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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*

Reference number
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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).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

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.

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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 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]).

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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.

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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

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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

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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)).

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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.

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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.

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8 Procedure

8.1 Experimental design

8.1.1 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) are prepared by mixing measured quantities with a 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). Each treatment is replicated at least four times.

8.1.2 Range-finding test

A test to find the range of mixture ratio affecting Enchytraeidae is optional. If it is necessary to determine the range of concentrations (dilutions) for use in the definitive test, perform a range-finding (acute) test in a large range of concentrations (dilutions) of the contaminated soil, e. g. 0 % – 1 % – 5 % – 25 % – 50 % – 75 % – 100 %, 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 standard soil (5.2.2) and a control using 10 worms per container.

The range-finding test is conducted without replication.

8.1.3 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 a comparison of the biological effects for the soil(s) to be tested with the effects 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.[14]

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 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 EC*x* approach, 12 concentrations should be used. Two replicates for each concentration plus at least six controls are recommended. The spacing factor can be variable; 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 at least eight controls are recommended. This mixed approach allows a NOEC as well as an EC*x* evaluation.

A limit test can be sufficient if in the range-finding test no toxic effect was observed. In the limit test only the soil to be tested without any dilution and the control (or the soil to be tested vs. the control soil) shall be tested with at least four replicates each.

If the soil to be tested has properties that are quite different from the usual 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 can, however, be less good than ‘usual’ and quality control of the test is not possible on the basis of the reference soil. For assessing toxicity of the soil to be tested, it is important however to have such a reference soil, which can also be used to prepare dilution series of the soil to be tested. For test quality assessment it is essential in such case to include both a reference and a standard control soil.

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8.2 Preparation of test mixtures

8.2.1 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 equal to 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 according to Annex C. In some cases e.g. when testing waste materials, higher percentages are required. This can be easily tested by compressing the substrate by the fist and looking for water coming through the fingers.

Determine the pH for each test mixture (one container per concentration) according to ISO 10390 at the beginning and end of the test (do not adjust the pH).

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

8.2.2 Testing substances added to the test substrate

For each test container (6.1), the quantity of substrate used shall be equivalent to 20 g (dry mass). Standard soil (5.2.2) is used as test substrate. 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 required for the replicates of each concentration (or that portion of it necessary to wet the soil) in order to meet the requirements of 5.2.2, and mix it thoroughly with the soil before introducing it into a test container.

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) and 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 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.

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. Place the mixture, the remainder of the soil (5.2.2) and the water into the test container (6.1) and mix thoroughly.

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 substrate.

Proceed simultaneously with at least four replicates per concentration and the control(s).

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Determine the pH for each test mixture (one container per concentration) according to ISO 10390 at the beginning and end of the test (do not adjust the pH).

8.2.3 Preparation of control container

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 according to Annex C).

Use one control container for the range-finding test and four to eight control containers, depending on the test design (see 8.1.3).

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 8.2.2), use an additional control prepared with solvent but without the test substance. Cover the containers as indicated in 6.1.

8.3 Addition of the biological material

Into each test container and the control container 10 Enchytraeidae (5.1) are placed carefully on the test mixture 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.

Cover the container as indicated in 6.1 and place it in the incubator or small room with air-conditioner (6.10).

8.4 Test conditions and measurements

The duration of the first part of the test is 21 d (assessment of mortality). The adult worms are fed once a week with 50 mg at the beginning of the test and afterwards with 25 mg dry mass rolled oats per vessel. If food consumption is low, reduce feeding to a minimum to avoid fungal growth or moulding. After 21 d, the soil to be tested is carefully searched manually (e.g. using a jeweller's tweezers, a hook or loop, or a small brush with a hook) for the adult worms, which are then removed and counted. Morphological and behavioural changes of the adult worms are recorded. If mortality is the main end point of the test, the whole procedure is stopped at this point.

The same soil to be tested to which the adult worms were exposed, including cocoons deposited during the first three weeks of the test, is incubated under the same test conditions for another three weeks. The juvenile worms hatched in the second half of the definitive test are fed with 25 mg dry mass rolled oats per vessel per week (except after four weeks). Again, over-feeding shall be avoided (see 5.3). After total test duration of six weeks, the juveniles hatched in the meantime are counted by staining with Bengal red (5.4). Wet (but not heat) extraction techniques have also proved to be suitable (see Annex D). The first method is recommended, since wet extraction is difficult to use with artificial soil because the clay particles make the water turbid.

NOTE Annex D gives examples of two suitable methods, including one which allows counting of cocoons.

8.5 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 organism does not change statistically over time. It is advisable to test a reference substance at least twice a year or, when testing is carried out in lower frequency, in parallel to the determination of the toxicity of a test substance. Boric acid is recommended as a reference substance. If the compound is mixed into the substrate, observe the effects on reproduction ($\alpha = 0{,}05$) at concentrations of between 400 mg and 600 mg boric acid per kilogram dry mass of substrate.

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9 Calculation and expression of results

9.1 Calculation

For each dilution or concentration, determine the percent mortality, of the adults and number of offspring produced in the definitive test.

Compare means by suitable statistical methods, e.g. Williams, Dunnetts or Student-*t*-test and test for significance (α = 0,05) of differences from control(s).

9.2 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 test mixture ratio 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 series were performed indicate:

— in % 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 lethal concentration of the test substance, which reduces the number of juvenile worms to 50 % compared to the control within the test period and

— the 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 reproduction (p < 0,05).

10 Validity of the test

The results are considered to be valid if the following conditions are met in the control:

a) The mortality of the adult worms should not exceed 20 % on average at the end of the range-finding test and after the first three weeks of the definitive test.

b) In the definitive test, the average number of juveniles should be higher than 25 per test vessel at the end of the definitive test, assuming that 10 adult worms (with eggs in the clitellum region) per test vessel were introduced at the beginning of the test.

c) The coefficient of variation calculated for the reproduction data should be not higher than 50 % at the end of the definitive test.

11 Statistical analysis

11.1 General

Analyses of the results differ according to the purposes and particular designs of the test. Standard statistical procedures (e.g. Analysis of variance and multiple comparisons) generally are sufficient for analysing the results.

11.2 Range-finding test

If a clear dose-response is obvious, 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.

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11.3 Definitive test

Analysis of variance (ANOVA) involving multiple comparisons of end-point data derived for undiluted soils to be tested (single-concentration test) including field replicates of field-collected soil from more than one sampling location is commonly used for statistical interpretation of the significance of findings from soil toxicity tests (see Annex E). This is a hypothesis-testing approach, and is subject to appreciable weaknesses.[7] 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. [7],[8]

In cases where various dilutions (concentrations) of each sample of field-collected soil with control soil are tested, data can be analysed in two ways:

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

First of all 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 and 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 Mann-Whitney-U-test procedure should be used.

— EC*x* (effect concentration)-approach

The 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 EC*x*-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 EC*x* is obtained by inserting a value corresponding to *x* % of the control mean into the equation found by regression analysis. Since EC50 values have smaller confidence limits compared with smaller effect concentrations (e.g. EC20), it is recommended to determine EC50 values.

In any case the results of the statistical evaluation should be interpreted, taking the biological knowledge on the morphology and behaviour of the worms into account.

12 Test report

The test report shall include the following information:

a) a reference to this Inernational Standard: ISO 16387;

b) a full description of the experimental design and procedures, including a description of the standard soil and test equipment used;

c) chemical identification of the test substance according to IUPAC nomenclature, batch, lot and CAS number, structural formula, and purity of the test substance;

d) properties of the soil to be tested;

e) properties of the test and reference substance (e.g. stability in soil);

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f) method of application;

g) identification of the test organism and description of stock cultures;

h) description of the culturing conditions;

i) source of supply of the test organism;

j) description of the test conditions, including water content and pH value of the standard soil at the start and end of the test;

k) mortality of the adults at the end of the range-finding test;

l) mortality of adults after three weeks and the average number of juveniles at the end of the definitive test;

m) description of obvious physical or pathological symptoms or distinct changes in behaviour observed in the test organisms;

n) statistically calculated values (LC50, NOEC and/or ECx) including 95 % confidence limits, method of calculation, plot of the dose-response relationship;

o) all information, including all measured raw data, developed during all phases of testing with the test and reference substances;

p) discussion of the results;

q) all details not specified in this International Standard or which are optional, as well as any incident which may have affected the results.

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Annex A
(informative)

Conditions for culture of *Enchytraeus* sp

A.1 Culture conditions

Enchytraeidae of the species *Enchytraeus albidus* (as well as other *Enchytraeus* sp.) can be bred in large plastic boxes (e.g. 30 cm × 60 cm × 10 cm) filled with a mixture of artificial soil and natural, uncontaminated garden soil. Compost material should be avoided since it can contain toxic test substances such as heavy metals (untreated garden compost without household material is also suitable). Every breeding substrate should be defaunated before use. Pure artificial soil is also possible, but reproduction can be slower compared to mixed substrates. The substrate should have a pH of 6,0 ± 0,5. The culture is kept in an incubator at a temperature of 15 °C to 20 °C without light. In any case, a temperature higher than 23 °C shall be avoided. The artificial/natural soil moisture is moist but not wet. When the soil is gently pressed by hand, only small drops of water should appear. In any case, anoxic conditions shall be avoided (e.g. if a lid is used, the number of lid holes shall be sufficient). Additionally, the breeding substrate should be aerated by carefully mixing it once a week.

The worms are fed approximately twice a week with a proper amount of rolled oats, which are strewn on the soil surface or carefully mixed into the substrate. If food from the last feeding date remains on the soil surface, the amount of food given shall be adjusted accordingly. If fungi grow on the remaining food, it should be replaced by a new quantity of rolled oats. From time to time, the rolled oats can be supplemented with milk and cod-liver oil. After three months, the animals are transferred into a freshly prepared culture or breeding substrate. The rolled oats, which shall be stored in sealed vessels, should be autoclaved or heated before use in order to avoid infection by flour mites (e.g. *Glyzyphagus* sp., Astigmata, Acarina) or predacious mites [e.g. *Hypoaspis* (*Cosmolaelaps*) miles, Gamasida, Acarina]. After disinfection, the food is ground so that it can easily be strewn on the soil surface. Another possible food source is baker's yeast or fish food. If a culture is infected by mites, their number can be reduced by keeping the substrate slightly dry for some days so that the worms retreat in deeper layers; afterwards, the uppermost centimetres of the substrate (including most of the mites) can be removed mechanically. Absolutely mite-free cultures are only available if the substrate is put in water and adult worms are selected manually into a newly made fresh substrate, preferably using a binocular lens since small mites can be transported by hanging onto the worm cuticle.

In general, the culture conditions are adequate if worms

— do not try to leave the substrate,

— of different ages are visible,

— move quickly through the soil,

— are more or less whitish-coloured,

— exhibit a shiny outer surface without soil particles clinging to it.

Finally, worms can be considered to be healthy if they reproduce continuously.

A.2 Life cycle data of *E. albidus*

Based on data from several sources, some life cycle data of *E. albidus* are summarized in Table A.1.

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Table A.1 — Life-cycle data of *E. albidus*

Parameter	Data (see[10])	Data (see[28])
Temperature	18 °C	12 °C
Eggs per cocoon	10	7
Non-developed cocoons	50 %	60 %
Embryonic development	approximately 12 days	approximately 18 days
Juvenile development	approximately 21 days	approximately 56 days
Total development cycle	approximately 33 days	approximately 74 days
Hatching length	1,5 mm to 3 mm	1 mm to 2 mm
Adult length	15 mm to 41 mm	15 mm to 35 mm
NOTE Smaller *Enchytraeus* sp. have shorter but also temperature-dependent life cycles.		

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Annex B
(informative)

Test procedure using other *Enchytraeus* species

B.1 Selection of species

Species other than *E. albidus* may be used, but the test procedure and the validity criteria should be adapted to provide suitable test conditions. Many *Enchytraeus* species are readily available and can be satisfactorily maintained in the laboratory. Therefore, the most important criterion for selecting an *Enchytraeus* species other than *E. albidus* is ecological relevance and, additionally, comparable sensitivity. There can also be formal reasons for a change of species. In countries where *E. albidus* does not occur and cannot be imported (e.g. due to quarantine restrictions), other Enchytraeus species may be used.

B.2 Potential candidates

Enchytraeus crypticus (Westheide and Graefe 1992): in recent years, this species is very often used in ecotoxicological studies due to the simplicity of its breeding and testing.[2],[30] However, its individual size is small (3 mm to 12 mm), which makes handling more difficult than with *E. albidus* (especially before implementation of the staining method).

Enchytraeus buchholzi (Vejdovsky 1879): this name probably covers a group of closely related species, which are morphologically difficult to distinguish (length 5 mm to 10 mm). Therefore, its use in a test is not recommended until the animals have been clearly described. From an ecological standpoint, these animals are usually found in meadows and disturbed sites such as roadsides.

Enchytraeus luxuriosus ([32]) was formerly known as *E. "minutus"*. This species was found for the first time by U. Graefe (Hamburg) in a meadow close to St. Peter-Ording (Schleswig-Holstein, Germany). Because of its relatively large size (8 mm to 13 mm) in comparison to the other members of this genus (except *E. albidus*), it is easy to handle.

Enchytraeus bulbosus (Nielsen and Christensen 1963): this species has hitherto been reported in German and Spanish mineral soils, where it is common but usually not very abundant (length approximately 5 mm). In comparison to other small species of this genus, it is relatively easy to determine. Up to now, however, nothing is known about its behaviour in laboratory tests and about its sensitivity to substances.

B.3 Breeding conditions

All *Enchytraeus* species mentioned above can be kept and bred in the same substrate as *E. albidus*. The size of the breeding vessels can be smaller. They can also be fed the same food (i.e. rolled oats), but due to their smaller individual size, the amount of food per feeding shall be adjusted. In general, it should be kept in mind that the life-cycle of these animals is shorter, which means e.g. that feeding should be done more often.

B.4 Test conditions

The conditions are the same as in the case of *E. albidus*, except for the following aspects.

— The size of the test vessel may, but need not, be smaller.

— In tests with small species, it is not necessary to remove the adults since their – in relation to the number of juveniles – small number does not influence the overall results of the test.

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— The duration of the definitive test may, but need not, be shorter, i.e. four weeks instead of six weeks. The duration of the range-finding test should not be changed.

NOTE Experience in different laboratories has shown that with small test species like *E. crypticus* a test period of four weeks is sufficient and that no removal of adults is needed.

— Due to the small individual size of the juvenile worms, the use of the staining method is strongly recommended for counting them.

The validity criterion “number of juveniles per test vessel in the control” should be changed to “50”.

The reproduction is species-dependent, meaning that small species (such as *E. crypticus*) produce more juveniles per time than larger species (such as *E. albidus*).

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Annex C
(informative)

Determination of maximum water-holding capacity

C.1 The following method has been found to be appropriate for laboratory samples of soils to be tested and standard soils

C.2 Apparatus

C.2.1 Glass tube, approximately 20 mm to 50 mm diameter and at least 100 mm in length.

C.2.2 Water bath at room temperature.

C.2.3 Filter paper.

C.2.4 Drying oven set to (105 ± 5) °C.

C.2.5 Balance, capable of weighing with an accuracy of ± 0,1 g.

C.3 Method

Plug the bottom of the tube with filter paper, and after filling with the artificial soil substrate 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 is used for the soil substrate is satisfactory.

Weigh the sample, dry it to constant mass at 105 °C and reweigh it.

C.4 Calculation of the water-holding capacity (*WHC*)

$$WHC = \frac{m_S - m_T - m_D}{m_D} \times 100 \qquad \text{(C.1)}$$

where

WHC 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.

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Annex D
(informative)

Detailed description of extraction techniques

D.1 Staining with Bengal red

This method, originally developed in limnic ecology, was first proposed for the counting of juvenile Enchytraeidae in this test by de Coen (University of Ghent, Belgium). Independently, a modified version (Bengal red mixed with formaldehyde instead of ethanol) was developed by Posthuma et al.[25] At the end of the definitive test (i.e. after six weeks), transfer the artificial soil in the test vessels to a shallow container [e.g. a plastic vessel or a photo basin with ribbed bottom (6.12)] and fix the juveniles with ethanol (approximately 5 ml per replicate). Then fill the vessels with water up to a depth of 1 cm to 2 cm. Then add a few drops (200 µl to 300 µl) of Bengal red (1 % solution in ethanol), and mix the two components carefully. After 12 h, the worms are completely reddish-coloured and now very easy to count because they are lying on the surface of the substrate.

Another possibility is to pass the substrate/Ethanol mixture through a sieve of mesh size: 0,250 mm before counting the worms. The kaolinite clay, the peat and some sand grains are removed and the reddish-coloured worms are easier to see. The use of illuminated lenses (lens dimensions at least 100 mm × 75 mm; magnification factor × 2 to × 3) also facilitates counting the already reddish juveniles.

Due to these improvements, the counting time can be reduced to a few minutes per vessel. Using the staining method, the vessels of one test can be assessed by a single person within one day (maximum two days), starting several hours or days after the end of the test.

D.2 Wet extraction

The wet extraction process should be started immediately after the end of the test. The artificial soil from each test vessel is placed into a common plastic sieve. The sieves are put in plastic bowls without touching the bottom. The bowls are carefully filled with water until the samples in the sieves are completely under the water surface. No water shall be poured directly over the soil to be tested. As they are in constant motion, the worms fall through the sieve openings (1 mm diameter). To ensure a recovery rate of more than 90 %, the soil extraction time should be three days at (20 ± 2) °C. At the end of the extraction time, the sieves are removed and the water (except for a small amount) is slowly decanted. The sediment at the bottom of the bowls should not be disturbed. Then the plastic bowls are shaken slightly to suspend the sediment in the supernatant water, which is transferred to a Petri dish. After clarification of the water (i.e. the soil particles have settled), the Enchytraeidae can now be collected from the Petri dish under a stereomicroscope, using a soft steel forceps or a small brush.

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Annex E
(informative)

Overview of the statistical assessment of data (NOEC determination)

Figure E.1 gives an overview of the statistical assessment of data (NOEC determination).

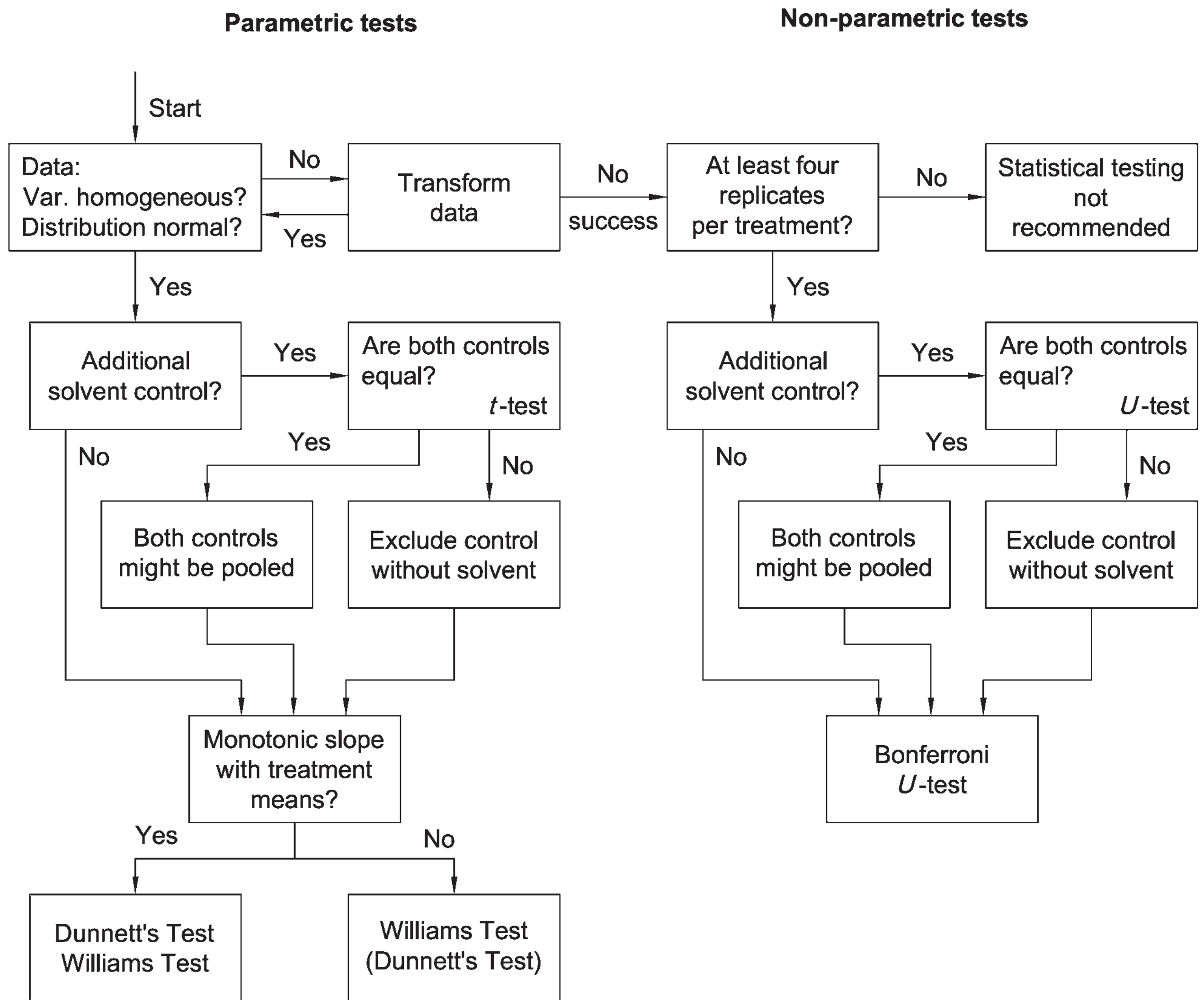

Figure E.1 — Statistical assessment of data

Bibliography

[1] Achazi R.K., Chroszcz G., Pilz C., Rothe B., Steudel I., Throl C. Der Einfluss des pH-Werts und von PCB52 auf Reproduktion und Besiedlungsaktivität von terrestrischen Enchytraeen, in PAK-, PCB- und Schwermetallbelasteten Böden. Verh. Ges. f. Ökol. 26, 1996, pp. 37-42

[2] Achazi R.K. Einfluss von anthropogenen Schadstoffen (PAK und PCB) auf terrestrische Invertebraten urbaner Ökosysteme. In: Bodenökologische Untersuchungen zur Wirkung und Verteilung von organischen Stoffgruppen (PAK, PCB) in ballungsraumtypischen Ökosystemen. Abschlussbericht für das BMBF. Kratz, W. & Brose, A. (eds.), GSF, München. S. 1997, pp. 109-120

[3] Achazi R.K., Chroszcz G., Mierke W. Standardisation of test methods with terrestrial invertebrates for assessing remediation procedures for contaminated soils. ECOINFORMA 12, 1997

[4] Augustsson A.K., & Rundgren S. The Enchytraeid *Cognettia sphagnetorum* in Risk Assessment: Advantages and Disadvantages. *Ambio*. 1998, **27** pp. 62–69

[5] Becker, H. Bodenorganismen — Prüfungskategorien der Forschung. UWSF — Z. Umweltchem. Ökotox., 3, 1991, pp. 19-24

[6] Bell A.W. The anatomy of *Enchytraeus albidus*, with a key to the species of the genus *Enchytraeus*. Ann. Mus. Novitat., 1902, 1958, pp. 1-13

[7] Bouguenec V., & Giani N. Deux nouvelles especes d´*Enchytraeus* (Oligochaeta, Enchytraeidae) et rediscription d´*E. bigeminus*. Remarques sur le genre Enchytraeus. *Ann. Limnol.* 1987, **23** pp. 9–22

[8] Brüggl G. Populationsentwicklung von *Enchytraeus crypticus* (Enchytraeidae, Oligochaeta) und Einfluss subletaler Pestizidbelastungen unter Laborbedingungen. Diss. Univ. Osnabrück, 1994, 101 pp

[9] Didden W.A.M. Ecology of terrestrial Enchytraeidae. *Pedobiologia (Jena)*. 1993, **37** pp. 2–29

[10] Dirven-Van Breemen E., Baerselmann R., Notenboom J. Onderzoek naar de Geschiktheid van de Potwormsoorten *Enchytraeus albidus* en *Enchytraeus crypticus* (Oligochaeta, Annelida) in Bodemecotoxicologisch Onderzoek. RIVM Rapport Nr. 719102025, 1994, 46 pp

[11] Dunger W., & Fiedler H.J. *Methoden der Bodenbiologie*. G. Fischer Verlag, Stuttgart, New York, 1997

[12] Dunnett C.W. A multiple comparison procedure for comparing several treatments with a control. *Amer. Statist. Ass. J.* 1955, **50** pp. 1096–1121

[13] Dunnett C.W. New tables for multiple comparisons with a control. *Biometrics*. 1964, **20** pp. 482–491

[14] Finney D.J. *Probit Analysis*. Cambridge Univ. Press, Third Edition, 1971, pp. 19–76.

[15] Finney D.J. *Statistical Method in Biological Assay*. Charles Griffin & Company Ltd, London, 1978

[16] Hamilton M.A., Russo R.C., Thurston R.V. Trimmed Spearman-Karber method for estimating lethal concentrations in toxicity bioassays. Environ. Sci. Technol.; 11, 1977, pp. 714-719. *Correction: Environ. Sci. Technol.* 1978, **12** p. 417

[17] Holm S. A simple sequentially rejective multiple test procedure. *Scand. J. Stat.* 1979, **6** pp. 65–70

[18] Kasprzak K. Review of enchytraeid community structure and function in agricultural ecosystems. *Pedobiologia (Jena)*. 1982, **23** pp. 217–232

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[19] Korinkova J., & Sigmund J. The colouring of bottom-fauna samples before sorting. *Vestnik Ceskoslovensko Spolecnosti Zoologicke*. 1968, **32** pp. 300–305

[20] Martikainen E. Toxicity of Dimethoate to some soil animal species in different soil types. *Ecotoxicol. Environ. Saf.* 1996, **33** pp. 126–128

[21] Nielsen C.O., & Christensen B. The Enchytraeidae, critical revision and taxonomy of European species. *Natura Jutlandica*. 1959, **8-9** pp. 1–160

[22] OECD. (Organisation for Economic Co-Operation and Development) (2008): Predatory mite (*Hypoaspis* (*Geolaelaps*) *aculeifer*) reproduction test in soil. Guideline for the testing of chemicals 226. Paris, France

[23] Guideline No O.E.C.D. 202, 2004, Daphnia sp., Acute Immobilisation Test, Organization for Economic Cooperation and Development, Paris

[24] Guideline No O.E.C.D. 220, 2004, *Enchytraeid Reproduction Test,* Organization for Economic Cooperation and Development, Paris

[25] Posthuma L., Baerselmann R., Van Veen R.P.M., Dirven-Van Breemen E.M. Single and joint toxic effects of copper and zinc on reproduction of Enchytraeus crypticus in relation to sorption of metals in soils. *Ecotoxicol. Environ. Saf.* 1997, **38** pp. 108–121

[26] Puurtinen H.M., & Martikainen E.A.T. Effect of Soil Moisture on Pesticide Toxicity to an Enchytraeid Worm, Enchytraeus sp. *Arch. Environ. Contam. Toxicol.* 1997, **33** pp. 34–41

[27] Römbke J. Entwicklung eines Reproduktionstests an Bodenorganismen — Enchytraeen. Abschlußbericht des Battelle-Instituts e.V. Frankfurt für das Umweltbundesamt (Berlin), FE-Vorhaben 106 03 051/01, 1989

[28] Römbke J. Entwicklung eines Reproduktionstests an Bodenorganismen — Enchytraeen (2nd edn). Abschlußbericht des Battelle-Instituts e.V. Frankfurt für das Umweltbundesamt (Berlin), FE-Vorhaben 106 03 051/01, 1991

[29] Römbke J. Enchytraeen (Oligochaeta) als Bioindikator, UWSF — Z. Umweltchem. Ökotox. 7, 1995:pp. 246-249

[30] Römbke J., & Federschmidt A. Effects of the fungicide Carbendazim on Enchytraeidae in laboratory and field tests. *Newsletter on Enchytraeidae*. 1995, **4** pp. 79–96

[31] Römbke J., & Moser Th. Report on an International Ringtest with the Enchytraeid Reproduction Test. *UBA-Text*. 1999, **4/99** p. 373

[32] Schmelz R.M., & Collado R. Enchytraeus luxuriosus sp.nov., a new terrestrial oligochaete species (Enchytraeidae, Clitellata, Annelida). *Carolinea*. 1999, **57** pp. 93–100

[33] Thompson W.R. Use of moving averages and interpolation to estimate median-effective dose. I. Fundamental formulas, estimation of error, and relation to other methods. *Bact. Reviews*. 1947, **11** pp. 115–145

[34] Westheide W., & Bethge-Beilfuss D. The sublethal enchytraeid test system: guidelines and some results. In: *Modern Ecology: Basic and Applied Aspects*, (Esser G., & Overdieck D. eds.). Elsevier, Amsterdam, 1991, pp. 497–508.

[35] Williams D.A. A test for differences between concentration means when several dose levels are compared with a zero dose control. *Biometrics*. 1971, **27** pp. 103–117

[36] Williams D.A. The comparison of several dose levels with a zero dose control. *Biometrics*. 1972, **28** pp. 519–531

[37] ISO 10390:1994, *Soil quality — Determination of pH*

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[38] ISO 10694:1995, *Soil quality — Determination of organic and total carbon after dry combustion (elementary analysis)*

[39] ISO 11260:1994, *Soil quality — Determination of effective cation exchange capacity and base saturation level using barium chloride solution*

[40] ISO 11272:1992, *Soil quality — Determination of dry bulk density*

[41] ISO 11277:1993, *Soil quality — Determination of particle size distribution in mineral soil material — Method by sieving and sedimentation*

[42] ISO 14240-1:1997, *Soil quality — Determination of soil microbial biomass — Part 1: Substrate-induced respiration method*

[43] ISO 15799, *Soil quality — Guidance on the ecotoxicological characterization of soils and soil materials*

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