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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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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

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.

Ten adult worms per test vessel should be used. 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 the worms do not consume the food, feeding should be minimized in order to avoid fungal growth or moulding. After 21 d, the test substrate is carefully searched manually (e.g. using a jeweller's tweezer, 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 noted. If mortality is the main endpoint of the test, the whole procedure is stopped at this point.

The same test substrate to which the adult worms were exposed, including cocoons laid down during the first 3 weeks of the test, is incubated under the same test conditions for another 3 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 4 weeks). Again, over-feeding shall be avoided (see 5.3).

After a total test duration of 6 weeks, the juveniles hatched in the meantime are counted by staining with Bengal red. Wet (but not heat) extraction techniques have also proved to be suitable (see Annex A). The first method is recommended, since wet extraction is difficult to use with artificial soil because the clay particles make the water turbid.

8.5 Reference substance

The NOEC and/or the ECx of a reference substance shall be determined once a year or parallel to the determination of the toxicity of a test substance, as a means of assuring that the laboratory test conditions (including the condition and sensitivity of the test organisms) are adequate and have not changed significantly. A suitable reference substance is carbendazim, which has been shown to affect mortality and reproduction of *Enchytraeidae*. The EC50 (reproduction) should be in the range of 1,2 mg ± 0,8 mg carbendazim/kg dry mass using an ECx design as determined in an international ring test (see Bibliography).

If testing of a positive control in parallel to each individual test is required, it should be done as for the untreated (water or negative) control; i.e. eight replicates are used for one concentration (preferably two to three concentrations covering the range of expected concentrations should be tested). It is recommended that the reference substance carbendazim be used as a positive control. If tested as a liquid formulation, a concentration of 1,2 mg a.i./kg dry mass should cause a decrease in the number of juveniles in comparison to the untreated control of approximately 50 % ± 20 %.

8.6 Summary and timetable of the test

The individual steps of this test can be summarized as follows, where AS is the artificial soil and WHC is the water-holding capacity:

Period	Range-finding test	Definitive test
Day -7	Preparation of artificial soil (mixing of dry constituents)	Preparation of artificial soil (mixing of dry constituents)
Day -5	Check of pH of prepared AS Measurement of WHCmax	Check of pH of prepared AS Measurement of WHCmax
Day -3 to Day -1	Sorting out of worms for acclimatization	Sorting out of worms for acclimatization
Day -1	Premoistening of AS and division into batches	Premoistening of AS and division into batches
Day 0	1 Preparation of stock solution 2 Application of test substance 3 Weighing of test substrate into the test vessels 4 Mixing of food into the soil 5 Introduction of worms 6 Measurement of pH and moisture	1 Preparation of stock solution 2 Application of test substance 3 Weighing of test substrate into the test vessels 4 Mixing of food into the soil 5 Introduction of worms 6 Measurement of pH and moisture
Day 7	Check of moisture	Check of moisture; feeding
Day 14	Determination of mortality Estimation of number of juveniles Measurement of pH and moisture	Check of moisture; feeding
Day 21		1 Removal of adults 2 Check of behaviour 3 Determination of mortality 4 Check of moisture; feeding
Day 28		Check of moisture
Day 35		Check of moisture; feeding
Day 42		Counting of juvenile worms Measurement of pH and moisture

9 Calculation and expression of results

9.1 General

It is recommended that a statistician be involved in the analysis of the test since in this International Standard, specific guidance on statistical procedures is given only in limited detail (for an overview, see Annex G).

9.2 Range-finding test

The test parameter (endpoint) is mortality after 14 d of exposure. Changes in behaviour (e.g. inability to burrow into the soil; lying motionless against the glass wall of the test vessel) and morphology (e.g. open wounds) of the worms should also be recorded. Probit analysis should be applied to determine the LC50 (see Bibliography). In case of failure (e.g. if less than three concentrations with partial kills are available), alternative methods can be used, such as the trimmed Spearman-Kärber method, moving averages after Thompson, or simple interpolation (e.g. geometrical mean of LC0 and LC100, as computed by the square root of LC0 × LC100).

9.3 Definitive test

9.3.1 General

The test parameter (endpoint) is reproduction (number of juveniles present during the experimental period) and adult mortality after 21 d of exposure. As in the range-finding test, all other signs of toxic impact should be recorded.

The data should be presented in tabular form, indicating the mean number of adults and juveniles for each concentration. Further statistical testing will depend on

a) whether the NOEC or the ECx approach has been chosen, and

b) whether the replicate values are normally distributed and are homogeneous regarding their variance.

It should be kept in mind that the proposed statistical methods are not appropriate in case of hormetic effects (see Annex G for details on how to proceed).

9.3.2 NOEC approach

For each concentration, a statistical analysis of the homogeneity and normality of replicate results shall be made, e.g. by using Kolmogoroff-Smirnov's and Bartlett's test procedures respectively. With normally distributed and homogeneous data, an appropriate statistical analysis, e.g. multiple t-tests such as the Dunnett or Williams test (α = 0,05, one-sided) should be performed. If these requirements are not fulfilled, it is recommended to use non-parametric methods, e.g. the Mann and Whitney U-test or the Bonferroni U-test.

If a limit test has been performed and the prerequisites (normality, homogeneity) of parametric test procedures are fulfilled, the pair-wise Student t-test or the Mann and Whitney U-test procedure should be used.

9.3.3 ECx approach

To compute any ECx value, the treatment means are used for regression analysis after an appropriate dose-response function has been found. A desired ECx is obtained by inserting a value corresponding to x % of the control mean into the equation found by regression analysis. Confidence limits can be calculated according to Fieller [15].

Alternatively, treatment results may be expressed as percentages of the control result or as percentage inhibition relative to the control. The normal (logistic) sigmoid can then be fitted to the results by means of the probit regression procedure.

10 Validity of the test

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

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

— 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.

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

11 Test report

The test report shall refer to this International Standard and shall contain a summary of the results obtained, the methods and parameters used during the study. The test report shall provide the following information:

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

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

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

d) method of application;

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

f) description of the culturing conditions;

g) source of supply of the test organism;

h) description of the test conditions, including moisture content and pH value of the artificial soil at the start and end of the test;

i) mortality of the adults and the number of juveniles at the end of the range-finding test;

j) mortality of adults after 3 weeks and the average number of juveniles at the end of the definitive test;

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

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

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

n) discussion of the results;

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

Annex A
(informative)

Detailed description of extraction techniques

A.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.* [23].

At the end of the definitive test (i.e. after 6 weeks), transfer the artificial soil in the test vessels to a shallow container [e.g. a Bellaplast 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) or alternatively 0,5 % eosin, 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 press the substrate/alcohol 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 1 d (maximum 2 d), starting several hours or days after the end of the test.

A.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. As they are in constant motion, the worms fall through the sieve openings (∅1 mm). To ensure a recovery rate of more than 90 %, the soil extraction time should be 3 d at 20 °C ± 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.

A.3 Flotation

Alternatively, according to Kuperman (US Army Edgewood Chemical Biological Center), the following procedure is also possible: after fixing the content of a test vessel with ethanol, the artificial soil is flooded with Ludox [AM-30 colloidal silica, 30 % (mass fraction) suspension in water] up to 10 mm to 15 mm above the soil surface. After thoroughly mixing the soil with the flotation agent, the juvenile worms floating on the surface can easily be counted after 2 min to 3 min. To ensure that all juveniles have emerged on the surface of the solution, stirring should be repeated once or twice.

Annex B
(normative)

Determination of the effects of contaminated soil on *Enchytraeidae* reproduction

B.1 General

The method described in this International Standard can be adapted to compare *Enchytraeidae* reproduction in a number of soils. Different species of the genus *Enchytraeus* can be used to assess the quality of a specific soil (sample). In order to distinguish between effects (e.g. mortality or a decrease in reproduction) of a toxic test substance or of soil parameters, two prerequisites shall be fulfilled:

— a control soil shall be available in order to evaluate effects of the soil to be tested;

— the ecological requirements of the selected species shall be known.

Concerning a control soil, only three choices a), b) and c) below are possible [18]. The best option is to use a) and c) in parallel. If a) is not available (which is probably often the case), b) and c) should be used accordingly.

a) a field soil uncontaminated but in every other respect pedologically comparable to the soil sample being tested;

b) a well-characterized field soil, such as the German standard soils[1)], which are often used as a test substrate in pesticide registration studies;

c) a standardized artificial soil (for example in accordance with OECD 1984 or ISO 11268-2); however, one natural component (the peat) is often difficult to handle since its properties can vary considerably.

Depending on the test species, different control soils can affect the worms even without any contamination (e.g. due to pH preferences). Therefore, it is very important to identify the ecological requirements of those *Enchytraeus* species which are potential test organisms for soil quality assessment. In any case, it is recommended to look for a test species (here, different *Enchytraeus* species) of which the range of ecological requirements comprises the relevant soil properties (i.e. the pH and organic matter content). Any change in the soil properties can influence the bioavailability of chemicals. In 4.2 the ecological requirements of two *Enchytraeus* species are presented. Testing should be restricted to soils whose relevant properties are covered by the ecological requirements of the test species recommended.

B.2 Principle

The effects on mortality and reproduction of adult worms (*Enchytraeus albidus* or other *Enchytraeus* sp.) are determined in the test soil (including dilution series of contaminated soil mixed into control soils) and in a control soil. If the objective is to assess the effect of a suspected contamination, the control soil should be as similar as possible to the test soil in all characteristics other than the presence of contaminant(s). In any case an additional control soil of known good acceptable properties with respect to the ecological requirements of the selected test species should be included.

1) German standard soils are supplied by Landwirtschaftliche Untersuchungs- und Forschungsanstalt, Postbox 1629, D-67326 Speyer. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

B.3 Apparatus

Use the same apparatus as described in Clause 6.

B.4 Procedure

B.4.1 Preparation of the test soils

If a “matched” soil is required, the following characteristics should be determined and reported:

a) pH in accordance with ISO 10390;

b) cation exchange capacity in accordance with ISO 11260;

c) organic matter content in accordance with ISO 10694;

d) particle size distribution in accordance with ISO 11277;

e) bulk density in accordance with ISO 11272;

f) water content in accordance with ISO 11465;

g) water-holding capacity as described in Annex D.

In addition, it is recommended to measure the microbial biomass in accordance with ISO 14240. At least five replicates of each soil and the control should be used. The soil should be used at 40 % to 60 % of its water-holding capacity. The pH and the water content of the soil should be determined at the start and the end of the test.

B.4.2 Ecological requirements of *Enchytraeus* species used as test organisms

At this moment, knowledge about the ecological requirements of *Enchytraeus* species concerning soil parameters is very limited. In the following compilation, suitable ranges for the soil parameters pH, organic matter content, soil type and moisture are given as extracted from literature. Data are available only for *E. albidus* and *E. crypticus*, since these species have been used relatively often in chemical tests. Based on these experiments, a suitable survival and reproduction was considered to be (taking artificial soil and standard German soil LUFA 2.2 as examples):

— *E. albidus*: nearly no mortality (< 10 %) and 25 juveniles per 10 adults after 3 weeks;

— *E. crypticus*: nearly no mortality (< 10 %) and 50 juveniles per 10 adults after 3 weeks.

A compilation of more detailed data and for other species (especially from the *E. buchholzi* complex) will be added when sufficient data are available. The final aim is to get a selection of three to four test species from the same taxonomical/physiological group but having differently ecological requirements (e.g. pH ranges). Such an inventory of test species is necessary, since otherwise it is not possible to test natural field soils with their very different properties: having only one test species with specific ecological requirements, it would often be impossible to distinguish effects linked to the contamination from those of the soil itself.

Since most of the tests conducted with these two species were performed in artificial soil (sand content 70 %), the influence of the various soil properties in this substrate is discussed. A second step attempts to extrapolate these experiences to natural field soils. In Table B.1 the effects of actual moisture, pH and the content of organic matter on the two *Enchytraeidae* species are presented [10].

Table B.1 — The effects of actual moisture, pH and the content of organic matter on the reproduction of *E. albidus* and *E. crypticus*

Soil parameter	Number of juveniles per worm and week	
	E. albidus	*E. crypticus*
Water content		
35 %	0,36 ± 0,32	3,80 ± 0,65
55 %	1,82 ± 0,89	2,65 ± 0,69
65 %	1,62 ± 0,58	1,43 ± 0,28
90 %	1,38 ± 0,67	2,11 ± 0,60
pH value		
3,2	0,01 ± 0,01	0,64 ± 0,53
3,6	0,01 ± 0,02	3,27 ± 0,51
4,0	0,30 ± 0,42	5,49 ± 2,35
5,3	2,79 ± 1,19	4,78 ± 2,15
6,8	5,75 ± 2,11	9,54 ± 1,55
7,0	7,63 ± 1,14	8,25 ± 0,98
Org. matter (%)		
5	6,4 ± 1,7	4,4 ± 0,6
10	8,8 ± 2,2	4,0 ± 1,1
20	8,2 ± 2,4	4,1 ± 1,4

Considering the relatively low number of data, these data indicate that the ecological ranges of both species are different:

— *E. crypticus* has a lower moisture optimum than *E. albidus* (35 % to 55 % versus 55 % to 65 % water content);

— *E. albidus* is clearly acidophobic (optimum: pH 6,8 to 7,0), whereas *E. crypticus* avoids only very acid soils (pH < 4,0; even at pH 3,6 considerable reproduction is possible);

— no correlation between number of juveniles and the organic matter content (between 5 % and 20 %) of the artificial soil is observable;

— in addition (data not presented), both species have clearly different temperature optima: *E. albidus* has an optimum of 15 °C to 20 °C, whereas for *E. crypticus* it is 25 °C to 30 °C.

These results are in good agreement with data from laboratory experiments with field soils.

For example, a preliminary evaluation of tests with *E. albidus* in standard German soil LUFA 2.2 and related soils shows that good survival and reproduction values were often reached [1], [26], [27]:

— 60 % WHC is usually sufficient (no actual moisture data available);

— high numbers of juveniles are found at pH values between 5,8 to 6,0;

— an effect on reproduction was observed below 3 % organic matter content;

— even a very high percentage of sand (77 % to 93 %) does not negatively affect reproduction.

However, the reproduction of *E. albidus* is slow in soils where combinations such as high sand content, low organic matter and a relatively low pH (4,8 to 5,6) and in one case a high pH (7,4) are present. This is for example the case for the standard German soils LUFA 2.1 and 2.3 [27]. These results are in very good agreement with the occurrence of this species in the field.

In the case of *E. crypticus*, the available data can be summarized as follows [1], [2], [8], [21], [25] (in the latter two references the species determination was not absolutely clear):

— no problems were found when using WHC of 60 % to 70 %;

— high juvenile numbers were recorded between pH values of 4,8 and 6,5, but the optimum is probably between pH 5,9 and 6,5. A drop to low numbers occurs at pH < 4,85 and below pH 4 nearly no juveniles were found [higher pH values (up to pH 7,7) usually did not have negative effects];

— the organic matter content varied very much (2,8 % to 12,1 %) in the successful tests; in rare cases even 0,3 % did not negatively affect the worms;

— even at a percentage of 93 % sand high juvenile numbers are possible.

In those tests where the reproduction success was low, a very low organic matter content (1,2 %) might partly be responsible, but sometimes no obvious reason is identifiable (e.g. for low juvenile numbers in a clayey soil of pH = 6,6; organic matter content 6,5 [21]). No comparison with field data is possible, since this species has not yet been found in the field.

The data presented here indicate that the two species which up to now were used mainly in single chemical testing, could be used for soil quality testing too. Due to the fact that their ecological ranges are different, they cover relatively moist, mainly sandy and moderately acid to basic soils (pH 4,8 to > 7,0) with an organic matter content of 3 % and higher. Not all combinations of these factors are equally suited for these two species. In some cases (e.g. acid soils) obviously more test species with a preference for soils with a pH < 4,5 are urgently needed, whereas in other cases it is simply not known whether *Enchytraeidae* would be suitable test organisms (e.g. in very organic or clayey soils).

The following conclusions can be drawn from the data presented:

— both *Enchytraeus* species discussed here are suitable test organisms for artificial soil and the most common German standard soil (LUFA 2.2); the same is probably true for other agricultural soils with comparable properties;

— if the soil to be tested does not fall into the ecological range of the two species, another test system should be used until other *Enchytraeus* (e.g. *E. norvegicus* for moderately acid soils) or even other genera (e.g. *Cognettia sphagnetorum* as proposed in [4]) have been validated for being used as test organisms.

B.4.3 Test procedure

To each test container, the worms should be added in accordance with Clause 8. In all respects the test performance should follow the instructions given in Clause 7 and Clause 8 accordingly. As mentioned in B.4.2, no adjustment of soil parameters is recommended.

When using other species than *E. albidus*, the conditions are the same, except for the following aspects.

— The size of the test vessel may, but need not be smaller (the same is true for the amount of substrate needed).

— The duration of the definitive test may, but need not be shorter, i.e. 4 weeks instead of 6 weeks (depending on the duration of the life cycle of the selected species). The duration of the range-finding test should not be changed.

— The change in test duration is especially obvious if a smaller species with a higher optimum temperature is chosen.

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

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

B.5 Expression of results

For each soil, the percent mortality of the adults and the number of juveniles produced should be determined. The mean values of the control and test soils (including dilution series, given as percent of contaminated soil in the test mixture) should be compared by suitable statistical methods. Significant differences ($\alpha = 0,05$) from the control(s) should be tested. In addition, any behavioural changes (e.g. escape reactions) should be recorded.

Annex C
(informative)

Determination of the pH value

The following method has been found to be appropriate. It is based on the description in ISO 10390.

A defined quantity of test substrate is dried at room temperature for at least 12 h. A suspension of soil (at least 5 ml) is made up in 5 times its volume in a solution of potassium chloride [c(KCl) = 1 mol/l, recognized analytical grade] in water {alternatively, a solution of calcium chloride [$c(CaCl_2)$ = 0,01 mol/l] is possible}. The suspension is shaken thoroughly for 5 min and left to stand for at least 2 h but not longer than 24 h (preferably overnight). The pH is measured using a pH-meter which has been calibrated before each measurement series using different buffer solutions (e.g. pH 4,0 and 7,0).

Annex D
(informative)

Determination of maximum water-holding capacity

The following method has been found to be appropriate for determination of the water-holding capacity of artificial soil, in accordance with ISO 11268-2:1996, Annex C.

Take a defined quantity (e.g. 5 g) of the test soil substrate, using a suitable device (auger tube, etc.), sufficient to achieve saturation with water. Close the bottom of the auger tube with filter paper, and after filling with soil, place the tube on a rack in a water bath. The water level should first be beneath the upper lid of the tube and later above this lid. Leave the soil substrate sample in the water for about 3 h. As not all water absorbed by the soil substrate capillary can be retained, the sample should be placed for a period of 2 h on very wet finely ground quartz sand in a closed vessel for draining. Weigh the sample, dry it to constant mass at 105 °C and re-weigh it.

Calculate the water-holding capacity (WHC) as follows:

$$WHC = \left(m_S - m_T - m_D\right) \times 100/m_D$$

where

WHC is the water-holding capacity of the soil, expressed as % of dry mass;

m_S is the mass of water-saturated substrate + mass of tube + mass of filter paper;

m_T is the tare (mass of tube + mass of filter paper);

m_D is the dry mass of substrate.

Annex E
(informative)

Conditions for culture of *Enchytraeus* sp.

E.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 could 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 could 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 should 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 should be avoided (e.g. if a lid is used, the number of lid holes should 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 should 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 vitamins, milk and cod-liver oil. After 3 months, the animals are transferred into a freshly prepared culture or breeding substrate. The rolled oats, which are 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 commercial 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.

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

Based on data from several sources, some life cycle data of *E. albidus* are summarized in Table E.1 [30].

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

Parameter	Literature	Römbke[26]
Temperature	18 °C	12 °C
Eggs per cocoon	10	7
Non-developed cocoons	50 %	60 %
Embryonic development	≈ 12 d	≈ 18 d
Juvenile development	≈ 21 d	≈ 56 d
Total development cycle	≈ 33 d	≈ 74 d
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.		

Annex F
(informative)

Test procedure using other *Enchytraeus* species

F.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.

F.2 Potential candidates

Enchytraeus crypticus (Westheide & Graefe 1992): in recent years, this species is very often used in ecotoxicological studies due to the simplicity of its breeding and testing [2], [28]. 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). Additionally, it was only described from earthworm cultures. Since this species has not been found to exist with certainty in the field up to now, its ecological requirements are not known.

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 (Schmelz and Collado [30]) 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. Additionally, *E. bulbosus* seems to be easy to culture (E. Belotti, pers. comm.). Up to now, however, nothing is known about its behaviour in laboratory tests and about its sensitivity to chemicals.

F.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 should 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.