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

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

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This draft has been developed within the International Organization for Standardization (ISO), and processed under the **ISO-lead** mode of collaboration as defined in the Vienna Agreement.

This draft is hereby submitted to the ISO member bodies and to the CEN member bodies for a parallel five-month enquiry.

Should this draft be accepted, a final draft, established on the basis of comments received, will be submitted to a parallel two-month approval vote in ISO and formal vote in CEN.

Member bodies are strongly encouraged to provide their comments on the draft in time for the September 2012 ISO/TC 190/SC 4/WG 11 meeting so that the group in charge of the development of this standard could work on these comments straight away, i.e. by **2012-09-08**.

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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.

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The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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ISO 16387 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

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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 endpoint reproduction) is proposed.

ISO 16387 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 may be used for testing chemicals 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 part of ISO 16387 has been drawn up taking into consideration test procedures recommended by the Organization for Economic Cooperation and Development (see references in the Bibliography).

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

1 Scope

This part of ISO 16387 specifies one of the methods for evaluating the habitat function of soils and determining effects of soil contaminants and chemicals 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 test soil and in a control soil. According to the objective of the study, the control and dilution substrate (dilution series of contaminated soil) should be either an uncontaminated soil comparable to the soil sample to be tested (reference soil) or a standard soil (e.g. artificial soil).

Information is provided how to use this method for testing chemicals 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.

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

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 11268-1, *Soil quality — Effects of pollutants on earthworms — Part 1: Determination of acute toxicity to *Eisenia fetida*/*Eisenia andrei**

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 six weeks incubation under the specified test conditions

3.2

reproduction rate

mean numbers of offspring hatched from the cocoons and surviving until the end of the test period per adult enchytraeid remaining alive after six weeks

3.3

EC_x

effect concentration

concentration of test item or percent dilution of contaminated soil estimated to reduce the reproduction rate at the end of the test to x % compared to the control within the test period (x is the percentage (10, 20, 25, 50) of this effect)

3.4

LOEC

Lowest Observed Effect Concentration

lowest tested mixture ratio of a test soil in a reference or a standard control soil at which a statistically significant effect is observed or lowest tested concentration of a test substance at which a statistically significant effect is observed compared with the control. All test mixtures above the LOEC have a harmful effect equal or greater than those observed at the LOEC

3.5

NOEC

No Observed Effect Concentration

soil mixture ratio immediately below the LOEC or highest tested concentration of a test substance which when compared to the control has no statistically significant lethal or other effect such as weight alteration ($p < 0,05$)

3.6

test mixture

mixture of the test substance with the standard soil, mixture of contaminated soil with the control soil or mixture of contaminated soil with an uncontaminated soil comparable to the soil sample to be tested

3.7

test mixture ratio

ratio between the contaminated soil and the control soil in a test mixture

3.8

contaminant

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

[ISO 15176]

3.9

reference soil

uncontaminated site-specific soil (e.g. collected in the vicinity of a contaminated site) with similar properties (nutrient concentrations, pH, organic carbon content and texture) as the test soil

3.10

standard soil

field collected soil or artificial soil whose main properties (e.g. pH, texture, organic matter content) are within a known range, e.g. Euro soils [15], artificial soil [20], LUFA Standard soil

[11268-1]

Note 1 to entry: The properties of standard soils may differ from the test soil.

3.11

control soil

reference or standard soil used as a control and as medium for preparing dilution series with test soils or a reference substance, that allows the presence of enchytraeids (at least the validity criteria shall be fulfilled).

Note 1 to entry: All effect-concentrations are expressed in milligrams of test substance per kilogram (dry mass) of the test substrate (5.2). Soil mixtures are given in per cent based on soil dry weight.

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) preliminary 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:

- a preliminary acute toxicity 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 final test can then be selected;
- the definitive test on reproduction to determine sublethal effects of (dilutions of) contaminated soil or the concentration of a chemical 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 reference soil is an essential requirement to demonstrate the present status of the test population, and to avoid misinterpretation of results.

If the soil to be tested has properties that are quite different from the normal standard control soils (e.g. OECD artificial soil or LUFA 2.2 soil), for instance a rather low or high pH, or very low or high organic matter or clay contents, it is essential to have a reference soil that has similar properties. In that case, control performance of the enchytraeids may however, be less good than 'normal' and quality control of the test is not possible on the basis of the reference soil. For assessing toxicity of the test soil, it is important however to have such a reference soil, which may also be used to prepare dilution series of the test soil. It is therefore essential in such case to include both a reference and a control soil.

5 Reagents

5.1 Biological material

The recommended test species is *Enchytraeus albidus* Hence 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 Bibliography). It can be easily recognised 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 may consist 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 Bibliography). 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 waste 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 can 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 normally (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 OECD Guideline 202) should preferably be used, since demineralized water or tap water (risk of copper contamination) could 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 soils

5.2.1 Field soil

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

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

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

- a) pH in accordance with ISO 10390;