vessels shall also be aerated periodically (e.g., in combination with feeding) by removing the lids for ≥1 minute.

A.1.4 Climatic conditions

Cultures are maintained at a temperature of (20 ± 2) °C with a light intensity of 400 lux to 800 lux and a light/dark cycle ranging from 12-h light:12-h dark to 16-h light:8-h dark.

A.1.5 Food

Granulated dry yeast is used as a food supply for maintaining cultures and for the test. Food should be added to the culture vessels twice per week, divided into several piles or sprinkled over the substrate surface. The quantity of food added depends on mite density and developmental stage (i.e., based on food consumption). Old unconsumed yeast can be removed before new food is added to avoid fungal and bacteria growth, however this shall be done carefully as eggs and newly hatched larva are often found underneath the yeast. The yeast should be hydrated to activate (i.e., added after the substrate is hydrated or with a few drops of water).

A.1.6 Handling and Transfer

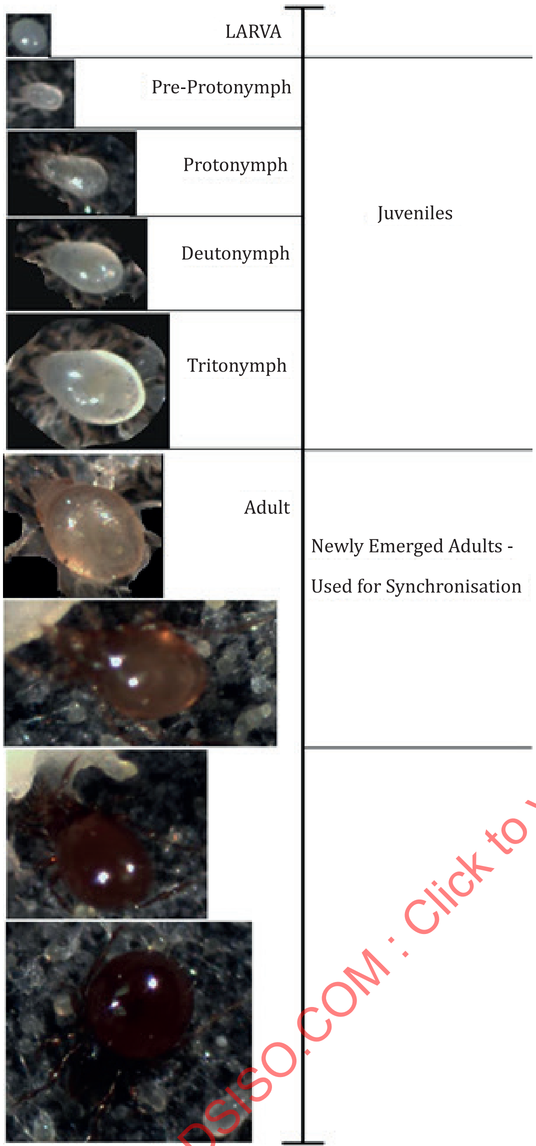
Mites should be handled as little as possible, to avoid damage and stress. Mites can be moved using a fine-tipped paintbrush or by gently tapping one vessel over another. Alternatively small pieces of plaster of Paris (see Section A.1.3) can be baited with food (i.e., yeast), and after 24 h (i.e., once the mites are concentrated around the food), moved to a new container. A loading density of about 5 to 15 adult mites per cm^2 is suggested (i.e., mites do well when slightly crowded[24]).

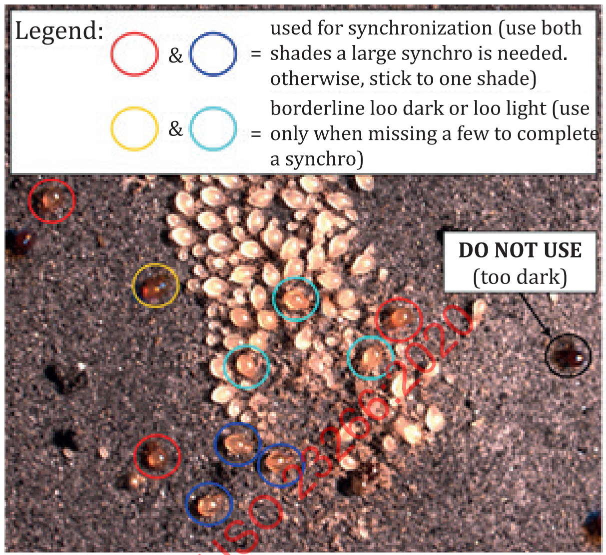
The substrate in each culture vessel should be renewed every 4 to 6 months as required, regardless of organism density. Alternating cultures between plaster of Paris and soil substrates helps maintain culture health and stimulate culture growth (i.e., reproduction). The change of substrate and/or the addition of organic matter to the surface of the plaster of Paris may stimulate oviposition[24].

A.2 Synchronization of cultures

Organisms that are used in the test shall be of similar age ((i.e., aged 8 to 10 d after ecdysis to adult stage). Age-synchronized cultures are established by selecting recently emerged adults from existing cultures and transferring them to a new fresh vessel containing plaster of Paris substrate. Newly emerged adults can be identified based on the appearance of their integument which ranges in colour from pink to light orange/amber and is almost translucent (see A.3). Newly emerged adult mites are selected and added to this culture for up to three days to achieve the number of organisms needed for testing. Once the required number of mites has been added, the culture is allowed to mature for 8 to 10 days before being used in the test. During the age-synchronizing period, mite cultures shall be fed, hydrated, and maintained under similar climatic conditions as those to be used in a test.

A.3 Illustrative photographs of *Oppia nitens*, taken from [24]





Shades of *O.nitens* appropriate for use in age synchronized testing.

Mites with an opaque 'milky' appearance should not be used in testing.

Figure A.1 — Illustrative photographs of *Oppia nitens*

Annex B
(normative)

Determination of water holding capacity

B.1 General

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

B.2 Apparatus

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

B.2.2 Water bath, at room temperature.

B.2.3 Filter paper.

B.2.4 Drying oven, set to (105 ± 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, and after filling with the control or test sample to a depth of 5 cm to 7 cm, place the tube on a rack in a water bath. Gradually submerge the tube until the water level is above the top of the soil, but below the upper edge of the tube. Leave the substrate sample in the water for about 3 h.

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

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

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

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

where

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

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

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

m_D is the dry mass of substrate.

Annex C
(informative)

Guidance on adjustment of pH of artificial soil

To estimate how much $CaCO_3$ is needed to obtain the desired pH (6,0 to 7,5), artificial soil is prepared by mixing peat, sand, kaolin and water as described in 5.2.2. Small portions are taken and mixed with different amounts of $CaCO_3$, e.g. corresponding with concentrations of 0,2 %, 0,4 %, 0,6 %, 0,8 % and 1,0 % dry mass. After a suitable equilibration period (i.e., 3 d), the pH is determined from these portions 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 to 7,5 can be estimated. The optimal pH range for the *O. nitens* test is 7,0 ± 0,5 pH units.

Annex D
(informative)

Extraction and counting of *Oppia nitens*

D.1 General

For micro-arthropods heat extraction is an appropriate method to separate specimens from the soil/substrate. The method is based on the activity of the organisms, so only mobile specimens can be recorded. The principle of the heat extraction is to make conditions for the organisms gradually worse (i.e., dried out and hot) in the sample, so that they leave the substrate and fall into a separate collection vessel. Crucial points are the duration of the extraction and the gradient of good to moderate to bad conditions for the organisms. The temperature and moisture conditions in the sample should always be in a range that allows the mites to move. The heating of the soil/substrate sample leads to desiccation; however, if the rate of desiccation is too quick some mites may also desiccate before they manage to escape.

The following procedure for heat extraction has been shown to be effective for use with *O. nitens* and is proposed herein[24][35]; however, other extraction methods are possible, such as the use of a controlled temperature gradient extractor based on principles developed by MacFayden[35] or Tullgren-type extracting devices[36]. In principle, a gradient of light, temperature and moisture is created over time. Therefore, the heat extraction apparatus consists of an upper vessel, containing the test soil/substrate to be extracted, and a lower vessel, within which the organisms are collected for enumeration. The two vessels are separated by a fine mesh through with the organisms can move, while preventing soil particles from falling or passing through. The micro-arthropods react to the heat and subsequent dessication gradient by moving downwards through the mesh, into the lower collection vessel. The organisms within the collection vessel can then be enumerated by traditional flotation methods, or preserved (e.g., using ethanol (70 % v/v.)).

All equipment, apparatus and construction materials should be made of non-toxic material, and the use of toxic materials including copper, zinc, brass, galvanized metal, lead, and natural rubber shall be avoided. The facility should be well ventilated and free of fumes as well as isolated from any contaminants that might affect the test organisms and isolated from areas for sample preparation and storage.

The extraction efficiency of the chosen method should be validated once or twice a year in controls with known numbers of adults and juveniles; alternately, the extraction efficiency may also be determined by flotation of the heat-extracted soil once the heat extraction method is complete. Efficiency should be ≥90 % on average combined for all developmental stages. The heat extraction technique should also be optimized to ensure the extraction efficiency within a reasonable amount of time. The following procedures recommend either a 48-h or 120-h extraction time.

D.2 Environment and Climate Change Canada Procedure

Instructions for the construction of the heat-extraction unit are provided in [24]. In brief, two polypropylene cups (133 mL) are fitted together, with a fine mesh (lined with cheese cloth) separating the upper vessel from the lower collection vessel (see Figure D.1 and Figure D.2).

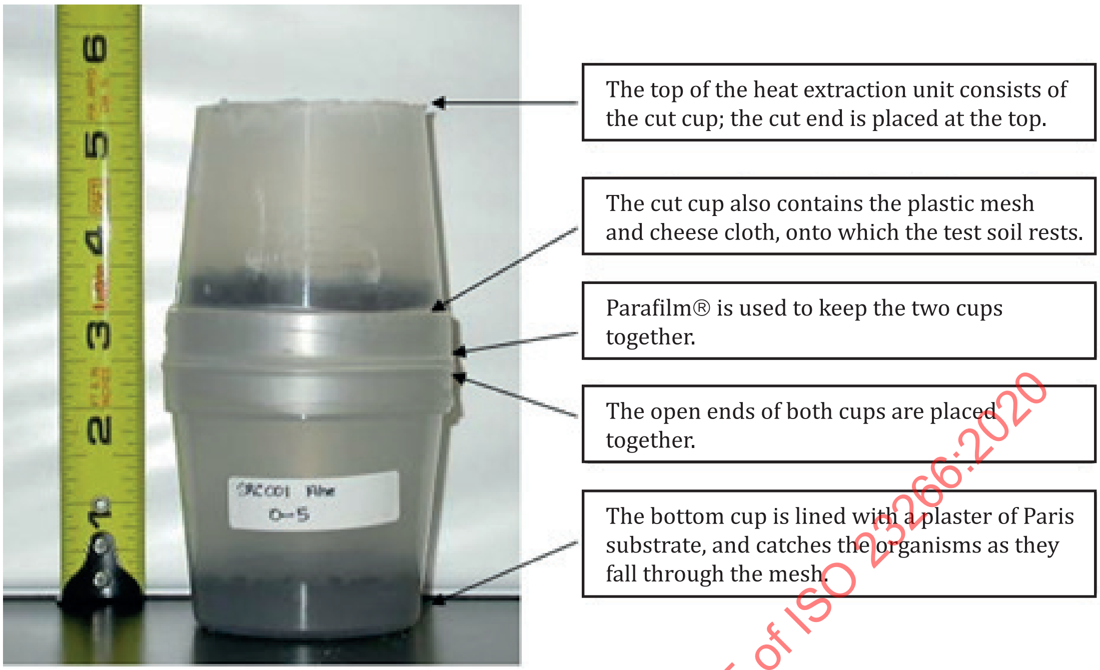


Figure D.1 — Assembly of the heat extraction unit

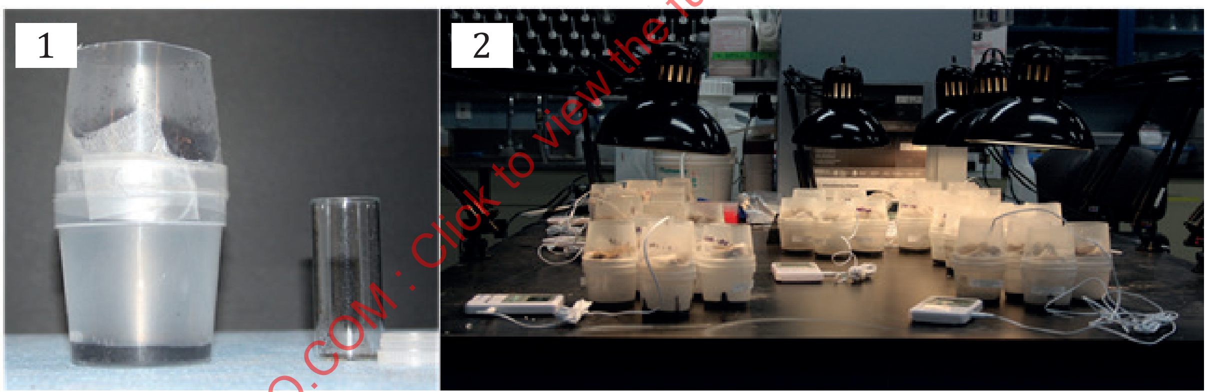


Figure D.2 — (1) Soil is transferred from the test vessel to the heat extraction unit. (2) The heat extraction units are placed underneath the lamps with 60-watt bulbs for 48 h

The heat gradient is generated from a lamp fitted with a 60-watt lightbulb and is regulated by the distance of the lightbulb from the surface of the soil in the heat extraction unit. One heat extraction unit is prepared for each test vessel. At the end of the test (Day 28), the soil from each test vessel is transferred into a heat extraction unit (see Figure D.2). The soil surface is gently smoothed out evenly over the plastic mesh (lined with cheese cloth), using a spoon or a scoopula. The heat extraction units are placed underneath the lamps, limiting the number of units per lamp to no more than five or six, so that the heat and light are kept consistent for each unit. The bottom of the lightbulb is adjusted to ~25 cm above the top of the soil and an electronic thermometer is set up within one of the units to monitor temperature changes throughout the extraction. The temperature should be checked every few hours along with the hydration of the soil (with lamps being raised during periods when technicians are not present to monitor the units (e.g., over-night, to prevent the soil from drying completely). The lamp height does not need any adjustment, and the temperature should reach ~32 °C after 48 h. At the end of the extraction period (i.e., 48 h), the lamps should be turned off and the Parafilm®[1]) connecting the two halves of the heat extraction unit removed. The organisms that have dropped down through the

1) Parafilm® is a trademark of Bemis Company, Inc. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named.

mesh to the plaster of Paris substrate can be counted immediately, either manually or through image analysis, or they can be preserved for enumeration at a later date.

The heat extraction method described above was recommended for use in the international ring test (see Annex E). The heat extraction efficiency for tests conducted by Environment and Climate Change Canada, ranged from 0,0 (±0,0) to 1,5 % (±3,4) adults remaining in the heat-extracted soil, and from 0,0 (±0,0) to 8,0 (±3,9) % juveniles remaining in the heat-extracted soil, for negative control soils (e.g., artificial soil, a field (VSL) soil, and LUFA standard soil). For other soil types, the heat extraction efficiency should be tested prior to or as part of, the test.

D.3 Alternative Heat Extraction Techniques

Alternative heat extraction techniques are available[35][36] and the following two approaches proved successful throughout the inter-laboratory ring testing program.

D.3.1 First Alternative Technique

This method is simply a variation of that provided in Section D.1 in that a 25-watt lightbulb is recommended, rather than a 60-watt lightbulb. The height of the 25-watt lightbulb is started at approximately 30 cm above the surface of the soil (instead of 25 cm). On Day 0 of the heat extraction, the temperature should reach 26 °C. On Day 1, the distance of the lamp to the soil is minimized to increase the temperature up to 28 °C. To achieve 32 °C on the last day (Day 2), the height of the lamp is adjusted to approximately 20 cm above the surface of the soil.

D.3.2 Second Alternative Technique

This procedure uses a MacFayden-type extractor, generating a high light and heat gradient to slowly force the organisms out of the test soil through a mesh screen, dropping onto a collection vessel below.

The equipment and reagents to be used include:

— MacFayden Extractor (including plastic crucibles with metal mesh at the bottom);

— Thin gauze cut-outs (for covering the metal mesh);

— Plastic petri dishes (for covering the vessels);

— Labels;

— Plastic spray bottle with water;

— Plastic spray bottle with Ethanol 70 %;

— Collecting vessels;

— Ethanol (70 % v/v.).

The soil samples are placed into (labelled) plastic crucibles containing a mesh and thin gauze cut-outs over a glass funnel. A collecting vessel is fixed below the funnel, containing ethanol (70 % v/v.) for the preservation of the animals. The soil samples are covered with petri dishes to prevent drying of the soil.

The extraction is carried out with stepwise daily temperature increases from room temperature to 32 °C. Throughout the extraction process, the soil is moistened two to three times per day according to demand with a plastic spray bottle. One day before the heat extraction is complete, the soil is no longer remoistened. The temperature regime is provided in Table D.1.

Table D.1 — Temperature region used for the MacFayden-type heat extraction technique

Time (hours)	Temperature (°C)
0 (Start)	22 (Start)
8	24
24	26
48	28
72	30
96	32
120 (Finish)	32 (Finish)

The duration of extraction and temperature gradient are tentative and should optimize the heat extraction efficiency. The temperature conditions are recorded using a data logger. After the heat extraction is complete, the plastic crucibles are removed and the funnels are rinsed two times with ethanol 70 % to ensure that all of the organisms are captured in the collection vessel (i.e., not remaining in the funnel). Furthermore, the rim of collection vessel is rinsed, to transfer all organisms stuck on the rim into the ethanol collection vessel. Otherwise, they will dessicate and are difficult to detect while counting.

Annex E
(informative)

Performance of the method

E.1 General

Test method validation for the Canadian test method[24] involved participation from 9 laboratories from five countries (Canada, Germany, The Netherlands, Portugal and Spain), of which results have been reported in [15]. A summary of performance of the method based on the results of 3 rounds of tests are provided within this Annex, including the overall test validity criteria (Section E.2). The ring test involved a period for establishment of cultures, an initial round of performance tests for participating laboratories to become familiar with the test species and methodology, followed by two rounds of chemical testing in control and field soils. The ring test was initiated in October 2015 and concluded in October 2018.

E.2 Validity criteria

Validity criteria for this standard is based on testing of 13 soil types and horizons (agricultural and forest soils) generated by Environment and Climate Change Canada (24) and the results of two of the three rounds of international inter-laboratory testing[15] (see E.3).

Criterion	Limit value
Mean percent mortality of adults in the controls at test end	≤30 %
Mean number of live juveniles in controls at test end	≥30
Coefficient of variation for reproduction in controls	≤40

E.3 International inter-laboratory testing (ring test)

The ring test involved laboratories from Canada, Germany, The Netherlands, Spain, and Portugal. Participants had varied levels of experience working with oribatid mites, although all laboratories were familiar with soil ecotoxicity testing based on existing OECD, ISO, and Canadian standard test methods.

Three phases were developed for the ring test: (i) performance testing in artificial (AS), agricultural (VSL) and forest soil (FS) (mainly for laboratories to become familiar working with the test species) (Round 1); (ii) a chemical test using boric acid in a field (VSL) soil (Round 2); and (iii) a chemical test using boric acid in the standard LUFA 2.2 test soil (Round 3). Details on the testing and results of the international ring test are available[15].

E.3.1 Results of control performance

All laboratories met the proposed control performance criteria of ≥70 % for adult survival (Figure E.1) (or <30 % adult mortality), with the exception of one laboratory from Round 1 in artificial soil (mean adult survival of 53 ± 19 %). Round 1 was intended for the laboratories to establish familiarity with working with the test species. Test performance for adult survival was consistent or increased with each Round of testing. In all cases, with the exception of two cases in Round 1, all laboratories demonstrated a coefficient of variation for adult survival <30 % across all Rounds of testing. For juvenile production, test performance also improved from Round 1 to the subsequent Rounds. Within Round 1, for one field soil (VSL), two laboratories could not meet the proposed validity criteria, with an observed end-

