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Soil quality — Effects of contaminants on *Enchytraeidae* (*Enchytraeus* sp.) — Determination of effects on reproduction

Qualité du sol — Effets des contaminants sur les Enchytraeidae *(*Enchytraeus *sp.) — Détermination des effets sur la survie et la reproduction*

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Contents

Page

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

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

This second edition cancels and replaces the first edition (ISO 16387:2004), which has been technically revised.

Introduction

Ecotoxicological test systems are applied to obtain information about the effects of contaminants in soil and are proposed to complement conventional chemical analysis. ISO 15799 includes a list and short characterization of recommended and standardized test systems. 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. For the latter, a standardized test system using Enchytraeidae (a chronic test with end-point reproduction) is proposed.

This International Standard describes a method that is based on the determination of acute and sublethal effects of contaminated soils to adult Enchytraeidae of the genus *Enchytraeus*. Optionally, the method can be used for testing substances added to standard soils (e.g. artificial soil) for their sublethal hazard potential to Enchytraeidae.

Soil-dwelling annelids of the genus *Enchytraeus* are ecologically relevant, i.e. they are abundant in many soils where earthworms are scarce, but can also reach high population densities in soils well inhabited by earthworms. Enchytraeidae can be used in laboratory tests as well as in semi-field and field studies. From a practical point of view, many *Enchytraeus* species are easy to handle and breed, and their generation time is significantly shorter than that of earthworms [the test duration for a reproduction test with Enchytraeidae is four weeks to six weeks, compared to eight weeks (12 weeks including synchronization) with earthworms]. In addition, a much smaller volume of soil is needed in the enchytraeid test compared to the amount needed in earthworm tests.

This International Standard has been drawn up taking into consideration test procedures recommended by the Organization for Economic Cooperation and Development (see[22],[24]).

Soil quality — Effects of contaminants on *Enchytraeidae* (*Enchytraeus* sp.) — Determination of effects on reproduction

1 Scope

This International Standard specifies one of the methods for evaluating the habitat function of soils and determining effects of soil contaminants and substances on the reproduction of *Enchytraeus* sp. by dermal and alimentary uptake in a chronic test. It is applicable to soils and soil materials of unknown quality, e.g. from contaminated sites, amended soils, soils after remediation, agricultural or other sites under concern and waste materials.

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

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

The method is not applicable to volatile substances, i.e. substances for which *H* (Henry's constant) or the air/water partition coefficient is greater than 1, or for which the vapour pressure exceeds 0,013 3 Pa at 25 °C.

NOTE No provision is made in the test method for monitoring the persistence of the substance under test.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

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

ISO 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*

3 Terms and definitions

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

3.1
reproduction
mean number of offspring per test vessel after incubation under the specified test conditions

Note 1 to entry: The test period for the determination of the reproduction (definitive test) is six weeks.

Note 2 to entry: offspring = hatched juvenile enchytraeids

3.2
reproduction rate
mean number of offspring produced per a number of adults over the test period

Note 1 to entry: The test period for the determination of the reproduction (definitive test) is six weeks.

Note 2 to entry: offspring = hatched juvenile enchytraeids

3.3
EC*x*
effect concentration for *x* % effect
concentration (mass fraction) of a test substance that causes *x* % of an effect on a given endpoint 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.

3.4
LOEC
lowest observed effect concentration
lowest test substance concentration that has a statistically significant effect (probability $p < 0,05$)

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

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

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

3.6
test mixture
mixture of contaminated soil or test substance (e.g. chemical, biosolid, waste) with control soil

3.7
test mixture ratio
ratio between the soil to be tested and the control soil in a test mixture

3.8
contaminant
substance or agent present in the soil as a result of human activity

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

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

EXAMPLE Euro soils, artificial soil, LUFA Standard soil

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

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

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

4 Principle

The effects on survival and reproduction of adult Enchytraeidae (*Enchytraeus* sp.) exposed to a dilution range of contaminated soil or range of concentrations of a test substance are determined. Test mixtures are prepared at the start of the test and are not renewed within the test period.

The test can be divided into two distinct steps: a short (two weeks) test in which the range of toxic effects (mainly mortality) is determined, and a long-term (six weeks) definitive test in which the survival of parental worms and the fecundity (number of juveniles) are measured. The results obtained from the tests are compared with a control and are used to determine the dilutions or concentrations which cause no effects on survival and reproduction (NOEC) and the concentration (dilution) resulting in x % reduction of juveniles hatched from cocoons compared to the control (ECx, 42 d).

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

— an acute toxicity test (range-finding test) is carried out, to give an indication of the effect dilution/concentration, and the dilution/concentration giving no mortality (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 offsprings hatched from cocoons compared with the control (NOEC), and the lowest concentration causing effects (LOEC).

NOTE The use of a suitable 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, recommended test species is *Enchytraeus albidus* Henle 1837 (white potworm; Enchytraeidae, Oligochaeta, Annelida). *E. albidus* is one of the largest enchytraeid species, measuring 15 mm to 40 mm, and has a world-wide distribution (see e.g.[21],[31]). It can be easily recognized by two characteristics: four setae per bundle ventrally, and the very long seminal duct in the clitellum region as well as some segments behind it. The species can be found in marine, limnic and terrestrial habitats, mainly in decaying organic matter (seaweed, compost) and only rarely in meadows. This broad ecological tolerance and some morphological variations indicate that the species probably consists of several races (or ecotypes). *E. albidus* can be obtained commercially, since it is sold as food for fish. It should be verified whether such a culture is contaminated by other, usually smaller species (see e.g.[7],[10],[32]). If contamination of the culture occurs, all worms are washed in water in a Petri dish. With the help of a stereomicroscope, large adult specimens of *E. albidus* are selected to start a new culture. All other worms

of the original culture are discarded. *E. albidus* can be bred easily in a wide range of organic materials (see Annex A) and has a short life cycle, reaching maturity between 33 d (at 18 °C) and 74 d (at 12 °C). Only cultures which have been kept in the laboratory for at least five weeks (one generation cycle) without problems shall be used for testing purposes.

Other species of the genus *Enchytraeus*, especially the true soil-inhabiting but smaller species *E. crypticus* Westheide and Graefe 1992 or *E. buchholzi* Vejdovsky 1879, are also suitable as test organisms (see Annex B). If other species of *Enchytraeus* are used, they shall be clearly identified and the rationale for the selection of the species as well as deviations of the experimental method should be reported in this case. The worms used in the tests should be adult with eggs (white spots) in the clitellum region and should have approximately the same size (approximately 15 mm). A synchronisation of the breeding culture is not necessary. The Enchytraeidae should be acclimatised in untreated artificial soil under test conditions for at least 24 h prior to testing. During this period, the same food which is used as a food source in the test should be given in sufficient amount.

For one test, an excess number of adult clitellate worms should be taken from the culture box without observing them in detail in order to get enough suitable worms. At the end of the acclimatization period, only worms with eggs and behaving as usual (e.g. not trying to leave the artificial soil) are selected for the test. This selection is made by placing the worms in a Petri dish filled with a small amount of water under a stereomicroscope, and discarding the animals without eggs. A freshwater medium (e.g. reconstituted water as described in[23]) should preferably be used, since demineralized water or tap water (risk of copper contamination) can harm the Enchytraeidae. During this process, other organisms living in the cultures, such as mites, are also removed from the worms.

NOTE An example of culturing *Enchytraeus* sp. is given in Annex A.

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

5.2.1 Field-collected soil or waste

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

The fields 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 soils to be tested should be as short as possible. The soil shall be stored in accordance with ISO 10381-6 using containers that minimize losses of soil contaminants by volatilisation and sorption to the container walls. Soil pH should not be corrected as it can influence bioavailability of soil contaminants.

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

a) pH in accordance with ISO 10390;

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

c) water content in accordance with ISO 11465;

d) water holding capacity according to Annex C;

e) cation exchange capacity in accordance with ISO 11260;

f) organic carbon in accordance with ISO 10694.

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

5.2.2 Control soil, either a) reference (3.9) or b) standard soil (3.10) that allows the presence of Enchytraeidae (at least the validity criteria shall be fulfilled). Control soil and soil used for dilution shall not differ in one test (either a) or b)).

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

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

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

	Percentage expressed on dry mass basis
– Sphagnum peat finely ground and with no visible plant remains	10 %
– Kaolinite clay containing not less than 30 % kaolinite	20 %
– Industrial quartz sand (dominant fine sand with more than 50 % of particle size 0,05 mm to 0,2 mm)	69 %

Approximately 0,3 % to 1,0 % calcium carbonate ($CaCO_3$, 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.[22]

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

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

NOTE 2 Allowance should be made for any water that is to be used for introducing the test substance into the soil.

5.3 Food

Rolled oats, preferably autoclaved (heating is also possible) before use to avoid infection with other organisms, were found to be suitable[31]. The first feeding is made by mixing 50 mg of ground rolled oats per test vessel into the soil (after application of the test substance but before adding the worms); additional feedings (25 mg per vessel per week except after 28 d) are made only on the surface to avoid harming the worms. Since the need for food can vary in the different vessels, feeding should be adjusted to demand (i.e. over-feeding shall be avoided). Some soil particles should be placed on top of the flasks in order to reduce fungal growth.

5.4 Reagents

5.4.1 Bengal red.

5.4.2 Ethanol.

5.4.3 Boric acid, suitable as reference substance.

6 Apparatus

Usual laboratory equipment and the following.

6.1 Test container, of capacity 0,20 l to 0,25 l, with a diameter (e.g. 5 cm) enabling a depth of 1,5 cm to 2 cm of soil, with lids (e.g. glass or perforated plastic film). The beakers shall be suitable as test vessels, containing an amount of artificial soil corresponding to 20 g dry mass. The lids shall permit gaseous exchange between the soil substrate and the atmosphere.

6.2 Drying cabinet.

6.3 Stereomicroscope.

6.4 Balances with a weighing range of 50 g to 32 kg; precision at least 1 g.

6.5 Analytical balance with a weighing range of 10 mg to 200 g; precision at least 1 mg.

6.6 pH-meter.

6.7 Temperature registration (e.g. temperature/humidity recorder).

6.8 Lux meter.

6.9 Mixer.

6.10 Incubator or small room with air-conditioner.

6.11 Jeweller's tweezers, hooks, loops or a small brush.

6.12 Photo basins with ribbed bottoms.

7 Test environment

Cover the test vessels (6.1) with glass lids to prevent the test substrate from drying, and keep under test conditions for two weeks (range-finding test) or six weeks (definitive test). The test temperature shall be (20 ± 2) °C; higher temperatures can affect reproduction. Carry out testing in a controlled light-dark cycle of long-day conditions, preferably 16 h to 8 h at 400 lx to 800 lx in the area of the test vessels, to prevent the worms from escaping from the soil.

Weigh the vessels at the beginning of the test and thereafter once a week. Replenish the mass loss with the appropriate amount of deionized water. This loss can be minimized by maintaining a high humidity (>80 %) in the test incubator (6.10). Place all test vessels in the test incubator in a random order, which should be changed every week.

At the beginning and the end of both the range-finding test and the definitive test, the water content and the pH should be measured. To facilitate checking of the pH and water content of the test substrate, use of additional containers (replicates) for each concentration and for the control is recommended.

