INTERNATIONAL STANDARD

ISO 15952

First edition
2006-02-15

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*

Reference number
ISO 15952:2006(E)

PDF disclaimer

This PDF file may contain embedded typefaces. In accordance with Adobe's licensing policy, this file may be printed or viewed but shall not be edited unless the typefaces which are embedded are licensed to and installed on the computer performing the editing. In downloading this file, parties accept therein the responsibility of not infringing Adobe's licensing policy. The ISO Central Secretariat accepts no liability in this area.

Adobe is a trademark of Adobe Systems Incorporated.

Details of the software products used to create this PDF file can be found in the General Info relative to the file; the PDF-creation parameters were optimized for printing. Every care has been taken to ensure that the file is suitable for use by ISO member bodies. In the unlikely event that a problem relating to it is found, please inform the Central Secretariat at the address given below.

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

Published in Switzerland

Contents

Page

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.

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.

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.

ISO 15952 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

Introduction

Because of the limited amount of data available concerning toxicity of contaminants on soil organisms, the problems of assessing 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 [5]] and three other standards assess sublethal effects of soil contaminants on reproduction (earthworms [2], Collembola [1], *Enchytraeidae* [3]). 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 [33].

Snails are pertinent ecological indicators for assessing the quality of soils [15], 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.) [6], [17], [26]. 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) [6], [17], [28]. 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 [8, 10 to 14, 16, 17, 19, 21, 26, 27, 35 to 43]; their ecological and physiological characteristics are well-known [6], [9], [29]; and they are now easy to breed under controlled conditions [19], [23, [29]. Their susceptibility to common contaminants of their environment has been demonstrated [10 to 15, 18 to 27, 32, 33, 36 to 42].

This International Standard describes a method for determining the effects on survival and growth of young snails of substances, preparations, 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”). 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 [9].

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 [6], [26]. So, for specific testing purposes (evaluation of the toxicity of a pesticide, for example), another test design, which is focussed on exposure via food uptake, is optionally available (Annex F and Reference [4]).

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

1 Scope

This International Standard specifies a semi-static method for the determination of 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 or preparations;

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

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 volatile substances, i.e. substances for which the Henry constant, H, or the air/water partition coefficient is over 1, or for which the vapour pressure is over 0,013 3 Pa at 25 °C.

This test takes into account the possible change in the test substance, preparation, soil or waste material because the test mixture is prepared and renewed every 7 days during the 28-day test period.

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 10381-6, *Soil quality — Sampling — Part 6: Guidance on the collection, handling and storage of soil for the assessment of aerobic microbial processes 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 11268-1, *Soil quality — Effects of pollutants on earthworms (*Eisenia fetida*) — Part 1: Determination of acute toxicity using artificial soil substrate*

ISO 11269-2, *Soil quality — Determination of the effects of pollutants on soil flora — Part 2: Effects of chemicals on the emergence and 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*

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.

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

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

NOTE It is expressed in the form of a growth coefficient.

3.5
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.

3.6
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

3.7
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 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.

3.8
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 The NOEC is the concentration just below the LOEC.

NOTE 2 For 3.5, 3.6, 3.7 and 3.8, results are given:

— in dry mass of test substance or preparation per dry mass of the test substrate;

— 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 days to a test mixture containing the test substance, preparation or matrix at different concentrations. The test mixture is freshly prepared and renewed every 7 days.

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

The snails are fed during the test with uncontaminated food.

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

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 days with respect to the fresh mass [$EC_{50,m}$ (28 days)] 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 days)].

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

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.

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 lux to 100 lux.

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 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 by 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 box (7.1) that has been moistened with water (6.1). The bottom of this box either can be 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 to 3 days), 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 (6.3.2) or a suitable natural soil (6.3.3) is used as test substrate.

NOTE 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 assess 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 by ISO 11268-1).

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 two days 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 two days to equilibrate acidity. To determine pH and the maximum water-holding capacity, the dry artificial soil shall be pre moistened one or two days 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 according to 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 according to ISO 11274 or to Annex A of ISO 11269-2.

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 Annex A of ISO 11269-2;

— 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[1)]. 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[2)] 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 Containers for food

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.

---

1) The snail feed “Helixal” manufactured and distributed by Établissements Chays Frères, 6, rue du Collège, BP 21, 25800 Valdahon, France, or the INRA formulation snail feed manufactured and distributed by Établissements Berton SARL, Lieu-dit Berton / Départementale 23, 85510 Le Boupère, France, or the snail feed manufactured and distributed by UCAAB, rue de l'Église, BP 19, 02400 Château-Thierry Cedex, France, are examples of suitable products available on the market. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

2) The disposable transparent polystyrene mouse boxes referenced E1DBBAC001 distributed by Charles River Laboratories France, BP 0109, 69592 L'Arbresle Cedex, France, are examples of suitable products available on the market. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

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 10381-6.

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 (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 according to 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 Final 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 provoked effect is between 10 % and 90 %.

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

9.1.2 Preparation of the test mixtures

9.1.2.1 General

The test mixture (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 (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 Annex A of ISO 11269-2).

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 Annex A of ISO 11269-2), 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 (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 to obtain the desired concentration. Pour the thus obtained mixture into a container containing the dry or raw test substrate (6.3) (except the 10 g used for the contamination). Mix carefully.

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 Annex A of ISO 11269-2), 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.

9.1.2.5 Solid matrixes

Increasing proportions of test matrix are mixed to the dry or raw test substrate (6.3) (e.g. 0 %; 12,5 %; 25 %; 50 %; 100 %).

The control treatment corresponds to 0 % of test matrix, i.e. 100 % of artificial soil (6.3.2) or natural soil (6.3.3).

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 Annex A of ISO 11269-2), then mix carefully. (The added water corresponds to the volume of water required in order to rehydrate the quantity of test substrate of the mixture and to the volume of water required in order to rehydrate the quantity of matrix of the mixture.)

If it is necessary to reduce the humidity of the solid matrixes, do it by dehydration outdoors or in a drying oven at a temperature not exceeding 30 °C, in order to limit the loss of volatile products.

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

Continue the test as specified in 9.2.

9.2 Distribution of the test mixture

In preparation for the test, add sufficient test mixture (9.1.2) into the test containers (7.1) to fill the bottoms of the test containers to a depth of at least 1 cm.

NOTE If the test substrate (6.3) is artificial soil, the quantity of test mixture is about 140 g (dry mass) for each test container.

Smooth the surface of the test mixture and compact the soil slightly.

9.3 Introduction of the feed

Place the container (7.2) containing the feed (6.4) on the bottom of the test container (7.1). The feed shall be provided *ad libitum*.

9.4 Introduction of the biological reagent

Select five snails (6.2) randomly for each per test container (7.1).

9.5 Handling during the tests

9.5.1 General

Cover the containers with a transparent perforated sheet [e.g. in polyalkylmethacrylate (Plexiglas) of approximate dimensions 26,5 cm × 13,5 cm] held in place by any appropriate device during the first two weeks of the test. During the following two weeks, use, to form the lid, a second container (7.1) turned upside down. This arrangement doubles the volume of the test chamber thus avoiding a negative group effect on the growth of the snails (see Figure B.2).

NOTE The plate and the container used to cover the test containers can be perforated by 3 to 4 holes with a diameter smaller than 2 mm.

Place the test containers with the snails in conditions of the test (5). Observe them regularly and note any anomaly that could interfere with the conducting of the test.

9.5.2 Three times a week

Three times a week (for example Monday, Wednesday and Friday) perform the following operations for each test container.

— Using a spatula, regularly remove the excrement on the test mixture in order to avoid its accumulation and the development of mould.

— Clean the side walls of the container with absorbent paper moistened with water (6.1) and wash the lid with tap water, then dry it and remoisten it with water (6.1).

— Moisten the test mixture (9.1.2) by spraying it with water (6.1) so that it is at 50 % to 60 % of its water-holding capacity. To ensure that the moisture content of the test mixture remains at 50 % to 60 % throughout the test duration, it is possible to prepare a container without snails which will be weighed regularly in order to estimate the quantity of water to be sprayed into the test containers.

— Renew the feed (6.4).

NOTE It is advisable to carry out the operations described above at regular times, if possible (morning or afternoon).

Note the mortality, if any.

9.5.3 Every 7 days

If effects need to be assessed weekly (optional, see Clause 4), snails are weighed and measured every week. If not, snails are weighed and measured only at the end of the test (28 days).

Every 7 days:

— prior to changing the feed and cleaning the side walls and the lid, weigh the snails individually (with a precision of 0,1 g) and measure the shell diameter (with a precision of 0,1 mm); see Figure 1;

— renew the test mixture (9.1.2) by a freshly-prepared one.

Figure 1 — Measure of the shell diameter, i.e. the longest size that can be measured
(white arrow on the photo)

The mass of the snails may be uncertain if they have test substrate on their shell or their foot. Before each weighing, using a spatula, remove the substrate from the shell or the foot. It is possible to leave the snails to move about on the clean container lid or on slightly moist paper so that they get rid of the substrate adhering to their foot.

Note the obvious or pathological symptoms (e.g. excessive production of mucus, extended oedematous body, drooping eyestalk like those described in References [11] or [41]), or any noticeable modifications in behaviour (e.g. lethargy on the test mixture, lack of feeding), observed on the snails.

At the end of the test, measure the pH of a control container and of a container for each concentration in accordance with ISO 10390. It is recommended to measure the pH of any containers in which the mortality rate or growth rate is unusual.

Optional: at the end of the test (28 days), after final weighing and measurement of the shell diameter, let the snails of each container fasted during 48 h in moist boxes without test substrate (until they no longer excrete), then deep-freeze them in view of possibly analysing the concentration of contaminants present in their tissues or organs.

10 Reference substance

A control test is regularly conducted with a reference substance to check the occurrence of changes in the sensitivity of the test organisms.

NOTE Experience has shown that cadmium chloride is a suitable reference substance.

On a regular basis, determine the values of $EC_{50,m}$ (28 days) and $EC_{50,d}$ (28 days) of the cadmium chloride by applying the protocol described in this International Standard.

WARNING — Appropriate precautions should be taken when dealing with cadmium chloride which is a harmful substance.

The $EC_{50,m}$ (28 days) of the $CdCl_2$ shall be between 350 mg and 650 mg of Cd per kg of dry test substrate.

The $EC_{50,d}$ (28 days) of the $CdCl_2$ shall be between 500 mg and 800 mg of Cd per kg of dry test substrate.

Mention the obtained values and their obtaining dates in the test report.

Four laboratories participated in 2000 in an interlaboratory test concerning cadmium chloride. The results are given in Table 2.

Table 2 — Results of the interlaboratory test with *Helix aspersa aspersa* (substrate contamination; semi-static method)

Substance	$EC_{50,m}$ (28 days) mg Cd/kg		$EC_{50,d}$ (28 days) mg Cd/kg	
	Mean	Range	Mean	Range
$CdCl_2$	500	398 to 622	600	507 to 781

11 Calculations and expression of results

11.1 Calculations

For each concentration, determine the percentage of mortality, if any, at the time of the final test.

At the end of the test (28 days) (optional: week by week), calculate the mean masses and the mean shell diameters, and the associated standard deviations, of the snails for each container and for each concentration.

An example of a data table is given in Annex D.

For each container, calculate the biomass growth coefficient (designated $k_{GC,m}$) and the shell diameter growth coefficient (designated $k_{GC,d}$) according respectively to Equations (1) and (2).

$$k_{GC,m} = \frac{(\bar{m}_t - \bar{m}_{t0})}{\bar{m}_{t0}} \times 100 \quad (1)$$

where

$k_{GC,m}$ is the biomass growth coefficient;

$\bar{m}_{tn}$ is the mean mass of the snails per replicate at time tn, in grams (g);

$\bar{m}_{t0}$ is the mean mass of the snails per replicate at time $t0$, in grams (g).

$$k_{GC,d} = \frac{(d_{tn} - d_{t0})}{d_{t0}} \times 100 \quad (2)$$

where

$k_{GC,d}$ is the shell diameter growth coefficient;

d_{tn} is the mean shell diameter of the snails per replicate at time tn, in millimetres (mm);

d_{t0} is the mean shell diameter of the snails per replicate at time $t0$, in millimetres (mm).

For each concentration, calculate the mean percentage of biomass growth inhibition (designated $\bar{p}_{I,m}$) and of shell diameter growth inhibition (designated $\bar{p}_{I,d}$) according respectively to Equations (3) and (4).

$$\bar{p}_{I,m} = \frac{(\bar{m}_{0tn} - \bar{m}_{0t0}) - (\bar{m}'_{tn} - \bar{m}'_{t0})}{(\bar{m}_{0tn} - \bar{m}_{0t0})} \times 100 \quad (3)$$

where

$\bar{p}_{I,m}$ is the mean percentage of biomass growth inhibition;

$\bar{m}_{0tn}$ is the mean mass of the snails at time tn in the control, in grams (g);

$\bar{m}_{0t0}$ is the mean mass of the snails at time $t0$ in the control, in grams (g);

$\bar{m}'_{tn}$ is the mean mass of the snail per concentration at time tn, in grams (g);

$\bar{m}'_{t0}$ is the mean mass of the snail per concentration at time $t0$, in grams (g).

$$\bar{p}_{I,d} = \frac{(\bar{d}_{0tn} - \bar{d}_{0t0}) - (\bar{d}'_{tn} - \bar{d}'_{t0})}{(\bar{d}_{0tn} - \bar{d}_{0t0})} \times 100 \quad (4)$$

where

$\bar{p}_{I,d}$ is the mean percentage of shell diameter growth inhibition;

$\bar{d}_{0tn}$ is the mean shell diameter at time tn in the control, in millimetres (mm);

$\bar{d}_{0t0}$ is the mean shell diameter at time $t0$ in the control, in millimetres (mm);

$\bar{d}'_{tn}$ is the mean shell diameter per concentration at time tn, in millimetres (mm);

$\bar{d}'_{t0}$ is the mean shell diameter per concentration at time $t0$, in millimetres (mm).

11.2 Expression of results

For each concentration and at various times of measurement, present the percentage of mortality for each replicate, and for the parameters masses and diameter of shell:

— the calculated mean values and the standard deviation per replicate;

— the growth coefficient per replicate (see examples in E.1 and E.2);

— the mean percentage of growth inhibition (see examples in E.3 and E.4);

— a graphic presentation of the results of the test, giving a clear image of the dose-response relationship for the effects on growth.

It is possible (optional) to determine the LOEC using an appropriate multiple comparison and to deduce from it the NOEC, the next lower concentration to the LOEC.

Possibly, for the test substances, preparations or matrixes for which the mortality is dose-dependent, calculate, using any appropriate statistical method, the LC_{50} (28 days) and its 95 % confidence interval (the method used for determining growth inhibition is suitable).

In order to estimate the concentration which would cause x % of growth inhibition (EC_x, for instance EC_{10}, EC_{20} or EC_{50}), it is possible to fit a model to the test results ($k_{GC,m}$ or $k_{GC,d}$). For this, the values of EC_x and the parameters characterizing this model shall, if possible, be estimated with their confidence interval (e.g. 95 %).

The fitting of the model to the data shall be assessed either using a statistical test, or by graphic representation.

It is possible to use the logistic model which is generally suitable for the statistical analysis of the produced data.

NOTE 1 This model is used in the framework of the analysis of interlaboratory test results (see Clause 10).

This model is characterized by Equation (5):

$$Y = \frac{a}{1+\left(\frac{C}{EC_{50}}\right)^b} \tag{5}$$

where

Y is the growth coefficient of the live snails per replicate ($k_{GC,m}$ or $k_{GC,d}$);

C is the concentration under test (test variable).

The following parameters, characterizing the model, are estimated from the obtained data (e.g. by the least squares method).

EC_{50} is the concentration causing 50 % of growth inhibition;

a is the growth coefficient of the snails expected in the control;

b is a parameter which characterizes the slope of the curve.

The EC_x can then be estimated by Equation (6):

$$EC_x = EC_{50}\left(\frac{x}{100-x}\right)^{\frac{1}{b}} \quad (6)$$

where

x is the sought-after effect level for calculating the EC_x (initially fixed parameter).

Other models can be used. In this case, a description of the model used shall be given in the test report. For example statistical methods are described in Reference [44].

For the substances or preparations, express the values of $EC_{50,m}$ (28 days), $EC_{50,d}$ (28 days) and, if needed , NOEC and LOEC in mg of test substance or preparation per kilogram of test substrate (expressed in dry mass).

For soils and waste, express the values of $EC_{50,m}$ (28 days), $EC_{50,d}$ (28 days) and, if needed, NOEC and LOEC in mass percentage of soil or waste in the test mixture (expressed in dry mass).

NOTE 2 It is also possible (optional) to determine other values of EC_x (for example x = 10 %, 25 %, etc.). Various parameters (EC_x, NOEC, LOEC, LC_{50}) can also be determinate at other times of measurement (7, 14 or 21 days).

12 Validity of test for *Helix aspersa aspersa*

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

— the percentage of mortality observed in the control containers is less than or equal to 10 % at the end of the test;

— the coefficient of variation calculated for the growth in the controls is less than or equal to 40 %;

— the mean mass of the snails in the controls has been multiplied at least by 4 throughout the test duration;

— the shell diameter of the snails in the controls has been multiplied at least by 1,5 throughout the test duration.

13 Test report

The test report shall make reference to this International Standard and shall include the following information:

a) all data required for the identification of the test substance, preparation or matrix [in particular the origin, the type of matrix (if it is known) as well as that of the contaminants (if they are identified) which it contains, its pH, its water-holding capacity and its moisture content];

b) the pre-treatment, if any, of the waste or soil submitted to testing;

c) the complete description of the biological reagent used (species, age, mass, shell diameter, breeding conditions, storage time and conditions prior to starting the test, supplier, awakening conditions);

d) the type of feed used (brand name, date of manufacture, storage conditions, moisture content);

e) the method of preparation of the test substrate, the pH at the start and at the end of the test for one container per concentration;

f) parameters characterizing the natural soil, if it was used as test substrate;

g) the method of preparation of the test mixtures (with indication of the solvent used in case of a substance or preparation insoluble in water);

h) the environment conditions of the test;

i) a table indicating the percentage of mortality obtained in each container, for each concentration and for the control;

j) tables indicating, for each concentration and at the different times of measurement, for the parameters mass and diameter of the shell:

   1) the calculated mean values and the standard deviation per replicate,

   2) the growth coefficient per replicate,

   3) the mean percentage of growth inhibition,

   4) a graphical representation of the results of the test, giving a clear image of the dose-response relationship for the effects on growth;

k) the results of the test according to Clause 11, the method used for the calculation of EC_{50} (28 days) and the assessment of the 95 % confidence interval, the NOEC and the LOEC if determined;

l) the results obtained with the reference substance in accordance with Clause 10 as well as the obtaining date;

m) the description of the obvious or pathological symptoms, or of the noticeable modifications in behaviour, observed on the organisms submitted to testing;

n) all operating details not specified in this document and all incidents likely to have influenced the results.

Annex A
(normative)

Static method

A.1 General

The static method can be implemented as an option in addition to the semi-static method described in the main part of this document.

The test environment, the reagents used, the apparatus, the preparation of the samples, the calculations and expression of results, the validity criteria, the test report are identical to those described for the semi-static method.

NOTE The static method is appropriate for example for substances, preparations or matrix which are not applied regularly (e.g. weekly) like pesticides sprayed once during the season, or sludge added to agricultural soil one time in a year. This could also be used for substances for which it was checked that they are stable in the conditions of the test.

A.2 Procedure

Proceed in the same manner as for the semi-static method (see Clause 9) except for the renewal of the test mixtures. The mixtures (9.1.2) are prepared and added at the start of the test, but are not renewed during the course of the 28-day test period.

A.3 Reference substance

On a regular basis, determine the values of $EC_{50,m}$ (28 days) and $EC_{50,d}$ (28 days) of the cadmium chloride (10) by applying the protocol described in the main part of this International Standard.

The $EC_{50,m}$ (28 days) of the $CdCl_2$ shall be between 500 mg and 900 mg of Cd per kg of dry test substrate.

The $EC_{50,d}$ (28 days) of the $CdCl_2$ shall be between 700 mg and 1 000 mg of Cd per kg of dry test substrate.

Mention the obtained values and their obtaining dates in the test report.

Four laboratories participated in 2000 in an interlaboratory test concerning cadmium chloride. The results are given in Table A.1.

Table A.1 — Results of the interlaboratory test with *Helix aspersa aspersa* (substrate contamination, static method)

Substance	$EC_{50,m}$ (28 days) mg Cd/kg		$EC_{50,d}$ (28 days) mg Cd/kg	
	Mean	Range	Mean	Range
$CdCl_2$	650	534 to 877	863	758 to approximately 1 000

Annex B
(informative)

Breeding technique for snails

B.1 General

The young snails used for the toxicity tests are obtained by the so-called “out-of-ground” breeding technique, because it takes place in a building within a controlled environment, which allows to have snails available throughout the whole year. The different stages are shown in Figure B.1.

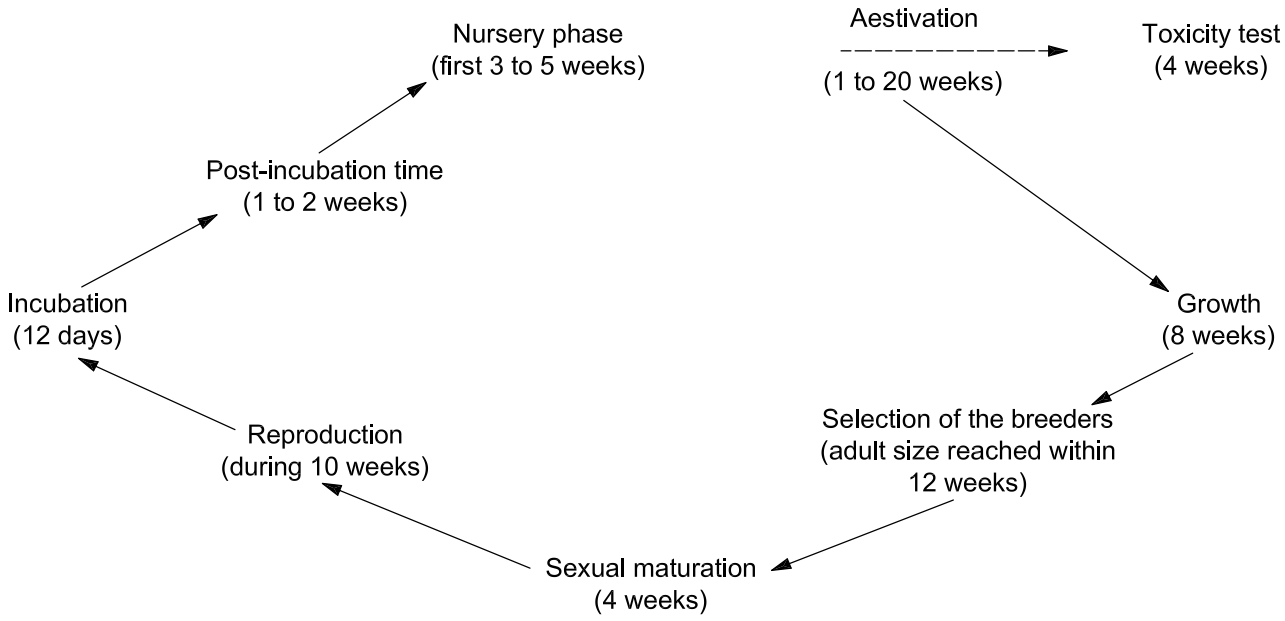


Figure B.1 — Breeding cycle under controlled conditions: example for *Helix aspersa aspersa*

As from the beginning of the nursery phase, three months are required in order to obtain snails with shells having a lip (shell edge curled over), i.e. which have reached adult size. At least one month is then necessary for these lipped shell snails to be capable of reproducing; this time corresponds to the maturation of the genital tract. The reproduction is monitored during 10 weeks. Indeed, not all the snails are ready to lay eggs in a synchronized manner and this time period allows to obtain 70 % to 120 % reproduction (the reproduction rate is defined by the ratio between the total number of obtained laying and the number of breeders at the start of reproduction).

To have homogeneous and synchronous batches of young snails and in order to avoid the maintaining of continuous rearing of snails in activity, it is possible to use breeders obtained by another rearing method, called “mixed”, in which the reproduction and incubation take place in buildings and the growth in outdoor enclosures from May to September. The future reproductive snails are collected from these enclosures and can be assigned to reproduction either directly (if they are sampled precociously, before the natural photoperiod diminishes, i.e. before August 15) or after a minimum period of hibernation of 5 months (if they are sampled from the beginning of September to mid October) at a temperature of around 7 °C.

At each of the stages of the biological cycle of the snails, it is possible to put them into dormancy (rest phase) by depriving them of moisture. These periods can range from a few months (one to three months) for young snails to a year for adults at 6 °C to 7 °C. For short periods (up to 5 months), it is possible to leave the snails under dry conditions at ambient temperature 15 °C to 20 °C in aestivation.

These techniques can be used for the breeding of different pulmonate Gastropoda.

The mainly used species for laboratory toxicity testing is currently the following:

— *Helix aspersa aspersa* Müller or snail (Petit-Gris) of which the adult mass is 8 g to 12 g. The strain maintained in a laboratory since several generations stems from the Cavaillon region (France).

B.2 Environment parameters

B.2.1 Lighting

Reproduction, incubation, nursery phase and growth take place under a long photoperiod of 18 h of lighting per 24 h (18 h of light and 6 h of dark). The lighting is supplied by daylight type fluorescent tubes [colour rendering index of 85 and luminous efficiency of 90 (quotient of luminous flux in lumen by radiant flux (electricity consumption) in watts]. Whatever the output of the light source, 10 watts to 15 watts per m^2 are required. The quantity of light measured in the breeding devices is 50 lux to 100 lux [depending on whether specific breeding cages for snails made of grey polyvinyl chloride (PVC) or transparent plastic containers are used].

B.2.2 Temperature

All the breeding stages are conducted at (20 ± 2) °C. The temperature lowers at the time of cleaning in a more or less pronounced manner depending on the season (the water temperature varies from 6 °C to 14 °C between winter and summer).

B.2.3 Hygrometry

The relative humidity is 80 % to 95 %.

If disposable plastic containers are used, moisture is ensured by moist absorbent paper laid over the bottom of the containers. During incubation, the incubation containers are placed in a room without an air humidification system.

If specific breeding cages for snails are employed, a moistening apparatus ensures the humidification of the premises where the cages are located (one moistening apparatus for approximately 150 m^3).

B.2.4 Feed

During the nursery period (corresponding to the initial three weeks to five weeks of breeding), use a “first” age feed (see 6.4 and Annex C).

NOTE The weight of the snails varies from 0,03 g on hatching to 1 g at four weeks with a “first” age feed.

During the growth and reproduction phases, use a “second” age feed (see 6.4 and Annex C).

B.2.5 Density

For reproduction in cages [Figure B.2 f)], use 45 breeders for three communicating cages, namely 30/m^2.

For reproduction in disposable mouse boxes [Figures B.2 a) to B.2 e)], use four breeders per double container, namely 30/m^2.

For growth in cages, use 50 post-nursery young snails per cage, namely 100/m^2.

For growth in disposable mouse boxes, use:

— five young snails (post-nursery) per box (one box and a flat lid) during the first two weeks of growth, namely 55/m^2;

— five snails per double container (two containers, one inverted over the other) during the following two weeks of growth, namely 38/m^2;

— four snails per double container from 8 to 16 weeks, namely 30/m^2.

For the nursery phase in cages, use 250 snails per cage, namely 500/m^2.

For the nursery phase in disposable mouse boxes, use 80 hatched young snails per container (one container and a lid), namely 888/m^2 during the first two weeks, then during the following two to three weeks, install a container turned upside down as a lid (namely 444/m^2) (two to three sorting operations after three, four and five weeks).

For the explanation of the sorting, see B.4.4.

B.3 Breeding equipment (Figure B.2)

B.3.1 Specific breeding cages for snails (BCS)

The cage material is made of grey, food-contact polyvinyl chloride. The door is made of transparent polycarbonate.

This equipment can be used for the three breeding stages: nursery, growth and reproduction. The cages are not recommended for the toxicity tests, because the water can trickle down from one level to the other and thus be at the origin of an uncontrolled contamination of the snails.

A cage has a bonding surface area of approximately 0,5 m^2 and a volume of 0,02 m^3. These cages can be equipped with a rotating axis which passes through the row of 6 or 12 cages; it is fitted with spraying nozzles having a flow rate of 1,3 l/min (for a series of 24 cages cleaned all together). In this case, the cages are said to be “semi self-cleaning”, since additional cleaning is carried out manually with a water jet after having operated the nozzle spraying system during 2 min to 5 min (depending on the size of the snails). This cage nozzle spraying system is optional; cleaning can also be carried out manually with a water jet. The cages of a same row can intercommunicate depending on whether growth or reproduction is being conducted. For reproduction, the cages communicate in threes; in the middle cage, the spraying is eliminated and a laying recipient (transparent polystyrene mouse box) is installed.

B.3.2 Egg-laying containers

If reproduction is carried out in cages (BCS), use transparent polystyrene mouse boxes (MB), having a volume equal to 1 600 cm^3, filled with compost (universal horticultural compost). In order to easily collect the eggs which have been laid, place a hard polystyrene sheet (thickness 3 cm) on the surface of the compost with 10 or so drilled holes (hole diameter 2,5 cm).

If reproduction is carried out directly in transparent polystyrene mouse boxes (two boxes, one inverted over the other), place egg-laying pots filled with compost in the bottom box. These pots are made of glass (volume 140 cm^3 to 180 cm^3). To collect the eggs which have been laid, turn over the glass pot then pick up the eggs with a spoon.

B.3.3 Incubation containers

For the incubation of several egg-layings together, use a horticultural container (approximate dimensions 34,5 cm × 21 cm × 5 cm) closed by a plastic lid with ventilation holes. On the bottom of the container, place slightly moistened absorbent paper in order to avoid dehydration of the laid eggs. Deposit a maximum of 18 to 25 layings per container, i.e. 90 g to 150 g of eggs.

For the incubation of individual layings, use Petri dishes (90 mm diameter × 15 mm high). Place one laying per dish, always on moistened absorbent paper; the cluster of eggs is opened up in order to be able to install the lid without crushing the eggs.

B.4 Maintaining of the breeding

B.4.1 Cleaning and food

Whether in BCSs or in MBs, cleaning is carried out 3 times a week (Monday, Wednesday and Friday). It is important to dispense treatment at set hours and at regular intervals, because the snails become accustomed to a rhythm of activity and feed; non compliance with this simple rule can compromise the success of the breeding.

In the cages equipped with automatic spraying, spray during 1 min to 5 min depending on the age and size of the snails. Then carry out additional manual cleaning with a water jet. Leave the cages to drip for around 15 min to 30 min, then deposit the feed on the bottom of the cages.

In the MBs, after having cleaned the walls of the box, place clean absorbent paper on the bottom and moisten it. Then deposit in the box a Petri dish containing fresh feed.

B.4.2 Reproduction

In BCSs, install the laying recipient during a maximum period of one week. On withdrawal from the cages, the laying recipients are covered over with a mouse box in order to collect those snails which are still in the egg-laying position and which will then be put back into their original batch. The layings are gathered up and deposited in the incubation container.

In MBs, proceed in the same manner, but use glass pots as egg-laying containers.

B.4.3 Incubation/hatching

During this period, the moisture content of the containers (B.3.3) should be monitored and it should be ensured that the absorbent paper does not dry up (the eggs dehydrate) or is not too moist (the eggs burst and/or become mouldy).

B.4.4 Nursery

The hatching sessions begin 12 days to 14 days after the eggs have been laid. The newly hatched snails remain first of all clustered together on the bottom of the container, then, after a few days, they start to migrate towards the lid. During this migration, they eat the paper which serves as an incubation substrate. A few days after this migration (6 days to 15 days), the newly born snails are more pigmented and can be “started off” for the initial breeding or “nursery” phase. At this stage, their mean mass is 25 mg to 40 mg for *Helix aspersa aspersa* Müller (whereas, for example, it is 25 mg to 50 mg for *Helix aspersa maxima*, another subspecies well adapted for snail breeding).

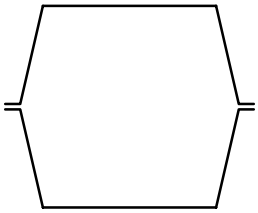
The nursery phase can take place either in BCSs or in MBs (500/m^2 and 888/m^2, then 444/m^2); carry out two to three sorting operations after three, four and five weeks of breeding.

See B.2 for the environment parameters.

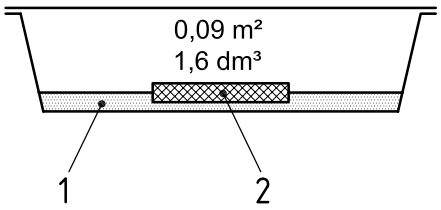
During the nursery phase, sort the snails having a mean mass equal to 0,7 g to 1,3 g in order to have the most homogeneous population possible, either for conducting toxicity tests on snails approximately one month old

(an initial sorting operation is generally carried out after three weeks of breeding; next, sorting operations are conducted after four and five weeks), or for the continuation of growth without any specific treatment. This sorting operation is necessary because the population is relatively heterogeneous at the end of the nursery period; on average, 15 % to 30 % of the young snails stemming from an egg-laying remain “dwarfs” and are not kept for the remainder of the breeding process.

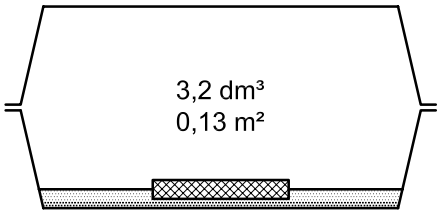
a) Single-use clear polystyrene “mouse box” — Front view [a]



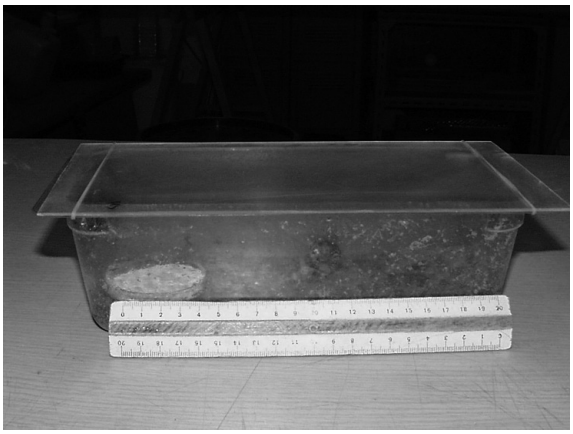
c) Single-use clear polystyrene “mouse box” — Front view [b]



b) Single-use clear polystyrene “mouse box” — Side view [a]



d) Single-use clear polystyrene “mouse box” — Side view [b]



e) Single-use clear polystyrene “mouse box” — Photo [c]

f) BCS specific breeding cage for snails — Photo

Key

1 soil (or wet absorbent paper)

2 petri dish with feed

[a] In Figures B.2 a) and B.2 b), the transparent Plexiglas cover is held in place by two rubber bands (weeks 1 and 2 of the test). The volume is 1,6 m^3.

[b] In Figures B.2 c) and B.2 d), the flat cover is replaced by another box up-side down (weeks 3 and 4 of the test). The volume is 3,2 dm^3.

[c] Photo of a “mouse box” which contains 5 snails for the test. The cover is held in place by two rubber bands.

Figure B.2 — Single-use clear polystyrene “mouse box” and BCS specific breeding cages for snails

Annex C
(informative)

Example of composition of snail feed

Table C.1 — Composition of the "Helixal" feed

Feed	Proteins	Crude fat content	Total cellulose	Total ash	Ca	Vitamins U.I./kg			Metals mg/kg					
	g/100 g					A	D3	E	Cu	Zn	Cd	Pb	Cr	Ni
"First age" feed	13,4	4,3	2,5	34,6	12,1	15 000	2 000	20	13	65	0,09	1,27	6,2	0,12
"Second age" feed	15,7	7,38	1,8	31	10,3	20 500	3 900	39	30	137	0,16	0,94	9,3	0,12

Annex D
(informative)

Example of table of data

Table D.1 — Example of data

Test of (date of start and end of testing)										
Conducted with one month old young snails in aestivation since (date); duration of aestivation =										
Conducted with “second age” feed dated (date of manufacture)										
Nursery phase conducted with “first age” feed dated (date of manufacture)										
m: fresh mass; d: largest diameter of the shell										
	Date		**Date**		**Date**		**Date**		**Date**	
Concentration	t = 0		t = 1 week		t = 2 weeks		t = 3 weeks		t = 4 weeks	
	m g	d mm	m g	d mm	m g	d mm	m g	d mm	m g	d mm
Replicate 1										
Standard deviation										
Mean										
Replicate 2										
Standard deviation										
Mean										
Replicate 3										
Standard deviation										
Mean										
Mean standard deviation of the replicates										
Mean of the replicates										

Annex E
(informative)

Example of results with *Helix aspersa aspersa*

Table E.1 — Example of growth coefficients of the biomass of *Helix aspersa aspersa*

	Mean mass g					Growth coefficient $k_{GC,m}$			
Concentration	t = 0	1 week	2 weeks	3 weeks	4 weeks	1 week	2 weeks	3 weeks	4 weeks
Control 1	0,98	1,72	2,86	4,12	5,74	76	192	320	486
Control 2	1,04	1,88	3,08	4,22	5,84	81	196	306	462
Control 3	1,00	1,72	2,98	4,34	5,62	72	198	334	462
Mean for controls	1,01	1,77	2,97	4,23	5,73	75	194	319	467
197 µg Cd/g Repl 1	1,04	1,92	2,84	3,96	5,28	85	173	281	408
197 µg Cd/g Repl 2	1,00	2,28	3,34	4,08	5,30	128	234	308	430
197 µg Cd/g Repl 3	0,94	1,84	3,06	4,32	5,72	96	226	360	509
Mean 197 µg Cd/g	0,99	2,01	3,08	4,12	5,43	103	211	316	448
296 µg Cd/g Repl 1	0,98	1,94	2,54	3,42	4,50	98	159	249	359
296 µg Cd/g Repl 2	1,00	2,16	3,34	4,42	5,76	116	234	342	476
296 µg Cd/g Repl 3	1,04	1,60	2,30	3,08	4,14	54	121	196	298
Mean 296 µg Cd/g	1,01	1,90	2,73	3,64	4,80	88	170	260	375
444 µg Cd/g Repl 1	1,02	1,80	2,32	3,04	3,46	76	127	198	239
444 µg Cd/g Repl 2	1,02	1,36	2,26	2,78	3,72	33	122	173	265
444 µg Cd/g Repl 3	1,02	1,54	2,36	2,86	3,52	51	131	180	245
Mean 444 µg Cd/g	1,02	1,57	2,31	2,89	3,57	54	126	183	250
667 µg Cd/g Repl 1	0,98	1,84	2,30	2,78	3,24	88	135	184	231
667 µg Cd/g Repl 2	1,02	1,66	2,04	2,26	2,76	63	100	122	171
667 µg Cd/g Repl 3	1,04	1,52	2,24	2,62	2,88	46	115	152	177
Mean 667 µg Cd/g	1,01	1,67	2,19	2,55	2,96	65	117	152	193
1 000 µg Cd/g Repl 1	1,00	1,52	1,70	2,16	2,52	52	70	116	152
1 000 µg Cd/g Repl 2	0,96	1,60	1,92	2,32	2,66	67	100	142	177
1 000 µg Cd/g Repl 3	1,00	1,32	2,20	2,18	2,52	32	120	118	152
Mean 1 000 µg Cd/g	0,99	1,48	1,94	2,22	2,57	49	96	124	160

Table E.2 — Example of growth coefficient of shell diameter of *Helix aspersa aspersa*

	Mean diameter mm					Growth coefficient $k_{GC,d}$			
Concentration	t = 0	1 week	2 weeks	3 weeks	4 weeks	1 week	2 weeks	3 weeks	4 weeks
Control 1	15,51	18,75	22,75	25,49	28,65	21	47	64	85
Control 2	15,70	18,47	22,15	25,91	29,35	18	41	65	87
Control 3	15,27	18,92	22,64	25,46	27,83	24	48	67	82
Mean for controls	15,49	18,71	22,52	25,62	28,61	21	45	65	85
197 µg Cd/g Repl 1	15,47	18,77	21,92	24,43	26,98	21	42	58	74
197 µg Cd/g Repl 2	15,54	18,67	21,63	24,56	26,92	20	39	58	73
197 µg Cd/g Repl 3	15,66	19,65	22,32	25,87	27,73	25	43	65	77
Mean 197 µg Cd/g	15,56	19,03	21,96	24,95	27,21	22	41	60	75
296 µg Cd/g Repl 1	15,47	18,72	20,75	23,05	25,10	21	34	49	62
296 µg Cd/g Repl 2	15,52	18,70	22,47	25,79	27,71	20	45	66	79
296 µg Cd/g Repl 3	15,67	18,41	20,37	22,68	23,83	17	30	45	52
Mean 296 µg Cd/g	15,55	18,61	21,20	23,84	25,55	20	36	53	64
444 µg Cd/g Repl 1	16,17	18,29	19,89	21,53	23,27	13	23	33	44
444 µg Cd/g Repl 2	15,41	17,07	19,04	21,00	22,55	11	24	36	46
444 µg Cd/g Repl 3	15,33	17,62	19,47	21,49	22,23	15	27	40	45
Mean 444 µg Cd/g	15,64	17,66	19,47	21,34	22,68	13	24	36	45
667 µg Cd/g Repl 1	15,45	18,14	20,43	21,38	22,22	17	32	38	44
667 µg Cd/g Repl 2	15,44	17,08	18,27	19,98	20,64	11	18	29	34
667 µg Cd/g Repl 3	15,77	17,55	19,31	20,73	21,17	11	22	31	34
Mean 667 µg Cd/g	15,55	17,59	19,34	20,70	21,35	13	24	33	37
1 000 µg Cd/g Repl 1	15,44	17,06	18,26	20,21	20,46	10	18	31	33
1 000 µg Cd/g Repl 2	15,68	17,50	19,15	20,53	21,01	12	22	31	34
1 000 µg Cd/g Repl 3	15,57	17,04	18,95	20,08	20,97	9	22	29	35
Mean 1 000 µg Cd/g	15,56	17,20	18,79	20,27	20,82	11	21	30	34

Table E.3 — Example of mean percentages of growth inhibition of the biomass of *Helix aspersa aspersa*

Concentration	t = 0 mass g	1 week mass g	Gain in mass g	% of inhibition 1 week	2 weeks mass g	Gain in mass g	% of inhibition 2 weeks	3 weeks mass g	Gain in mass g	% of inhibition 3 weeks	4 weeks mass g	Gain in mass g	% of inhibition 4 weeks
Control	1,01	1,77	0,76	0,00	2,97	1,96	0,00	4,23	3,22	0,00	5,73	4,72	0,00
197 µg Cd/g	0,99	2,01	1,02	–34,21	3,08	2,09	–6,63	4,12	3,13	2,80	5,43	4,44	5,93
296 µg Cd/g	1,01	1,90	0,89	–17,11	2,73	1,72	12,24	3,64	2,63	18,32	4,80	3,79	19,70
444 µg Cd/g	1,02	1,57	0,55	27,63	2,31	1,29	34,18	2,89	1,87	41,93	3,57	2,55	45,97
667 µg Cd/g	1,01	1,67	0,66	13,16	2,19	1,18	39,80	2,55	1,54	52,17	2,96	1,95	58,69
1 000 µg Cd/g	0,99	1,48	0,49	35,53	1,94	0,95	51,53	2,22	1,23	61,80	2,57	1,58	66,53

Table E.4 —Example of mean percentages of inhibition of the shell diameter of *Helix aspersa aspersa*

Concentration	t = 0 diameter mm	1 week diameter mm	Gain in diameter mm	% of inhibition 1 week	2 weeks diameter mm	Gain in diameter mm	% of inhibition 2 weeks	3 weeks diameter mm	Gain in diameter mm	% of inhibition 3 weeks	4 weeks diameter mm	Gain in diameter mm	% of inhibition 4 weeks
Control	15,49	18,71	3,22	0,00	22,52	7,03	0,00	25,62	10,13	0,00	28,61	13,12	0,00
197 µg Cd/g	15,56	19,03	3,47	–7,76	21,96	6,40	8,96	24,95	9,39	7,31	27,21	11,65	11,20
296 µg Cd/g	15,55	18,61	3,06	4,97	21,20	5,65	19,63	23,84	8,29	18,16	25,55	10,00	23,78
444 µg Cd/g	15,64	17,66	2,02	37,27	19,47	3,83	45,52	21,34	5,70	43,73	22,68	7,04	46,34
667 µg Cd/g	15,55	17,59	2,04	36,65	19,34	3,79	46,09	20,70	5,15	49,16	21,35	5,80	55,79
1 000 µg Cd/g	15,56	17,20	1,64	49,07	18,79	3,23	54,05	20,27	4,71	53,50	20,82	5,26	59,91

Annex F
(informative)

Determination of the effects on growth by food contamination

F.1 Introduction

This annex describes a method for determining the effects of contaminants on growth and survival with respect to young snails after food contamination (digestive exposure).

As many parts of this method are unchanged compared to the exposure of snails by soil contamination, only the special parts for contamination by food are described in this annex.

The method described is applicable to substances and preparations.

This method does not apply to volatile substances, i.e. substances for which the Henry constant, H, or the air/water partition coefficient is over 1, or for which the vapour pressure is over 0,013 3 Pa at 25 °C.

In order to limit the evolution of the substance, of the preparation to be tested, the test mixture is periodically renewed in the test containers (semi-static test).

F.2 Principle

Juvenile land snails (usually *Helix aspersa aspersa* Müller) are fed during a period of 28 days with a feed for snails containing the test substance, preparation or matrix at different concentrations.

The test substance, preparation or matrix is mixed in with the feed in a single operation at the start of the test. The obtained test mixtures are then divided up into aliquots, deep-frozen at − 20 °C and used progressively throughout the test.

The snails are placed in a test container containing artificial soil which is not renewed during the course of the test.

F.3 Reagents

F.3.1 Test substrate

The substrate used, is artificial soil (ISO 11268-1).

F.3.2 Feed

The feed is provided in the form of flour so as to form a homogeneous mixture with the test substance or preparation. The food is used in a dry (previously dehydrated in an oven at 30 °C) or raw state (in this case, dehydrate an aliquot portion in order to assess its moisture rate).

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.

F.4 Procedure

F.4.1 Preparation of the test mixtures

F.4.1.1 General

The test mixture made up of feed and of test substance or preparation is prepared in a single operation at the start of the test, then share in aliquot portions in the containers for test mixtures. Cover the containers for test mixture, then place them at the freezer at a temperature of − 20 °C in order to limit the evolution of the substance or the preparation to be tested. The aliquot portions of the test mixture are progressively used during the test (envisage three aliquot portions of test mixture a week and per replicate, i.e. 12 aliquot portions by replicate for the duration of the test).

CAUTION — If the feed is used in the raw state, take into account its moisture rate so as to express the concentrations in milligrams of substance or of preparation per kilogram of dry feed.

F.4.1.2 Water-soluble or emulsifiable substances and preparations

For each examined concentration, dissolve in water (6.1) the quantity of test substance or preparation required for obtaining the desired concentration. Spray the obtained solution over the dry or raw feed (F.3.2). Mix carefully. The final moisture content of the test mixture is a mass fraction of 20 %.

Proceed likewise for the control test by moistening, with water (6.1), the dry or raw feed (F.3.2) so that the final moisture content of the mixture is equal to a mass fraction of 20 %.

For the final test, prepare at least 200 g of test mixture (dry mass) for each concentration (quantity required for feeding three replicates of five snails during 28 days). Prepare 36 aliquot portions of the test mixture according to the quantities indicated in Table F.1.

Table F.1 — Preparation of the aliquot portions per tested concentration for the final test

Week	Number of required aliquot portions	Approximate dry mass of each aliquot portion g
1st week	9	3
2nd week	9	4
3rd week	9	7
4th week	9	8

Store the test mixtures as indicated in F.4.1.1 and continue the test as specified in F.4.2.

F.4.1.3 Water-insoluble substances and preparations, but soluble in organic solvents

Dissolve in a volatile solvent (e.g. methanol or acetone) the quantity of test substance or preparation required for obtaining the desired concentration. Spray the obtained solution into a container containing the dry or raw feed (F.3.2). Carefully mix the totality and leave the organic solvent to evaporate under a fume cupboard 24 h.

Spray with water (6.1) so that the final moisture content of the test mixture is equal to a mass fraction of 20 %. Mix carefully.

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

For the final test, prepare at least 200 g of test mixture (dry mass) for each concentration (quantity required for feeding three replicates of five snails during 28 days).

Prepare 36 aliquot portions of the test mixture according to the quantities indicated in Table F.1.

Store the test mixtures as stated in F.4.1.1 and continue the test as specified in F.4.2.

F.4.1.4 Substances and preparations insoluble in both water and organic solvents

In the case of a substance or preparation that is insoluble in a volatile solvent, prepare a mixture of 5 g of industrial quartz sand (F.3.1) and of the quantity of test substance or preparation required in order to obtain the desired concentration. Pour the obtained mixture into a container containing the dry (F.3.2) (195 g of dry feed) or raw food. Mix carefully.

Spray with water (6.1) so that the final moisture content of the test mixture is equal to a mass fraction of 20 %. Mix carefully.

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

For the final test, prepare at least 200 g of test mixture (dry mass) for each concentration (quantity required for feeding three replicates of five snails during 28 days).

Prepare 36 aliquot portions of the test mixture according to the quantities indicated in Table F.1.

Store the test mixtures as stated in F.4.1.1 and continue the test as specified in F.4.2.

F.4.2 Distribution of the test substrate

At the start of the test, distribute the test substrate (F.3.1) into the test containers at the rate of 140 g of dry test substrate per container. Moisten the substrate with ultra pure or distilled water up to 50 % to 60 % of its total water-holding capacity.

Mix carefully, smooth the soil surface and compact the soil slightly.

At the start of the test, measure the pH of the substrate of one container according to ISO 10390.

F.4.3 Introduction of the test mixture

Place the container containing the test mixture (F.4.1), the latter having been previously defrosted (at least 1 h at room temperature), on the bottom of the test container.

F.4.4 Handling during the tests

Same as effects on growth by soil contamination.

Three times a week (for example, Monday, Wednesday and Friday) perform the following operation for each test container:

— renew the test mixture (F.4.1) complying with the quantities indicated in Table F.1.

F.5 Reference substance

Five laboratories participated in 2000 in an interlaboratory test concerning cadmium chloride. The results are given in Table F.2.

Table F.2 — Results of the interlaboratory test with *Helix aspersa aspersa* (food contamination)

Substance	$EC_{50,m}$ (28 days) mg Cd/kg dry feed		$EC_{50,d}$ (28 days) mg Cd/kg dry feed	
	Mean	Range	Mean	Range
$CdCl_2$	106	68 to 139	133	102 to 159

F.6 Expression of results

For the substances or preparations, express the values of $EC_{50,m}$ (28 days), $EC_{50,d}$ (28 days) and, if needed, NOEC and LOEC in milligrams of test substance or preparation per kilogram of dry feed.

Annex G
(informative)

Test performance with other snail species

Other snails than *Helix aspersa aspersa* Müller may be used as soon as they can be reared under laboratory conditions, but the test procedure ought to be modified to provide suitable test conditions and the validity criteria ought to be adapted.

Potential candidates are

— *Helix aspersa maxima* [19], [23], [24], [27],

— *Helix lucorum* [6], [19],

— *Helix pomatia* [6], [8], [16], [17], [35],

— *Helix engadensis* [42],

— *Arianta arbustorum* [7], [8], [16], [17], [30], [31], [34], [35],

— *Cepaea nemoralis* [17], [30],

— *Cepaea hortensis* [17], [30], [43].

Therefore, the most important criterion for the selection of another snail genus or species than *Helix aspersa aspersa* Müller is ecological relevance. Other formal reasons may also exist, e.g. the fact that *H. aspersa aspersa* Müller cannot be imported in rare countries where it does not occur or where some other species has been ordered by the operator in advance.

Bibliography

[1] ISO 11267:1999, *Soil quality — Inhibition of reproduction of Collembola (*Folsomia candida*) by soil pollutants*

[2] ISO 11268–2:1998, *Soil quality — Effects of pollutants on earthworms (*Eisenia fetida*) — Part 2: Determination of effects on reproduction*

[3] ISO 16387, *Soil quality — Effects of pollutants on Enchytraeidae (*Enchytraeus sp.*) — Determination of effects on reproduction and survival*

[4] NF X 31-255-2:2001, *Soil quality — Effects of contaminants on juvenile land snails (Helix aspersa) — Part 2: Determination of the effects on growth by soil contamination*

[5] NF X 31-260:1999, *Soil quality — Effects of pollutants on insect larvae (Oxythyrea funesta) — Determination of acute toxicity using artificial soil substrate*

[6] BARKER, G.M. *The Biology of terrestrial molluscs*. Barker, G.M.(Ed). Publisher: C.A.B. International. Wallingford, U.K., 2001, pp. 331-355

[7] BAUR, B and BAUR, A. Reduced reproductive compatibility in *Arianta arbustorum* (Gastropoda) from distant populations. *Heredity,* 1992, **69**, pp. 65-72

[8] BERGER, B. and DALLINGER, R. Terrestrial snails as quantitative indicators of environmental pollution. *Environmental Monitoring and Assessment*, 1993, **25**, pp. 65-84

[9] CHEVALLIER, H. La variabilité de l'escargot Petit-Gris *Helix aspersa* Müller. *Bulletin du Muséum national d'histoire naturelle*, 3ème série, n° 448, *Zoologie*, 1977, **311**, pp. 425-442

[10] COEURDASSIER, M., GOMOT-DE VAUFLEURY, A. and BADOT, P.M. Dose-dependent growth inhibition and bioaccumulation of hexavalent chromium in the land snail *Helix aspersa aspersa*. *Environmental Toxicology and Chemistry*, 2000, **19** (10), pp. 2571-2578

[11] COEURDASSIER, M., SAINT-DENIS, M., GOMOT-DE VAUFLEURY, A., RIBERA, D. and BADOT, P.M. The garden snail (*Helix aspersa*) as bioindicator of organophosphorus exposure: effects of dimethoate on survival, growth and acetylcholinesterases activity. *Environmental Toxicology and Chemistry*, 2001, **20**, pp. 1951-1957

[12] COEURDASSIER, M., GOMOT-DE VAUFLEURY, A. and BADOT, P.M. Laboratory and field methods to use terrestrial land snails as bioindicator of pollution. *23rd SETAC North-America Congress, 16-20 November 2002, Salt Lake City, Utah, USA,* 2002

[13] COEURDASSIER, M., SAINT-DENIS, M., GOMOT-DE VAUFLEURY, A., RIBERA, D., BADOT, P. M. The garden snail (*Helix aspersa aspersa*) as a bioindicator of organophosphorus exposure: effects of dimethoate on survival, growth and acetylcholinesterase activity. *Environmental Toxicology and Chemistry*, 2001, **20**, pp. 1951-1957

[14] COEURDASSIER, M., GOMOT-DE VAUFLEURY, A., LOVY, C. and BADOT, P.-M. Is the epithelial cadmium uptake from soil important in bioaccumulation and toxic effects for snails. *Ecotoxicology and Environmental Safety*, 2002, **53**, pp. 425-431

[15] CORTET, J., GOMOT-DE VAUFLEURY, A., POINSOT-BALAGUER, N., GOMOT, L., TEXIER, C. and CLUZEAU, D. The use of invertebrate soil fauna in monitoring pollutants effects. *European Journal of Soil Biology*, 1999, **35**(3), pp. 115-134

[16] DALLINGER, R. and WIESER, W. Patterns of accumulation, distribution and liberation of Zn, Cu, Cd and Pb in different organs of the land snail *Helix pomatia* L. *Comp. Biochem. Physiol.*, 1984, **79C**, pp. 117-124

[17] DALLINGER, R., BERGER, B., TRIEBSKORN, R., KÖHLER, H.. Soil biology and ecotoxicology. In: *The Biology of terrestrial molluscs*. Chap. 9. Editor: G.M. Barker. Publisher: C.A.B. International. Wallingford, U.K., 2001, pp. 331-355

[18] GOMOT-DE VAUFLEURY, A. and COEURDASSIER, M. Normalisation of a new protocol using snails to evaluate the effects of pollutants. 11th Annual Meeting SETAC Europe, Madrid, Spain, 6-10 May 2001

[19] GOMOT, A. Contribution à l'étude de la croissance d'escargots du genre *Helix*: influence de facteurs de l'environnement. Nutrition et composition biochimique. Contrôle neuro-endocrine. *Doctorat Sciences de la Vie*, n° 398, Université de Besançon, 1994

[20] GOMOT, A. Dose-dependent effects of cadmium on the growth of snails in toxicity bioassays. *Archives of Environmental Contamination and Toxicology*, 1997, **33**, pp. 209-216

[21] GOMOT, A. Effet des métaux lourds sur le développement des escargots. Utilisation des escargots comme bioindicateurs de pollution par les métaux lourds pour la préservation de la santé de l'homme. *Bulletin de l'Académie nationale de médecine de Paris*, 1997, **181**, pp. 59-75

[22] GOMOT-DE VAUFLEURY, A. Standardised growth toxicity testing (Cu, Zn, Pb and pentachlorophenol) with *Helix aspersa*. *Ecotoxicology and Environmental Safety*, 2000, **46**, pp. 41-50

[23] GOMOT-DE VAUFLEURY, A. Regulation of growth and reproduction. In: *The Biology of terrestrial molluscs*. Chap. 9. Editor: G.M. Barker. Publisher: C.A.B. International. Wallingford U.K., 2001, pp. 331-355

[24] GOMOT-DE VAUFLEURY, A. and BISPO, A. Methods for toxicity assessment of contaminated soil by oral or dermal uptake in land snails.1. Sublethal effects on growth. *Environmental Sciences & Technology*, 2000, **34**, pp. 1865-1870

[25] GOMOT-DE VAUFLEURY, A. and KERHOAS, I. Effects of cadmium on the reproductive system of the land snail *Helix aspersa*. *Bulletin of Environmental Contamination and Toxicology*, 2000, **64**, pp. 434-442

[26] GOMOT-DE VAUFLEURY, A. and PIHAN, F. Growing snails used as sentinels to evaluate terrestrial environment contamination by trace elements. *Chemosphere*, 2000, **40**, pp. 275-284

[27] GOMOT-DE VAUFLEURY, A. and PIHAN, F. Methods for toxicity assessment of contaminated soil by oral or dermal uptake in land snails: metal bioavailability and bioaccumulation. *Environmental Toxicology and Chemistry*, 2002, **21**, pp. 820-827

[28] GOMOT, A., GOMOT, L., BOUKRAA, S. and BRUCKERT, S. Influence of soil on the growth of the land snail *Helix aspersa*. An experimental study of the absorption route for the stimulating factors. *Journal of Molluscan Studies*, 1989, **55**, pp. 1-8

[29] GOMOT, L. and DERAY, A. Les escargots. *La Recherche*,1987, **186**, pp. 302-311

[30] HEIM, M., OEHLMANN, J., SCHULTE-OEHLMANN, U., MARKERT, B. Entwicklung eines terrestrischen biotests mit Scnecken. In: *Ökotoxikologie – ökosystemare Ansätze und Methoden*. OEHLMAN, J. und MARKERT, B. (Eds). Landsberg: ecomed, 1999

[31] HEIM, M., OEHLMANN, J., MARKERT, B. Use of snails for soil quality assessment. Global Environmental Issues in the 21st Century: Problems, Causes and Solutions, 3rd SETAC World Congress, 21 – 25 May 2000, Brighton, UK

[32] ISMERT, M., GUILLEMAIN, C., OSTER, T., BAGREL, D. Phase I and phase II enzyme activities in snail (*Helix aspersa*): sensitivity to atmospheric contamination by naphthalene. *17th International Symposium On Polycyclic Aromatic Compounds, Bordeaux, France*, 2000

[33] LASKOWSKI, R. et HOPKIN, S.P. Effect of Zn, Cu, Pb, and Cd on fitness in snails (*Helix aspersa*). *Ecotoxicology and Environmental Safety,* 1996, **34**, pp. 59-69

[34] LEDERGERBER, S., BAUR, A. and BAUR, B. Growth of juvenile *Arianta arbustorum* (Linnaeus, 1758) fed on white clover (*Trifolium repens* L.) grown under ambient or elevated atmospheric CO2. *Folia malacologia*, 1999, **7**, pp. 171-175

[35] RABITSCH, W.B. Metal accumulation in terrestrial pulmonates at a lead/zinc smelter site in Arnoldstein, Austria*. Bull. Environ. Contam. Toxicol.*, 1996, **56**, pp. 734-741

[36] RUSSELL, L.K., HAVEN DE, J.J. and BOTTS, R.P. Toxic effects of cadmium on the garden snail (*Helix aspersa*). *Bulletin of Environmental Contamination and Toxicology,* 1981*,* **26**, pp. 634-640

[37] SCHEIFLER, R., GOMOT-DE VAUFLEURY, A., TOUSSAINT, M.-L. and BADOT, P.-M. Transfer and effects of cadmium in an experimental food chain involving the snail *Helix aspersa* and the predatory carabid beetle *Chrysocarabus splendens*. *Chemosphere*, 2002, **48**, pp. 571-579

[38] SCHEIFLER, R., GOMOT-DE VAUFLEURY, A. and BADOT, P.-M. Transfer of cadmium from plant leaves and vegetable flour to the snail *Helix aspersa*: bioaccumulation and effects. *Ecotoxicology and Environmental Safety*, 2002, **53**, pp. 148-153

[39] SCHEIFLER, R., BEN BRAHIM, M., GOMOT-DE VAUFLEURY, A., CARNUS, J.-M. and BADOT, P.-M. A field method using microcosms to evaluate transfer of Cd, Cu, Ni, Pb and Zn from sewage sludge amended forest soils to *Helix aspersa* snails. *Environmental Pollution*, 2003, **122**, pp. 343-350

[40] SCHEIFLER, R., SCHWARTZ, C., ECHEVARRIA, G., GOMOT-DE VAUFLEURY, A., BADOT, P.-M. and MOREL, J.-L. "Non available" soil cadmium is bioavailable to snail: evidence from isotopic dilution technique. *Environmental Science and Technology*, 2003, **37**, pp. 81-86

[41] SCHUYTEMA, G.S., NEBEKER, A.V. and BOTTS, R.P. Effects of dietary exposure to forest pesticides on the brown garden snail *Helix aspersa* Müller. *Archives of Environmental Contamination and Toxicology,* 1994, **26**, pp. 23-28

[42] SWAILEH, K.M. and EZZUGHAYYAR, A. Dose-dependent effects of dietary Pn and Zn on feeding and growth rates of the landsnail *Helix engaddensis*. *Ecotoxicology and Environmental Safety*, 2001, **50**, pp. 9-14

[43] WILLIAMSON, P. Variables affecting body burdens of lead, zinc and cadmium in a roadside population of the snail *Cepaea hortensis* Müller. *Oecologia,* 1980, **41**, pp. 1 104-1 113

[44] ISO/TS 20281, *Water quality — Guidance on statistical interpretation of ecotoxicity data*

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

ICS 13.080.30

Price based on 33 pages