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

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

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ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

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Contents

Page

Foreword

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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*.

Introduction

This International Standard has been drawn up taking into consideration test procedures recommended by the International Organization for Standardization (ISO) and the Organization for Economic Cooperation and Development (OECD) (see Clause 2 and Bibliography).

The method described was developed for testing the effects of chemicals added to an artificial soil. An adaptation for testing or comparing soils to assess, for example, the effects of remediation treatments is given in Annex B. It can also be adapted for assessing sublethal effects and determining no-effect levels for pesticides.

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 4 weeks to 6 weeks, compared to 12 weeks (including synchronization) with earthworms].

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

1 Scope

This International Standard describes a method for determining the effects of substances or contaminated soils on reproduction and on survival of the worm *Enchytraeus albidus* (*Enchytraeidae*). The animals are exposed to the substances by dermal and alimentary uptake using a defined artificial soil substrate to which specified amounts of that substance are added, or by using a soil substrate of unknown quality.

This International Standard is applicable to test substances that are either insoluble or soluble in water, although the method of application differs. The method is not applicable to volatile test 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. The water solubility and the vapour pressure of the test substance should be known. Additionally, information on the persistence of the test substance in soil is desirable.

NOTE 1 Basic information on the ecology and ecotoxicology of *Enchytraeidae* in the terrestrial environment can be found in the bibliographic references.

NOTE 2 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 test substance.

NOTE 3 Recommendations for adapting the method to comparing or monitoring soil quality are given in Annex B.

2 Normative references

The following referenced documents are indispensable for the application 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 11268-2:1998, *Soil quality — Effects of pollutants on earthworms (*Eisenia fetida*) — Part 2: Determination of effects on reproduction*

OECD Guideline No. 207, 1984, *Earthworm Acute Toxicity Tests,* Organization for Economic Cooperation and Development, Paris

3 Terms and definitions

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

3.1
reproduction
increase in the mean number of juveniles per test vessel after 6 weeks

3.2
concentration lethal to 50 % of the test organisms
LC50
that concentration of the test substance which kills 50 % of the test animals within the period of the range-finding test or the definitive test

NOTE 1 The LC50 is expressed as mass of test substance per dry mass of the test substrate.

NOTE 2 It is the median lethal concentration.

3.3
lowest observed effect concentration
LOEC
lowest tested concentration at which the test substance is observed to have a statistically significant effect on reproduction (probability $p < 0,05$) when compared with the control

NOTE 1 The concentration is expressed as mass of test substance per dry mass of test substrate over a given exposure time.

NOTE 2 In addition, all test concentrations above the LOEC should have a harmful effect equal or greater than that observed at the LOEC. If these two conditions are not satisfied, a full explanation should be given for how the LOEC [and hence the NOEC (see 3.4)] has been selected.

NOTE 3 In this test, the effect on reproduction (number of juveniles) is used as test parameter.

3.4
no observed effect concentration
NOEC
test concentration immediately below the LOEC which, when compared with the control, has no statistically significant effect (probability $p > 0,05$) within a given exposure time

NOTE In this test, the effect on reproduction (number of juveniles) is used as test parameter.

3.5
effect concentration
ECx
concentration at which a specific effect is detected [where x is the percentage (10, 25, 50) of this effect, e.g. on reproduction, in relation to a control]

NOTE For example, EC50 means the concentration estimated to reduce the reproduction rate at the end of the test to 50 % compared to the control. All effect concentrations are expressed as mass of test substance per dry mass of the test substrate.

4 Principle

Adult *Enchytraeidae* worms are exposed to a range of concentrations of the test substance mixed in artificial soil. The test can be divided into two distinct steps: a short (2 weeks) range-finding test in which the range of toxic effects (mainly mortality) is determined, and a long-term (6 weeks) definitive test in which the survival of parental worms and the fecundity (number of juveniles) are measured. Therefore, the test is usually conducted as follows.

a) For test substances of unknown toxicity exposure, it is recommended to conduct a range-finding test for a period of 14 d, indicating the concentrations for total mortality and for the absence of mortality. The resulting dose-response relationship is important for the proper design of the definitive test.

b) The definitive test is designed to determine the concentration of a test substance mixed into the artificial soil that causes a defined significant or specified effect on reproduction. This test design includes the investigation of lethal effects on the parental *Enchytraeidae*. The total duration of the definitive test is 6 weeks (if another *Enchytraeus* species than *E. albidus* is used, it can be shorter). After the first 3 weeks, the adult worms are removed, the number of living worms and morphological changes (e.g. body lesions or fragmentation of the worm) are recorded. After another 3 weeks, the number of offspring hatched from the cocoons is counted. The NOEC and the ECx for reproduction are determined.

5 Materials

The test substrate and the test substance are set up with standard laboratory equipment and kept in glass vessels.

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 easily be 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 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 E) 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 5 weeks (one generation cycle) without problems can be used for testing purposes.

Other species of the genus *Enchytraeus*, e.g. the true soil-inhabiting but smaller species *E. buchholzi* Vejdovsky 1879 or *E. crypticus* Westheide and Graefe 1992, are also suitable as test organisms (see Annex F). 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.

5.2 Test substrate.

Artificial soil shall be prepared in accordance with OECD Guideline 207 and ISO 11268-2. It consists of the following components (based on dry mass):

— 10 % sphagnum peat [air-dried and finely ground (2 mm ± 1 mm)]; new batches of peat should be checked for toxicity to worms before use in tests;

— 20% kaolin clay (kaolinite content preferably above 30 %);

— approximately 69 % (depending on the amount of $CaCO_3$ needed) air-dried industrial quartz sand (predominantly fine sand, with more than 50 % mass fraction having particle size 0,05 mm to 0,2 mm).

Add approximately 0,3 % to 1,0 % calcium carbonate ($CaCO_3$, pulverised, analytical grade) to obtain a pH of 6,0 ± 0,5.

The amount of calcium carbonate required can vary, depending on properties of the individual batch (particularly of the peat), and should be determined by measuring sub-samples immediately before the test.

The artificial soil is prepared by thoroughly mixing the dry constituents listed above in a large-scale laboratory mixer approximately one week before starting the test. The mixed artificial soil shall be stored at room temperature for at least 2 d to equilibrate acidity.

To determine pH and the maximum water-holding capacity, the dry artificial soil is pre-moistened 1 d or 2 d before starting the test by adding enough deionized water to obtain approximately half of the required final water content (40 % to 60 % of the maximum water-holding capacity). The pH value is measured after mixing the soil with KCl solution [c(KCl) = 1 mol/l] in a ratio of 1 to 5 (see suggested method in Annex C). If the measured pH is not within the required range, a sufficient amount of $CaCO_3$ shall be added or a new batch of artificial soil shall be prepared. Parallel to determining the pH, the maximum water-holding capacity of the artificial soil shall be determined in accordance with Annex D.

Afterwards, the artificial soil is divided into as many batches as the number of concentrations plus controls that will be used in the test. Evaporation from the test substrate shall be avoided until the start of the test.

The final moisture content is reached by adding water together with, or in parallel to, the application of the test substance. The moisture content at the beginning and end of the test is determined by drying a small sample at 105 °C overnight and re-weighing. In any case, the substrate should be optimal for the worms (even if, due to the batch of peat used, these moisture values are not met). In case of doubt, the moisture should be checked by gently squeezing the soil by hand; only small drops of water should appear between the fingers.

5.3 Food source, of a quality shown to be capable of at least maintaining the *Enchytraeidae* population.

Rolled oats, preferably autoclaved (heating is also possible) before use to avoid infection with other organisms, were found to be suitable. 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 may 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 flakes in order to reduce fungal growth.

5.4 Bengal red, ethanol.

6 Apparatus

Usual laboratory equipment and especially the following materials are necessary.

6.1 Glass beakers, of capacity 0,20 l to 0,25 l, diameter approx. 6 cm, 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 25 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 2 weeks (range-finding test) or 6 weeks (definitive test). The test temperature shall be 20 °C ± 2 °C; higher temperatures may 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 moisture content and the pH should be measured. To facilitate checking of the pH and humidity of the test substrate, use of additional containers (replicates) for each concentration and for the control is recommended.

8 Procedure

8.1 Preparation of the test substrates

8.1.1 Water-soluble test substances

Immediately before starting the test, prepare an emulsion or dispersion of the test substance in deionized water in quantity sufficient for all replicates of one concentration. It is convenient to use the amount of water necessary to reach the final moisture content of the artificial soil as required in 5.2 (40 % to 60 % of the maximum water-holding capacity). Mix the emulsion or dispersion thoroughly with each batch of artificial soil (5.2) before introducing it into a test vessel.

8.1.2 Test 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) and mix it with a portion of the quartz sand required. Evaporate the solvent by placing the container in a fume hood for at least 1 h, add the remainder of the artificial soil (5.2) (allowing for the amount of sand used to prepare the test substance) and the water, and mix thoroughly before introducing it into the test vessels.

Ultrasonic dispersion, small amounts of organic solvents, emulsifiers or dispersants may be used to disperse substances with low water solubility. When such auxiliary substances are used, all test concentrations and an additional control should 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.

8.1.3 Test substances insoluble in water or organic solvents

A mixture of 10 g of finely ground quartz sand and the quantity of the test substance required to obtain the desired concentration is prepared. Afterwards, this mixture is mixed thoroughly with the pre-moistened artificial soil (5.2) and with the amount of deionized water necessary in order to obtain the final moisture level required before introducing it into the test vessels.

8.2 Preparation of test vessel contents

An amount of test substrate (8.1) corresponding to 20 g dry mass is placed into each test vessel (6.1).

Then the food source (5.3) is mixed in and 10 *Enchytraeidae* (5.1) are placed carefully on the test substrate surface, using a suitable device (6.11). The selection of the individual worms and their assignment to batches of 10 should be made in a randomized fashion.

8.3 Range-finding test

If it is necessary to determine the range of concentrations to be applied in the definitive test, a range-finding test is conducted at about five different concentrations of the test substance in the range of 0,1 mg/kg; 1,0 mg/kg; 10 mg/kg; 100 mg/kg and 1 000 mg/kg (dry mass of artificial soil). Test substances do not need to be tested at concentrations higher than 1 000 mg/kg dry mass of artificial soil. One test vessel (each containing 10 worms) for each concentration plus control is recommended. The test duration is 2 weeks, after which the mortality of the worms is determined. Worms are classified as dead if they do not respond to a gentle mechanical stimulus to the front end. Additionally, the presence of juveniles should be checked, using the staining method in Annex A, at the end of the test in order to obtain more information on the concentrations to be tested in the definitive test.

NOTE Due to the short test duration, only few juveniles can occur; therefore, this is primarily a qualitative evaluation.

Based on the mortality data from the range-finding test, the LC50 is roughly determined by calculating the geometrical mean. This value is used to determine the concentration range of the definitive test. For example, the NOEC or the EC10 is assumed to be lower than the LC50 by a factor of up to 10. However, it must be stressed that this is just an empirical relationship which might be different in any given case. Therefore additional information, such as the occurrence of juveniles, is helpful for the determination of the concentration range for the definitive test.

If no effects are observed, even at the highest concentration of 1 000 mg/kg, the definitive test can be designed as a limit test, comparing only eight control vessels with eight test vessels containing artificial soil with a concentration of 1 000 mg/kg.

8.4 Definitive test

The statistical design for the definitive test cannot be defined at this point since, on the one hand, the NOEC will still be required by regulatory authorities for the foreseeable future. On the other hand, statistical considerations and experiences with the ring test speak in favour of an ECx design. Additionally, practical reasons impose limits on replication and the number of concentrations that are feasible in the test. Therefore, three alternative designs are proposed until OECD recommendations concerning general rules on how to design a test are available.

For the definitive test, one of the following three designs is recommended (the concentrations shall be spaced by a factor not exceeding 2).

— For the NOEC approach, at least five concentrations in a geometric series should be used. Four replicates for each concentration plus eight controls are recommended.

— For the ECx approach, 12 concentrations should be used. Two replicates for each concentration plus six controls are recommended. The spacing factor may vary; being smaller at low concentrations, larger at high concentrations.

— For the mixed approach, six to eight concentrations in a geometric series should be used. Four replicates for each concentration plus eight controls are recommended. This mixed approach allows an NOEC as well as an ECx evaluation. It was originally proposed by the task force of the ring test (see Bibliography).

If mortality is the main endpoint of the test, the same options are possible but the concentrations (based on the results of the range-finding-test) shall be adjusted accordingly.