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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 reproduction*

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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

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

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 ~~for Project Committee~~ ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological characterization*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 444, *Environmental characterization*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This third edition cancels and replaces the second edition (ISO 16387:2014), which has been technically revised.

The main changes are as follows:

- correction of the vapor pressure value to update Clause 1 according to the recommendations of ECHA/OECD;
- addition (in Annex D) of an extraction method of enchytraeids with colloidal silica, suitable for *Enchytraeus crypticus*.

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. ISO 15799^[38] 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 document 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 document has been drawn up taking into consideration test procedures recommended by the Organization for Economic Co-operation and Development (OECD^{[24],[25]}).

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

1 Scope

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

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

The method is not applicable to substances, for which the air/soil partition coefficient is greater than 1, or to substances for which the vapour pressure exceeds 300 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 are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

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

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

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

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

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

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

3 Terms and definitions

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

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

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <https://www.electropedia.org/>

3.1 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 =refers to hatched juvenile enchytraeids.

3.2 EC_x

effect concentration for x % effect

concentration (mass fraction) of a test substance that causes x % of an effect on a given 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.

3.3 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.

3.4 NOEC

no observed effect concentration

highest test substance concentration immediately below the *LOEC* (3.3) 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.

3.5 test mixture

mixture of contaminated soil or test substance (e.g. chemical, biosolid, waste) with *control soil* (3.10)

3.6 test mixture ratio

ratio between the soil to be tested and the *control soil* (3.10) in a *test mixture* (3.5)

3.7 contaminant

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

3.8 reference soil

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

3.9

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.

3.10**control soil**

reference (3.8) or *standard soil* (3.9) 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 (EC_x, 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 offspring 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~~ used to demonstrate the ~~present~~appropriate status of the test population, and to avoid misinterpretation of results.

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).

5 Reagents and material

5.1 Biological material, recommended test species is *Enchytraeus albidus* Henle 1837 (white potworm; *Enchytraeidae*, subclass: *Oligochaeta*, phylum: *Annelida*). *E. albidus* is one of the largest enchytraeid species, measuring 15 mm to 40 mm, and has a world-wide distribution (see, for example,

References [22] and [32]). 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, for example, References [7], [10] and [33]). 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 for *E. albidus*). A synchronisation of the breeding culture is not necessary. The *Enchytraeidae* should be acclimatised in the reference soil, the standard soil or the 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 Reference [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 18400-206 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~~ in accordance with Annex C;
- e) cation exchange capacity in accordance with ISO 11260;
- f) organic carbon in accordance with ISO 10694.

~~NOTE—It is also important to measure the~~ The water holding capacity of all mixtures used in the test should also be measured.

5.2.2 Control soil, either a) reference (3.8) or b) standard soil (3.9) 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 soil (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 $6,0 \pm 0,5$.

NOTE Taking the properties of highly non-polar ($\log K_{ow} > 2$, where K_{ow} is the octanol/water coefficient) or ionizing substances into account, 5 % of peat have proven to be sufficient for maintaining the desired structure of the artificial soil^[25].

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~~ in accordance with ISO 10390.

5.3 Food

Rolled oats, preferably autoclaved (heating is also possible) before use to avoid infection with other organisms, were found to be suitable^[32]. 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 rolled oats 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~~The usual laboratory equipment and the following shall be used.

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 with lids (6.1) 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.