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Soil quality — Effects of pollutants on juvenile land snails (Helicidae) — Determination of the effects on growth by soil contamination

Qualité du sol — Effets des polluants vis-à-vis des escargots juvéniles (Helicidae) — Détermination des effets sur la croissance par contamination du sol

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Foreword

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

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This document was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological characterization*.

This second edition cancels and replaces the first edition (ISO 15952:2006), which has been technically revised.

Introduction

Because of the limited amount of data available concerning toxicity of contaminants on soil organisms, the ecotoxicity of soils and waste are cause for serious concern at both national and international levels. Currently available tests use soil-fauna organisms restricted to annelid (earthworms and *Enchytraeidae*) and arthropod phyla (insects: Collembola and Coleoptera). Among the latter, two standards assess acute toxicity [earthworms (ISO 11268-1) and coleoptera larvae[6] and three other standards address sublethal effects of soil contaminants on reproduction (earthworms[3], Collembola[2], *Enchytraeidae*[4]). In the biological cycles of organisms, it appears that growth is, like reproduction, a fundamental ecophysiological parameter to be taken into consideration for the sustainability of species and ecosystems[38].

Snails are relevant ecological indicators for assessing the quality of soils(See References [16][18] to [20][32][33][40] to [42]), as they are characteristic of the soil surface layer (saprophagous and phytophagous) of which a large part of the biological cycle takes place in the soil (egg-laying, hatching, initial stages of development, hibernation, etc.)[7][18][29]. During the other phases of their cycle, they eat soil and are in contact with the soil via their moist pedal sole (foot) covered with mucus and participate in the permanent exchanges with the soil (water, mineral salts, excrement and finally shell and organic matter when they die)[7][18][31]. In addition, they constitute an important link between plants, fauna and soil microorganisms. They correspond fully to the criteria for a good biological indicator: easy to sample and identify, they are widely distributed; they accumulate contaminants (See References[9],[11] to [15],[17][18][22][24][29][30],[33] to [48]); their ecological and physiological characteristics are well-known[7][10][32]; and they are now easy to breed under controlled conditions[22][26][32]. Their susceptibility to common contaminants of their environment has been demonstrated (See References [11] to [16],[19] to [28],[30],[33] to [38],[37] to [48]).

This International Standard describes a method for determining the effects on survival and growth of young snails of substances, preparations (i.e. a mixture or solution composed of two or more substances), soils or waste materials added to an artificial or a natural soil. The described method is thus applicable to test contaminated soils or to compare different uncontaminated soils. The recommended species is *Helix aspersa aspersa* Müller (also commonly called: common garden snail, brown garden snail, garden snail, land snail, "Petit-Gris"; synonyms: *Cantareus aspersus, Cornu aspersum*[56]). Among land snails (stylommatophoran pulmonate gastropod molluscs of the *Helicidae* family), *Helix aspersa aspersa* Müller is the most ubiquitous. This palearctic species can be acclimated to regions with different types of climate: Mediterranean, oceanic temperate, midcontinental temperate and even tropical. *Helix aspersa aspersa* Müller is of European origin and has been introduced into all parts of the world. They are now on all continents except Antarctica[10].

Indeed, in their natural environment, snails integrate the contaminants by contact (with various substrates such as soil, soil leachates, plant litter), by ingestion (of plants and soil), as well as through the respiratory tract[7][29]. So, for specific testing purposes (evaluation of pesticide toxicity, for example), another test design, which is focussed on exposure via food uptake, is optionally available (Annex F and Reference [6]).

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Soil quality — Effects of pollutants on juvenile land snails (Helicidae) — Determination of the effects on growth by soil contamination

1 Scope

This document specifies a semi-static method for determining the effects of contaminants on growth and survival of young snails, usually *Helix aspersa aspersa* Müller. The animals are exposed via the cutaneous and digestive route using a test substrate (artificial or natural soil according to the objective of the study) to which defined amounts of the following are added:

— substances, mixtures or preparations;

— soils (contaminated or of unknown quality) or waste materials.

This test takes into account the possible changes in the test substance, preparation, soil or waste material because the test mixtures are prepared and renewed every week during the 28-day test period.

A static method may be implemented in addition to the semi-static method (optional). This method is described in Annex A.

This method does not apply to substances for which the air/soil partition coefficient is greater than one, or to substances with vapour pressure exceeding 300 Pa, at 25 °C.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 10390, *Soil quality — Determination of pH*

ISO 18400-206, *Soil quality — Sampling — Part 206: Guidance on the collection, handling and storage of soil for the assessment of biological functional and structural endpoints in the laboratory*

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

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

ISO 11269-2:2012, *Soil quality — Determination of the effects of pollutants on soil flora — Part 2: Effects of contaminated soil on the emergence and early growth of higher plants*

ISO 11274, *Soil quality — Determination of the water-retention characteristic — Laboratory methods*

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

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EN 14735, *Characterization of waste — Preparation of waste samples for ecotoxicity tests*

3 Terms and definitions

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

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

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at https://www.iso.org/obp

3.1
test substrate
artificial soil or natural soil used as control and dilution substrate

3.2
matrix
soil or waste material under test

3.3
test mixture
mixture of the test substance, preparation or matrix with the *test substrate* (3.1)

3.4
growth
<biomass> increase in the biomass, i.e. in the total fresh mass (body with shell) of the organisms between the start and completion of the test

Note 1 to entry: It is expressed as a biomass growth coefficient $k_{GC,m}$.

3.5
growth
<shell> increase in the maximum shell diameter, between the start and completion of the test

Note 1 to entry: It is expressed as a shell diameter growth coefficient $k_{GC,d}$.

3.6
effect concentration
EC_x
concentration at which a specific effect is detected; x is the percentage (10, 25, 50) of this effect, e.g. growth inhibition

EXAMPLE EC_{50} means the concentration estimated to reduce growth at the end of the test to 50 % compared to the control ($EC_{50,m}$ and $EC_{50,d}$ for biomass growth and shell growth respectively).

3.7
median lethal concentration
LC_{50}
concentration of the substance, of the test preparation initially present, or the concentration of the matrix causing the death of 50 % of the snails submitted to testing

3.8
lowest observed effect concentration
LOEC

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

Note 1 to entry: All test concentrations above the LOEC have a harmful effect equal to or greater than those observed at the LOEC. When these two conditions cannot be satisfied, a full explanation should be given for how the LOEC (and hence the NOEC) has been selected.

3.9
no observed effect concentration
NOEC
test concentration immediately below the LOEC, which, when compared with the control, has no statistically significant effect (p > 0,05) within a given exposure time

Note 1 to entry: The NOEC is the concentration just below the LOEC.

Note 2 to entry: For 3.6, 3.7, 3.8 and 3.9, results are given:

— in dry mass of test substance or preparation per dry mass of the *test substrate* (3.1);

— in mass percentage of the tested matrix in the test mixture (expressed in dry mass).

4 Principle

Juvenile land snails (usually *Helix aspersa aspersa* Müller) are exposed during a period of 28 d to test mixtures containing the test substance, preparation or matrix at different concentrations. The test mixtures are freshly prepared and renewed every 7 d.

According to the objectives of the study, the test mixtures may be prepared with artificial soil (see 6.3.2) or with a suitable natural soil (see 6.3.3).

The snails are fed during the test with uncontaminated food.

The effects on growth (biomass and shell diameter) and on survival are measured after 28 d of exposure (optionally, effects could be measured every 7 days during 28 d).

The results obtained during testing are compared with those of a control to determine the NOEC or LOEC and to allow the estimation of the concentration which reduces the growth of the snails by 50 % within 28 d with respect to the fresh mass [$EC_{50,m}$ (28 d)] and to the shell diameter [$EC_{50,d}$ (28 days)] or other values of EC_x.

If the concentrations selected result in lethal effects, the results obtained during testing are compared with those of a control and used for estimating the concentration which causes the death of 50 % of the snails [LC_{50}(28 d)].

For particular applications, various parameters (EC_x, NOEC, LOEC, LC_{50}) can be determined (optional) after exposure periods lower than 28 d (7 d, 14 d or 21 d).

The test is conducted in two stages:

— a preliminary test intended to indicate both the non-observed effect concentration, NOEC, and the complete growth inhibition. The resulting dose-response relationship is important for the proper design of the definitive test;

— a definitive test specifying the concentrations which cause between 10 % and 90 % of growth inhibition. It is not necessary to perform a final test where the preliminary test has not revealed any inhibitory effects at the maximum concentration tested.

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5 Test environment

The test shall be carried out at a temperature of (20 ± 2) °C under a day-night photoperiod of 18 h to 6 h. The illumination intensity (artificial light of daylight type), without any natural light in the test containers shall be 50 to 100 lx.

6 Reagents

6.1 Water, of purity at least deionized.

6.2 Biological material.

Test organisms shall be juvenile snails. The recommended species is *Helix aspersa aspersa* Müller (also known as *Cantareus aspersus* and *Cornu aspersum*) which shall be 3 to 5 weeks old, having a mean fresh mass of (1 ± 0,3) g and a shell diameter of (15,5 ± 1) mm.

NOTE The use of some other genus and/or species of *Helicidae* is possible (see examples and conditions in Annex G).

The snails shall be selected from synchronous breeding in order to form a population as homogeneous as possible with respect to size, mass and age. The breeding techniques for snails are described in Annex B. After a nursery period (3 to 5 weeks, see Annex B), the young snails shall be used after at least 1 week of aestivation and no more than 5 months. The aestivation is carried out in round wooden boxes (approximately 12 cm in diameter and 4 cm in height), with the snails under dry conditions, at a temperature of 17 °C to 20 °C.

Two to three days before starting the test, snails shall be woken by spraying water (6.1) into the boxes used for aestivation. The proportion of snails not woken shall be less than 10 %. As soon as they have resumed activity (snails not stuck to the walls of the box and which are beginning to move about), the snails shall be transferred to a test container (7.1) that has been moistened with water (6.1). The bottom of this box either is covered with absorbent paper that has also been moistened, or can contain some test substrate (6.3) moistened to 50 % to 60 % of its water-holding capacity. Between waking and the start of the test (2 d to 3 d), the snails shall be fed (6.4).

6.3 Test substrate.

6.3.1 General

According to the objectives of the study, either an artificial soil (see 6.3.2) or a suitable natural soil (see 6.3.3) is used as test substrate. The test substrate can be used dry or raw (i.e. without dehydration prior to use for natural soil).

Artificial soil may be used as a control and dilution substrate to assess the effect of a substance or of a preparation, or to compare different soils or waste, or to determine the effects of a contaminated soil.

Natural soil (field soil) may be used as a control and dilution substrate in order to assess, for example, the effect of the incorporation of wastewater treatment plant sludge into the field soil or to test the effect of a contaminated soil (in this case an uncontaminated soil comparable to the soil sample to be tested ought to be used).

6.3.2 Artificial soil

The artificial soil shall have the following composition (as defined in ISO 11268-1).

See Table 1.

Table 1 — Composition of artificial soil

Composition	Percentage expressed in dry mass
Sphagnum peat air-dried and finely ground (2 ± 1) mm without any visible plant remains.	10
Kaolinite clay, preferably containing not less than 30 % kaolinite.	20
Air-dried industrial quartz sand (predominantly fine sand with more than 50 % by mass of particle size 0,05 mm to 0,2 mm).	Approximately 69 (depending on the amount of $CaCO_3$ needed).
Calcium carbonate ($CaCO_3$, pulverised, analytical grade) to bring the pH of the wetted artificial soil to 6,0 ± 0,5.	Approximately 0,3 to 1,0

The artificial soil shall be prepared, at least 2 d prior to starting the test, by mixing the dry constituents listed above thoroughly in a large-scale laboratory mixer. The amount of calcium carbonate required might vary, depending on the properties of the individual batch (mainly the peat) and should be determined by measuring subsamples immediately before 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 one or 2 d before starting the test by adding deionized water to obtain half of the required final water content of 50 % to 60 % of the maximum water-holding capacity.

The pH value shall be measured in accordance with ISO 10390. 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. The maximum water-holding capacity of the artificial soil shall be determined in accordance with ISO 11274 or ISO 11269-2:2012, Annex C.

6.3.3 Natural soil

Determine the following parameters on the selected natural soil which shall be sieved through a 4-mm square mesh sieve to remove large fragments:

— pH, according to ISO 10390;

— water-holding capacity, according to ISO 11274 or ISO 11269-2:2012, Annex C;

— water content, according to ISO 11465;

— content of organic matter, according to ISO 10694.

It is also recommended to determine the cation exchange capacity, according to ISO 11260.

6.4 Feed.

The feed shall be provided in the form of flour at its natural moisture content (5 % to 10 %).

In order to obtain sufficient growth, it is recommended to carry out the tests with a flour-based feed comprising cereals, forage, mineral salts and vitamins which properly covers the needs of the snails). An example of feed composition is given in Annex C.

7 Apparatus

Use ordinary laboratory apparatus and the following.

7.1 Test containers.

Disposable mouse boxes made of transparent polystyrene) or any other container having a volume of approximately 1,6 l [advised approximate dimensions: 24 cm (length) × 10,5 cm (width) × 8 cm (height)].

7.2 Feed containers.

Petri dishes, approximately 5,5 cm in diameter and approximately 1 cm in height or any other containers of equivalent dimensions.

7.3 Calliper rule, having a precision of 0,1 mm.

7.4 Balances.

One analytical balance having a precision of at least 1 mg. Two other balances, one having a precision of 0,1 g, another having a precision of 1 g.

8 Storage and preparation of the samples

8.1 Soil to be tested

The soil samples received at the laboratory shall be stored in accordance with ISO 18400-206.

The soil sample submitted for testing shall be sieved through a 4-mm square mesh sieve to remove coarse fragments.

For each soil, the same characteristics than for natural soil (see 6.3.3) that can be used as control or dilution substrate, shall be determined.

8.2 Waste material

The samples of waste material received at the laboratory shall be stored in accordance with EN 14735 [less than 2 months at (4 ± 3) °C].

For conducting the tests, the grading of the waste shall be less than 4 mm. Where this condition is not fulfilled, the particle size of the waste material shall be reduced so that all of the particles pass through a 4-mm square mesh sieve.

9 Procedure

9.1 Preparation of the test

9.1.1 Selection of the concentrations to be tested

9.1.1.1 Preliminary test

This test is performed within a wide range of concentrations.

— Four concentrations of the substance or preparation and one control (e.g. 0 mg/kg; 50 mg/kg; 100 mg/kg; 500 mg/kg and 1 000 mg/kg of test substrate) with five snails per concentration and per container. The preliminary test may be conducted without replication.

— Four percentages of the matrix under examination and one control (e.g. 0 %; 12,5 %; 25 %; 50 %; 100 %) with five snails per percentage and per container. The preliminary test may be conducted without replication.

9.1.1.2 Definitive test

Select a range of at least five concentrations of the test substance, preparation or matrix according to a geometric progression, so as to cover and extend beyond the range of those concentrations or percentages which in the preliminary test did not have any effect on the growth or which inhibited it completely. The ratio of this geometric progression shall preferably not exceed 2.

If the ratio exceeds 2, it is necessary to have available two concentrations for which the observed effect is between 10 % and 90 %.

For the definitive test, three replicates are carried out per concentration.

9.1.2 Preparation of test mixtures

9.1.2.1 General

The test mixture (see 3.3) is made up of test substrate and of test substance, preparation or matrix. Prepare enough test mixture in order to cover the bottom of the test container with a layer of the test mixture of at least 1 cm.

If the test substance is used in the raw state (i.e. without dehydration prior to use), take into account its moisture rate so as to express the concentrations in milligrams of substance or of preparation per kilogram of dry test substrate and, for the matrixes, in mass percentage of matrix (expressed in dry mass) in the test mixture (expressed in dry mass).

9.1.2.2 Water-soluble or emulsifiable substances and preparations

For each examined concentration, dissolve the appropriate quantity of test substance or preparation required for obtaining the desired concentration in the same water (6.1) used for moistening the test substrate. Spray the solution over the dry or raw test substrate (6.3), then mix carefully.

The final test mixture shall have a moisture content corresponding to 50 % to 60 % of its total water-holding capacity (determined according to ISO 11274 or according to ISO 11269-2:2012, Annex C).

Measure the pH for each test concentration according to ISO 10390.

Proceed likewise for the control treatment apart from the addition of test substance or preparation.

Continue the test as specified in 9.2.

9.1.2.3 Water-insoluble substances and preparations, but soluble in organic solvents

Dissolve the quantity of test substance or preparation required for obtaining the desired concentration into a volatile solvent (e.g. methanol or acetone). Spray the obtained solution over the dry or raw test substrate (6.3). Carefully mix the totality and let the organic solvent to evaporate under a fume cupboard for 24 h.

Moisten the mixture with water (6.1) up to 50 % to 60 % of its total water-holding capacity (determined according to ISO 11274 or according to ISO 11269-2:2012, Annex C), then mix carefully.

Measure the pH for each test concentration according to ISO 10390.

Proceed likewise for the control treatment apart from the addition of test substance or preparation.

Continue the test as specified in 9.2.

9.1.2.4 Substances and preparations insoluble in both water and organic solvents

For a substance or preparation that is insoluble in a volatile solvent, prepare a mixture of 10 g of industrial quartz sand (see 6.3.2) (previously sampled from the quantity of sand required for the preparation of the test substrate) and of the quantity of test substance or preparation required in order