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Soil quality — Inhibition of reproduction of the soil mite (*Hypoaspis aculeifer*) by soil contaminants

*Qualité du sol — Inhibition de la reproduction de l'acarien prédateur (*Hypoaspis aculeifer*) par des contaminants du 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).

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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 and ISO 17616). 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.

Mites (Acari) are a world-wide and diverse group of arthropods belonging to the class 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 (see Reference [13]).

Among soil-inhabiting mites, the role of predation is ensured by, for example, *Hypoaspis* sp. (Laelapidae). Because they are exposed to chemical contamination, mites are already considered in the environmental risk assessment of pesticides, as non-target organisms (see Reference [10]). Indeed, among the data required for active substances of pesticides, effects on predatory mites are assessed, i.e. for the plant-inhabitant *Typhlodromus pyri* (Phytoseiidae) and the soil-inhabitant *Hypoaspis aculeifer* (Laelapidae) (see Reference [6]).

The first authors introducing *H. aculeifer* as a test organism in ecotoxicological studies[23][17] were later proposed a two-species test system in the European project SECOFASE (Sublethal Effects of Chemicals on Fauna in the Soil Ecosystem), including the collembolan *Folsomia fimetaria* as prey. In the context of the development of an ecotoxicological test for the assessment of plant protection products on non-target arthropods (see References [5][6]), a protocol on soil predatory mites using *H. aculeifer* was further proposed. More recently, a standard test protocol for the assessment of chemicals was developed for this species by OECD in 2008 and revised in 2016. The results of the associated international ring-test were published in Reference [25].

Among mites, the predator *Hypoaspis aculeifer* is the most studied species in laboratory. The reproduction end point was found in general to be more sensitive than mortality and avoidance. Compared to other soil meso-fauna invertebrates, mites were found in general less sensitive than or as sensitive as other test species, depending on the end points and chemicals studied. Considering semi-field studies, *H. aculeifer* was used as a top predator whereas other soil invertebrates, mainly springtails, were ranked in the grazer group. In these studies, mites showed to be quite tolerant towards anthropogenic contamination. This statement was also corroborated by field surveys. However, the applicability of laboratory test methods for the assessment of environmental samples (contaminated soils, wastes etc.) with mites is emphasized, as to date a limited number of studies are available.

This document describes a method that is based on the determination of lethal and sublethal effects of contaminated soils to adult predatory mites of the species *Hypoaspis aculeifer*. This species is considered to be representative of predatory soil arthropods. Background information on the ecology of these mites and their use in ecotoxicological testing is available in Reference [14].

Soil quality — Inhibition of reproduction of the soil mite (*Hypoaspis aculeifer*) by soil contaminants

1 Scope

This document specifies a chronic test method for evaluating the habitat function of soils and determining effects of soil contaminants and substances on the reproduction of *Hypoaspis aculeifer* by – mainly – alimentary uptake. This method 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 under concern and waste materials (e.g. dredged material, municipal sludge from a wastewater treatment plant, composed material, or manure, especially those for possible land disposal). The reproduction (= number of juveniles) is the measured parameter of the test. The test reflects the bioavailability of a mixture of contaminants in natural soils (contaminated site soils) to a species which represents a trophic level which is not covered by other ISO standards. This test is not intended to replace the earthworm (see ISO 11268-2) or Collembola (see ISO 11267) reproduction tests since this species belongs not only to a different trophic group but also a different taxonomic group (= mites; i.e. arachnids) than those used usually.

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. Depending on the objective of the study, the control and dilution substrate (dilution series of contaminated soil) are either an uncontaminated soil comparable to the soil to be tested (reference soil) or a standard soil (e.g. artificial soil).

This document provides information on how to use this method for testing samples (soils or substances) under temperate conditions.

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

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

2 Normative references

The following documents 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 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 for *x* % effect
EC_x
concentration (mass fraction) of a test sample that causes *x* % of an effect on a given end point within a given exposure period when compared with a control

EXAMPLE An EC_{50} 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 the 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
limit test
single concentration test consisting of at least four replicates each, the soil to be tested without any dilution or the highest concentration of test substance mixed into the control soil and the control

3.5
lowest observed effect concentration
LOEC
lowest test substance concentration that has a statistically significant effect (probability $p < 0,05$)

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

3.6
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

3.7
no observed effect concentration
NOEC
highest test substance concentration immediately below the *LOEC* (3.5) at which no effect is observed

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

3.8
no observed effect rate
NOER
lowest rate of a soil to be tested immediately below the *LOER* (3.6) which when compared to the control has no statistically significant effect (probability $p < 0,05$) within a given exposure period

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

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

EXAMPLE Euro soils, artificial soil, LUFA standard soil type 2.2.

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

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

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

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

3.13
test mixture ratio
ratio between the soil to be tested and the control soil in a test mixture

4 Principle

Adult females are exposed to the soil to be tested and the effects on reproduction measured are compared to those observed for females exposed to a control soil. If appropriate, effects based on exposure to a dilution range 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 test is started with 10 adult females per test vessel. Males are not introduced in the test, because experience has shown that females mate immediately or shortly after hatching from the deutonymph stage, if males are present. As the females are introduced into the test about 7 d after they have reached the adult stage, the females can be considered as already mated (Annex A and Annex E). The test runs until the first offspring have reached the deutonymph stage. At 20 °C the exposure time ends at day 14 after introducing the females (day 0), followed by two days of extraction. The number of surviving females and the number of juveniles per test vessel are determined. The reproductive output of the mites exposed to the test mixtures is compared to that of the controls in order to determine the concentrations which cause no effects on mortality and reproduction (NOER/NOEC) and the concentration resulting in x % reduction of juveniles hatched from eggs compared to the control (ER_x/EC_x) respectively, depending on the experimental design (see 7.1.3).

In case 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 on reproduction is carried out, to give an indication of the effect dilution/concentration, and the dilution/concentration giving no mortality (NOER/NOEC). Dilutions/concentrations to be used in the definitive test can then be selected;

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

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, *Hypoaspis (Geolaelaps) aculeifer*. adult female mites (7 d to 14 d after becoming adult; 28 d to 35 d after the start of the egg laying in the synchronisation) are required to start the test. The mites shall be selected from a synchronised cohort (see Annex E).

5.2 Test mixtures

5.2.1 Field-collected soil or waste. 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 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 or silt, clay) in accordance with ISO 11277;

c) water content in accordance with ISO 11465;

d) water-holding capacity according to Annex B;

e) cationic exchange capacity in accordance with ISO 11260;

f) organic carbon in accordance with ISO 10694;

g) percentage of material removed by the 4 mm sieve.

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

5.2.2 Control soil, either a) reference soil or b) standard soil that allows the presence of predatory 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 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 standard soil type 2.2.) 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	5 %
— 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)	74 %

Approximately 0,3 % to 1,0 % calcium carbonate ($CaCO_3$, pulverised, analytical grade) are necessary to get a pH of 6,0 ± 0,5.

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

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

Prepare the artificial soil at least three days prior to start the test, by mixing the dry constituents listed above thoroughly in a large-scale laboratory mixer. A portion of the deionized water required is added while mixing is continued. 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 (see Annex C). Store the mixed artificial soil at room temperature for at least two days to equilibrate acidity. To determine pH and the maximum water holding capacity, the dry artificial soil is pre-moistened one or two days before starting the test by adding deionised water to obtain 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 in accordance with Annex B, the pH shall be determined according to ISO 10390.

5.3 Reference substance

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

The NOEC and/or 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 needs 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 once or twice 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). Dimethoate as well as boric acid are suitable reference substances that have shown to affect reproduction[25].

The EC_{50} for dimethoate based on the number of juveniles should fall in the range between 3,0 mg a.s. (active substance)/kg soil (dry mass) and 7,0 mg a.s. (active substance)/kg soil (dry mass). Based on the results obtained with boric acid so far, the EC_{50} based on the number of juveniles should fall in the range between 100 mg/kg (dry mass) soil and 300 mg/kg (dry mass) soil.

5.3.2 Dimethoate (CAS 60-51-5), $C_5H_{12}NO_3PS_2$, to be tested as a formulation [e.g. Perfekthion[1)] (ca. 40 % dimethoate)].

5.3.3 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.

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

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

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

6.4 Suitable accurate balances.

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

6.6 Exhauster, for transfer of mites (see ISO 11267:2014, A.2).

6.7 Test environment.

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

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

6.8 Extraction apparatus, Tullgren funnel or comparable methods like e.g. McFadyen (see Annex D).

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).

1) Perfekthion 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 this product.

7.1.2 Range-finding test (preliminary test)

A preliminary test to find the range of mixture ratio affecting predatory mites is optional, 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 (see 5.2.2) and a control using 10 mites per container]. The preliminary test is conducted without replication. The duration of the range finding test is 14 d (exposure time), followed by an extraction time of two days. After a total 16 d, mortality of the adult mites and the number of juveniles is determined. Based on the results of the range finding test, the ER_{50}/EC_{50} is roughly determined by calculating the geometric mean of those two concentrations showing 0 % and 100 % mortality. The concentration/dilution range in the final test should preferably be chosen so that it includes concentrations at which juvenile numbers are affected while survival of the maternal generation is not. This, however, may not be possible for substances that cause lethal and sub-lethal effects at similar concentrations.

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[27]. The duration of the definitive test is 14 d (exposure time), followed by an extraction time of two days. After a total 16 d, mortality of the adult mites and the number of juveniles is determined.

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 NOER/NOEC approach, at least five concentrations or test mixtures in a geometric series should be used. Four replicates for each treatment plus eight controls are recommended.

— For the ER_x/EC_x approach, 12 concentrations or test mixtures should be used. Two replicates for each concentration plus six controls are recommended. The spacing factor can be variable; smaller at low concentrations, larger at high concentrations.

— For the mixed approach, 6 to 8 concentrations or test mixtures in a geometric series should be used. Four 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.

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 20 g dry mass of the test sample. To ensure easy migration of mites the substrate in the test container should not be compressed.

7.1.4 Limit test

If no effects are observed at the highest concentration in the range-finding test (i.e. 1 000 mg/kg or 100 %), the reproduction test can be performed as a limit test, using a test concentration of 1 000 mg/kg or undiluted soil. A limit test will provide the opportunity to demonstrate that the NOEC/NOER or the EC_{10}/ER_{10} for reproduction is greater than the limit concentration while minimising the number of mites used in the test. Eight replicates should be used for both the treated soil and the control.

7.2 Preparation of test mixtures

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 20 g (dry mass) 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 determined in accordance with Annex B. In some cases, e.g. when testing waste materials, higher percentages are required. A rough check of the soil moisture content can be obtained by gently squeezing the soil in the hand, if the moisture content is correct small drops of water should appear between the fingers.

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

Prepare the appropriate number of replicates per concentration/test mixture and the control(s) according to the selected approach (see 7.1.3).

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

7.2.2 Testing substances added to the test substrate

Standard soil (5.2.2) is used to prepare the test sample. For each test container (6.1), the mass of the substrate used shall be 20 g (dry mass). Substances are added to the test substrate and mixed thoroughly.

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

a) Water-soluble substance

— Immediately before starting the test, dissolve the quantity of the test substance in the water or a portion of it required to wet the soil samples for the replicates of one concentration in order to reach a final water content of 40 % to 60 % of the maximum water holding capacity, and mix it thoroughly with the soil before introducing it into the test containers.

b) Substances insoluble in water but soluble in organic solvents

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

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

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

c) Substances insoluble in water or organic solvents

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

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

Substances mixed into the substrate do not need to be tested at concentrations higher than 1 000 mg/kg mass of test sample.

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

Determine the pH for each test mixture (one container per concentration) in accordance with ISO 10390 at the beginning and end of the test.

7.2.3 Preparation of control containers

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

Perform one control container for the range-finding test and at least six 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 at least six additional control containers prepared with solvent but without the test substance. Cover the containers as indicated in 6.1.

7.3 Addition of the biological material

Ten adult females in 20 g dry mass of artificial soil are recommended for each control and treatment vessel. Test organisms should be added within 2 h after preparation of the final test substrate (i.e. after application of the test item). In specific cases (e.g. when ageing is considered to be a determining factor), the time between preparation of the final test substrate and the addition of the mites can be prolonged (for details of such ageing, see Reference [11]). However, in such cases a scientific justification shall be provided.

Mites 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 ISO 11267:2014, A.2. Before they are transferred to the test containers, organisms are counted and checked for damage both to reduce control mortality and to avoid systematic trial errors.

7.4 Test conditions and measurements

The test temperature should be (20 ± 2) °C. Temperature should be recorded at least daily and adjusted, if necessary. The test is carried out under 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. For reasons of comparability, these conditions are the same as in other soil ecotoxicological tests[19].

Gaseous exchange should be ensured by aerating the test vessels at least twice a week in case screw lids are used. If gauze covers are used, special attention should be paid to the maintenance of the soil moisture content.

The water content of the soil substrate in the test vessels is 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 Feeding of the mites

Cheese mites [*Tyrophagus putrescentiae* (Schrank, 1781)] have been shown to be a suitable food source. Small collembolans ([e.g. juvenile *Folsomia candida* (Willem, 1902) or *Onychiurus fimatus*[7] [22], enchytraeids (e.g. *Enchytraeus crypticus* Westheide and Graefe, 1992) or nematodes (e.g. *Turbatrix*

silusiae de Man, 1913)] may be also suitable. It is recommended to check the food before using it in a test. The type and amount of food should secure an adequate number of juveniles in order to fulfil the validity criteria (see Clause 9). For the prey selection, the mode of action of the test item should be considered (e.g. an acaricide may be toxic to the food mites too, see below).

Food should be provided ad libitum [i.e. each time a small amount (tip of a spatula)]. For this purpose, low suction exhauster as proposed in ISO 11267:2014, A.2 or a fine paint brush can also be used. Supplying food at the beginning of the test and two to three times a week will usually be sufficient. When the test item appears to be toxic to the prey, an increased feeding rate and/or an alternative food source should be considered.

7.6 Determination of surviving predatory mites

On day 14 the surviving mites are extracted from the soil via heat/light extraction (see Annex D). The numbers of juveniles (i.e. larvae, protonymphs and deutonymphs) and adults are counted separately at the end of the extraction (day 16 after starting the test). 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. Extraction efficiency shall be validated once or twice a year in controls with known numbers of adults and juveniles. Efficiency should be above 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.

8 Calculation and expression of results

8.1 Calculation

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

8.2 Expression of results

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

If dilution or concentration series were performed indicate:

— in the EC_x/ER_x – approach, the % soil to be tested based on dry mass or in milligrams per kilogram of dried soil substrate, the median percent dilution of contaminated soil or median concentration of the test substance, which reduces the number of juvenile mites to 50 % (EC_{50}) compared to the control within the test period; or

— in the NOEC/NOER – approach, the soil mixture ratio immediately below the LOEC/LOER or highest tested concentration/rate of a test substance which when compared to the control has no statistically significant lethal or other effect such as reproduction ($p < 0,05$).

9 Validity of the test

The results are considered to be valid, if:

— the mortality of the adults in the control(s) doesn't exceed 20 % at the end of the test;

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

— the coefficient of variation of reproduction in the control doesn't exceed 30 %.

10 Statistical analysis

10.1 General

Most of test methods with sub-lethal end points, e.g. reproduction, involve quantitative effects, e.g. counting juvenile mites. Quantal effects can also be measured in the same test, such as mortality after two weeks exposure.

Guidance given here for statistical evaluation of test results is intended to inform investigator about 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 reproduction) have different statistical methods. For sampling at several locations with field replication, analysis of variance (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[9].

An example of a single-concentration test for quantitative effects can be counting juvenile mites as end point of effects on reproduction after exposure to a sample of undiluted contaminated soil, compared to numbers of offspring exposed to a reference or standard soil. If there was only one mixture tested, and one control sample, without any replicates, results cannot be 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, 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 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.

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 ER_x/EC_x-approach or the NOEC-approach.

— ER_x/EC_x (effect concentration)-approach.

The ER_x/EC_x-approach can only be used if a clear dose response relationship is found. Wherever possible, the 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 ERx/ECx-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 ERx/ECx is obtained by inserting a value corresponding to x % of the control mean into the formula found by regression analysis. Since EC_{50} values have smaller confidence limits compared with smaller effect concentrations (e.g. ER_{20}/EC_{20}), it is recommended to determine ER_{50}/EC_{50} values.

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

First, a statistical analysis of the homogeneity of the variances shall be made, e.g. by using Cochran's test. With homogeneous data, an appropriate statistical analysis, e.g. a "One-Way Analysis of Variance (ANOVA)", followed by a one-sided Dunnett test (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, e.g. the U-test by Mann & Whitney or the Bonferroni-U-test can be used.

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

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

11 Test report

The test report shall include the following information:

a) a reference to this document, i.e. ISO 21285;

b) the results, expressed as in 8.2;

c) detailed description of the test sample and information on physical and chemical properties if helpful for the interpretation of the test result; similarly, detailed information on the soils used is needed:

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

— in case of testing soil material: a table with results of chemical analyses of the tested soil (e.g. including C_{org}, heavy metal contents), if available;

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;

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, the lowest concentration causing significant effects (LOEC), the highest concentration causing no observed effects (NOEC), EC_{10} and EC_{50} for the inhibition of reproduction and the method used for calculation (optional);

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

l) water content, pH and cationic exchange capacity (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 document, as well as any factors that may have affected the results.

Annex A
(informative)

Techniques for rearing and breeding of predatory mites

A.1 Rearing of *Hypoaspis (Geolaelaps) aculeifer*

Cultures can be maintained in plastic vessels or glass jars filled with plaster of Paris/charcoal powder (9:1) mixture. The plaster can be kept moist by adding few drops of distilled or deionised water if required. Rearing temperatures are optimal between (20 ± 2) °C, light/dark regime is not relevant for this species. Prey can be *Typrophagus putrescentiae* or *Caloglyphus* sp. mites (food mites should be handled with care since they could cause allergies in humans), but nematodes, enchytraeids and collembolans are also suited as prey items. Their source should be recorded. Population development can start with a single female because males develop in unfertilised eggs. Generations are largely overlapping. A female can live at least 100 d and can deposit approximately 100 eggs during its lifetime. A maximum oviposition rate is reached between 10 d and 40 d (after becoming adults) and amounts to 2,2 eggs female^{-1} day^{-1}. Developmental time from egg to adult female is approximately 20 d at 20 °C. More than one culture should be maintained and held beforehand.

A.2 Rearing of *Typrophagus putrescentiae*

The mites are kept in a glass vessel filled with fine brewer's yeast powder which is put in a plastic bucket filled with KNO_3-solution in order to avoid escaping. The food mites are placed on top of this powder. Afterwards, they are carefully mixed with the powder (which shall be replaced twice a week) using a spatula.

A.3 Synchronisation of cultures

Specimens that are used in the test should be of similar age (approximately 7 d after reaching the adult stage). At a rearing temperature of 20 °C, this is achieved by:

— transferring females to a clean rearing vessel and add sufficient food;

— allowing for two days to three days of egg laying, removing females;

— taking adult females for testing between the 28th day and 35th day after starting the placement of female adults in clean rearing vessels.

Adult females can be easily distinguished from males and other developmental stages by their larger size, bloated shape and their brown dorsal shield (males are slimmer and flat), immatures are white to cream coloured. The development of the mites follows approximately the pattern at 20 °C: egg 5 d, larva 2 d, protonymph 5 d, deutonymph 7 d, preoviposition period of female 2 d. Afterwards, the mites are adult.

The adult test animals are removed from the synchronised culture and introduced into the test vessels between the 28th day and the 35th day after the parental females have started egg laying (i.e. 7 d to 14 d after egg laying they became adult). This ensures that the test animals have already passed their preoviposition period and have been mated by males that are also present in the culture vessel. Observations in laboratory cultures suggest, that females mate immediately or shortly after becoming adult if males are present (Ruf, Vaninnen, pers. obs.). The period of seven days is chosen to facilitate integration in laboratory routine and to buffer individual developmental variability among mites. The oviposition should be started with at least the same number of females that is eventually needed for the test. If for example 400 females are needed in the test, at least 400 females should be allowed to oviposit for two days to three days. At least 1 200 eggs should be the starting point for the synchronised

population (sex ratio approximately 0,5, mortality approximately 0,2). To avoid cannibalism, it is more convenient to keep not more than 20 to 30 ovipositing females in one vessel.

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 (w_{wh})

$$w_{wh} = \frac{m_S - m_T - m_D}{m_D} \times 100 \qquad \text{(B.1)}$$

where

w_{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.