SLOVENSKI STANDARD
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Kakovost tal - Uporaba kletk s polži na terenu za oceno bioakumulacije onesnaževal (ISO/DIS 24032:2020)

Soil quality - In situ caging of snails to assess bioaccumulation of contaminants (ISO/DIS 24032:2020)

Bodenbeschaffenheit - In-situ-Käfighaltung von Schnecken zur Beurteilung der Bioakkumulation von chemischen Stoffen (ISO/DIS 24032:2020)

Qualité des sols - Encagement in situ descargots pour la mesure de la bioaccumulation de contaminants (ISO/DIS 24032:2020)

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Soil quality — *In situ* caging of snails to assess bioaccumulation of contaminants

Qualité du sol — Encagement in situ d'escargots pour la mesure de la bioaccumulation de contaminants

ICS: 13.080.30

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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

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Foreword

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For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

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

Introduction

Snails are ubiquitous soil macroinvertebrates living at the interface soil, plants and air. Those pulmonate gastropod molluscs are phytophagous and saprophagous (trophic level of primary consumers and detritivorous). They ingest vegetation and soil, crawl on the ground where they lay their eggs. Therefore, they integrate multiple sources and routes of contamination (see Annex A – Figure A.1). Snails participate in exchanges with soil and are preyed upon by various consumers (invertebrates: glow-worms, ground beetle larvae, or vertebrates: birds, small mammals such as shrews, hedgehogs and humans).

Among snail species, the recommended species is *Cantareus aspersus* O.F. Müller 1774 (synonyms: *Helix aspersa aspersa* (O.F. Müller, 1774), *Cornu aspersum;* https://inpn.mnhn.fr/espece/cd_nom/199863/tab/taxo) also known as common garden snail, brown garden snail, garden snail, land snail, nicked name in French “Petit-Gris”. This species is a stylommatophoran pulmonate gastropod molluscs of the Helicidae family, widely distributed across the world (Potts, 1975; Chevallier, 1977). This palearctic species can be acclimated to regions with different types of climate: Mediterranean, oceanic temperate, midcontinental temperate and even tropical. *Cantareus aspersus* (Müller, 1774) is of European origin and has been introduced into all parts of the world. It is now on all continents except Antarctica. On the other hand, the species is recognized as an agriculturally harmful snail in some countries and must be treated carefully.

Juvenile snails are already covered in ISO 15952 (2018) that describes how to assess *ex situ*, i.e. in laboratory conditions, toxic effect of chemicals or contaminated matrix on the survival and growth of juvenile (1 g fw).

Currently there is no standardized *in situ* bioassay allowing the assessment in the field of the transfer of contaminants from the environment to organisms of the soil fauna. Indeed, despite ISO 19204 (2017) (relative to the TRIAD approach) recommends the application of three combined lines of evidence (chemistry, ecotoxicology and ecology) and highlights the interest of bioindicators of effect and accumulation as additional tools for site-specific ecological risk assessment, few bioassays are available for this purpose. As described in Annex A of ISO 19204 (2017), measurements of bioaccumulation in plants or soil organisms are thus useful to:

— assess the effective bioavailability of soil contaminants to soil organisms;

— approach the food chain transfer and the risk of secondary poisoning of consumers.

In some cases, bioaccumulation can result in toxic effects but this is not always the case (see ISO 17402:2008).

Since farming is possible (see Annex in ISO 15952:2018), snails with a known biological past can be used on the field to analyse bioavailability of contaminants present in the habitats (soil, plants, air) by measuring their accumulation in individuals caged and exposed for a determined period of time.

C. aspersus can be used either in the field (Gomot-de Vaufleury and Pihan, 2000; Scheifler et al., 2006; Regoli et al., 2006; Gimbert et al., 2008a; Pauget et al., 2013; de Vaufleury et al., 2015; Mariet et al, 2017) or in the laboratory (Gimbert et al., 2008b; Pauget et al., 2011, 2012, 2017; Louzon et al., 2020) to assess the fate and transfer (i.e. environmental bioavailability, ISO 17402) of chemicals in soils. This soil bioindicator has been applied on numerous field sites (https://ecobiosoil.univ-rennes1.fr/ADEME-Bioindicateur/english/worksheet.php) to evaluate habitat and retention function of soils. This bioassay allows determining the bioavailability of chemicals to snails thanks to the measurement of their concentration in their visceral mass (which contain mainly the digestive gland and some other organs as described in Gomot-de Vaufleury and Pihan, 2002). The visceral mass is the main site of contaminant accumulation in snails.

This standard describes how to expose snails *in situ* for 28 days and how to prepare them until chemical analysis are performed to assess bioaccumulation in their viscera. This bioassay evaluates the transfer of contaminants from the environment to land snails.

This test is applicable in the field (e.g. contaminated sites, amended soils, soils after remediation, agricultural or other sites under concern and waste materials) by caging snails for 28 days on the studied site/soil/waste. Snails integrate chemicals of all terrestrial sources (soil, plant, air). After exposure, concentrations of chemicals are measured in the visceral mass of snails.

Optionally, the method can be used in the laboratory (*ex situ*) to evaluate bioaccumulation of chemicals of snails exposed only to soil (Annex I).

The results of a ring test performed *in situ* by six laboratories to assess the method of exposure and by four laboratories from exposure until to chemical analysis are shown in Annex H.

Soil quality — *In situ* caging of snails to assess bioaccumulation of contaminants

1 Scope

This document describes a method to assess the bioaccumulation of chemicals in snails, i.e. concentrations of metal(loid)s (ME) or organic compounds (e.g. PAHs and PCBs) accumulated in their tissues.

This document presents how to prepare snails for caging *in situ* for 28 days, the *in situ* test design and then how to collect and prepare the snails until conservation and further analysis. If a kinetic study of accumulation is necessary, sampling of snails at different time-points during exposure is possible as well (Gimbert et al., 2008a; Pauget et al., 2013; Mariet et al., 2017)

This standard excludes analytical methods: preparation (extraction and mineralization) of the samples and quantification of chemicals. These are not in the scope of the present document.

The method is applicable for soils under different uses (agricultural, industrial, residential, forests, before and after remediation, on potentially contaminated sites, etc.) and waste materials (de Vaufleury, 2015; Bourioug et al., 2015), and should be preferably used on soil with vegetation and/or humus cover.

The method is applicable subject to certain limits of temperature (frost-free period, i.e. mainly from April to October in temperate region).

Optionally (Annex A) the method can be used in the laboratory to evaluate the accumulation of contaminants (and optionally the Sum of Excess of Transfer (SET) index for ME, PAH, PCB) of snails exposed only to soil.

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 15952:2018, *Soil quality — Effects of pollutants on juvenile land snails (Helicidae) — Determination of the effects on growth by soil contamination*

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:

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

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

3.1
caging
closed microcosm allowing exposure of snails by various routes and several sources

3.2
bioaccumulation
phenomenon by which a chemical present in the medium accumulates in a living organism

Note 1 to entry: This phenomenon is observed when the rate of absorption exceeds the rate of elimination of the contaminant

3.3
inactive snails
snails without any activity, generally under dry conditions where they glue on the walls of the box in which they are placed (generally just due to a simple dried mucus ring)

3.4
aestivation
snails kept inactive, under dry conditions, at a temperature of 15 °C to 20 °C

3.5
plot
characteristic and representative sub-area of the site

Note 1 to entry: The geographical coordinates of each plot should be recorded

3.6
site
field place (or geographical entity) under study and where the microcosms will be placed to assess the bioavailability of contaminants to snails

Note 1 to entry: The site can present one or more plot(s) and land use, i.e., a field, a pasture, a forest, an industrial site, a discharge

4 Principle

Snails are caged in microcosms at the study site for 28 days. Fifteen sub-adult (5 ± 1 g of the body mass) garden snails shall be placed in each microcosm. From the end of their breeding to their placement on the soil, they can be stored in dry wooden boxes (round wooden boxes, approximately 12 cm in diameter and 4 cm in height, Figure 1 and B.1.),). They are awaken from aestivation by spraying them with water a few hours before they are placed in the microcosms. Here, they will be exposed to soil as well as plants that have grown on-site and ambient air in order to be under natural exposure conditions (climate hazards).

After exposure, the collected snails are brought back to the laboratory and starved for 48 hours. During the starvation, faeces are removed every 24 hours. Snails are then frozen at –80 °C. After thawing, the soft body is removed from the shell; the visceral mass and the foot (Annex B, Figure B.1) are separated and prepared for chemical analysis to determinate internal concentration of chemicals. Main steps are presented in Annex B.

5 Test organism and equipment

5.1 Biological material

Test organisms shall be sub-adult snails (to avoid mass change during the exposure duration and the consecutive dilution of the bioaccumulation per the mass gain during the growth or the transfers of compounds to the eggs during the reproductive stages). The recommended species is the land snail *Cantareus aspersus* (Müller, 1774) which shall be 7 to 12 weeks old, having a mean fresh mass of 5 ± 1 g (with min/max of 4/6 g).

NOTE 1 Optionally the shell diameter can be measured (mean ± SD of 25 mm ± 5 mm; min/max of 20/30 mm).

The snails shall be selected from synchronous breeding in order to form a population as homogeneous as possible with respect to mass and age. The breeding techniques for snails are described in Annex C. In summary, after a nursery and then a growth period (3 to 6 weeks followed by 4 to 6 weeks), the sub-adult snails shall be used directly or after an aestivation period that should not be more than 5 months (i.e. snail inactive, fixed on the wall of a dry box (plastic box must be avoided), in a temperature-controlled room between 15 and 20 °C). The aestivation is carried out in round wooden boxes (approximately of 12 cm in diameter and 4 cm in height; usually 15 snails per boxes, which is equal the number of snails per microcosm).

Snails shall be reared for the purpose of the project (see Annexes C and D) or be purchased from local snail farmers.

NOTE 2 The use of some other genus and/or species of *Helicidae* is possible (see examples and conditions in Annex G of ISO 15952:2018).

A control of the chemical quality of the subadult snails selected for the caging (i.e. unexposed snails) can be performed on 6 snails with respect to the initial concentrations of the chemicals of interest. These control snails can be selected at the same time as the snails used for snail caging. The analysis of the chemical quality of snails before caging can be done at the same time as the analysis of snails after exposure. It is not mandatory to make this control. Indeed, after exposure, all data are compared to the threshold guide value (TGV) (see 8.2.); however, if possible to get these data, it provides an indication that snails were uncontaminated before exposure. For chemicals for which no TGV are available, data can be compared to various values (see 8.2.2.3) among which are Csnail-t0.

The sub-adult snails used shall present usual concentrations in the visceral mass before caging (Annex E). For PAH and PCB data, as extraction are often made on fresh tissues, data of Table E.1. are in $\mu g.kg^{-1}$ fresh mass of viscera (these values can be converted in $\mu g.kg^{-1}$ dw on the basis of ≈ 15 % dry mass of the visceral mass); for metal(loids) in $mg.kg^{-1}$ dry mass of visceral mass..

5.2 Equipment

5.2.1 Microcosm

Stainless steel cylinders with 25 cm diameter and 25 cm height covered by a 0,5 or 1 cm mesh netting. An example is presented in Figure 1 and in Annex F.

NOTE 1 Other device could be used if the material that constitutes them cannot be a source of contamination; for some purpose (e.g. exposure of snails to chemicals sprayed in the field), fully screened microcosm can be used (see for example Druart et al. (2011) that used stainless steel cages of 25 × 25 × 15 cm (mesh size of grid: 1 cm) closed by a stainless steel grid of 30 × 30 cm (mesh size: 1 cm) held by four pickets (see Annex F, Figure F.2).

NOTE 2 In some cases, it could be necessary to protect the microcosm from predators or cattles (see examples in Annex F Figure F.3).

5.2.2 Netting

0,5 or 1 cm mesh netting, also stainless steel.

5.2.3 Pickets

Stainless steel picket (diameter 5 mm; length 46 to 72 cm) to maintain the mesh netting on the cage. Depending on the soil settlement or the presence of stones, the size of picket shall be adapted.

5.2.4 Pieces of tiles

See Figure 1 and Annex F.

5.2.5 Wooden storage

Inactive snails can be stored and transported before exposure in round wooden boxes (approximately 12 cm in diameter and 4 cm in height), with the snails under dry conditions, at a temperature of 15 °C to 20 °C (see Figure 1, Figure F.5 and Annex G).

5.2.6 Boxes for fasting, sampling

For the preparation of snails in the laboratory (e.g. to keep the snails before individual weighing), plastic containers (PCs) (e.g. made of transparent polystyrene or any other container having approximate dimensions: 24 cm (length) × 10,5 cm (width) × 8 cm (height)) can be used.

5.2.7 Calliper rule

For the measurement of the shell diameter, a calliper rule having a precision of 0,1 mm.

5.2.8 Balance

One analytical balance having a precision of at least 10 mg.

5.2.9 Water

Water, of purity at least deionized.

5.2.10 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 D.

5.2.11 Small material

Elastic strips to close wooden storage or boxes for fasting, sampling. Tape to label the wooden storage and boxes for fasting; indelible markers; Ziploc© bags.

6 Preparation of the organisms for the exposure

After the end of their growth (Figure C.1, growth 1, *i.e.* time needed to obtain sub-adults that reached the mass required for the test) snails shall be stored inactive in wooden box (see 3.5). Their mass will decrease during this storage period that's why in some cases (*i.e.* storage for more than 1 week) they shall be woken from aestivation few days before the start of the assay (see 6).

Depending on the duration of storage between the end of growth period (*i.e.* when reaching the mean mass requested, see 5.1.) and the start of the test in the field, snails shall or not be woken by spraying them with water into the box according to the following scenarios:

— if snails are used in the week following their weighing and distribution in homogeneous batch (15 snails for 1 microcosm) it is just necessary to awake them some hours before using in the field. They shall be sprayed with water. This facilitate their handling to remove them from the wood box and placed them in the microcosm once in the field.

— If they were stored for longer period (>1 week but < 5 months) before exposure in the field, they should be awaken and fed with snail feed (5.2.10) for 2 to 5 days in order they reach their initial mass. After being awaken by spraying water, they are placed in cages or plastic box (see Figure C.2 in Annex C) for 2-3 days, then again weighted and distributed in homogeneous batches (see example in Annex G, Table G.1, Figure G.1) used for brief storage (0 to 1 week).

The proportion of snails not woken shall be less than 20 %. As soon as they become active (snails not stuck to the walls of the box and starting to move), the snails shall be transferred into a box that has been premoistened with water.

All the snails needed for the assay shall be weighed, and distributed in distinct mass classes (e.g. group all snails from 4 g to 4,5 g, from 4,6 g to 5 g, from 5,1 g to 5,5 g, from 5,6 g to 6 g. Then, prepare group of 15 snails each as homogeneous as possible with respect to mass (same distribution of mean group mass, see example Annex G, Figure 1).

NOTE Optionally, the shell diameter could be measured.

Snails for the test shall be individually weighed and placed in wooden storage boxes; 15 individuals shall be stored per wood storage, since one microcosm shall contain 15 snails for exposure. The group of 15 snails shall be as homogeneous as possible with respect total fresh mass.

7 Exposure of the test organisms

The main steps of the bioassays are illustrated in Annex F (an example of table of data are given in Annex G, Table 1).

7.1 Beginning of exposure

Three microcosms shall be placed at each plot. Each microcosm should contain 15 snails that are exposed to soil, humus and vegetation under natural climatic conditions. Pieces of tiles shall be placed in the cage to provide a shelter and a bonding surface to snails.

The snails must be carefully removed from the wooden box, without pulling too hard to avoid braking the shell; they must not produce white mucus (like a white foam), which is a sign of mishandling.

NOTE 1 The number of microcosms per plot can be adapted depending on the number or mass of snail tissue needed for analysis. To consider soil heterogeneity in terms of intrinsic properties and contamination profiles, a minimum of 3 microcosms, per a certain plot area are used. This also allow to sample snails as described in 7.3.

NOTE 2 If there is no shade on site, a shade mesh could be placed above the netting to reduce the heat in the cage. Annex F, Figure F.4.

Once on the field, set up a microcosm on soil (remove stone to avoid space between microcosm and soil to ensure that the microcosm is sufficiently buried in the soil to avoid the nails from escaping, drive the cage in the top soil layer of 0,5 cm to 1 cm). Place the snails and the pieces of tiles used as shelters (Figure 1). Finally, cover the microcosms with the netting and fix the netting with the pickets. About 20 min are required for this step.

Figure 1 — *In situ* exposure: Active biomonitoring using microcosms where snails are exposed (from left to right: sub-adult snail, total fresh mass 4 g to 6 g); open microcosm; microcosms covered by a stainless steel netting (mesh size: 10 mm) securely fitted over the top of the microcosm by 4 pickets; microcosms on site)

7.2 End of the exposure — Starvation

All the snail from one microcosm are carefully removed and placed together, e.g. in the wood box used to store the snails before exposure.

Back in the laboratory, snails shall be cleaned, *i.e.* if necessary by removal of soil particles with a brush and water. Then, snails shall be placed for starvation in a plastic box easy to clean (e.g. as in Figure C.2). During starvation, snails shall be starved for two days (until they produce no more faeces). During this starvation period the faeces shall be removed every 12 hours to avoid that snails re-eat the faeces. It is recommended to weigh the snails at the end of exposure and after starvation before freezing.

NOTE 1 As the mass is influenced by the weather in the field, weighing the snails after starvation and an homogeneous hydration facilitates the comparisons between snails exposed under quite different meteorological conditions, or between experiments performed at different years). Optionally, the shell diameter could be measured.

Snails are then frozen at −80 °C. They can be frozen in Ziploc© bags or any other container that could be effectively closed.

NOTE 2 Optionally a −20 °C freezer can be used if no −80 °C freezer is available.

7.3 Sampling and preparation after exposure

For preparation of the visceral mass, the snails must be thawed. Depending of the temperature of the room, wait until the soft body is completely soft (without presence of ice in the body). After thawing, the snails shall be weighed, the soft body (*i.e.* foot + visceral mass) shall be removed from the shell and the visceral mass separated from the foot for analysis of chemicals (Annex B, step 3, Figure B.3).

NOTE 1 The removal of the visceral mass requires about 10 minutes for unskilled researcher and 2 minutes for skilled.

Two snails per microcosm shall be randomly sampled after 28 days of exposure. The total number of snails that shall be sampled for metal(loid)s analysis is two per microcosm, resulting in a total of six individuals per plot: three microcosms x two snail/microcosm). The remaining snails (13 snails (if no mortality occurred during exposure) could be stored frozen for further analysis.

NOTE 2 For the analysis of organic compounds, if the mass of the viscera is not sufficient for individual analysis, the visceral masses of two or more snails could be pooled to reach the required mass of sample for analysis.

NOTE 3 If only one microcosm is used on one plot (e.g. in a preliminary study), 6 snails are sampled in the microcosm.

8 Calculation and expression

8.1 General

Two different ways are currently possible: one for metal(loid)s for which guide values are available and other chemicals for which no guide value are available yet.

8.2 For metal(loid)s

8.2.1 Threshold guide value

For 14 metal(loid)s Threshold Guide Value (TGV; previously named internal concentrations of reference (CIRef), Pauget et al., 2013, 2015) have been determined in snails using the metal concentrations in snails exposed on unpolluted sites (n = 150) (see Table 1, Figure 2).

They allow to calculate the SET index (sum of the excess of transfer) to provide an evaluation of the abnormal transfer of metal(loid)s to snails. Briefly, the ME concentration in snails after 28 days exposure on the studied site are divided by the TGV for each ME to calculate the accumulation quotient (AQ); then the AQ-1 for each ME are added to provide the SET index.

Table 1 — Threshold Guide Value (TGV) of metal(loid)s in the viscera of snails after 1 month exposure on uncontaminated sites (Pauget et al., 2015). TGV are median value (see **Figure 2**)

ME	As	Cd	Co	Cu	Cr	Hg	Mo	Ni	Pb	Sb	Sn	Sr	Tl	Zn
TGV-*in situ* (mg kg^{-1})	0,307	2,27	6,676	184,7	2,01	0,198	4,428	5,249	12,9	0,076	0,058	125,7	0,259	1490

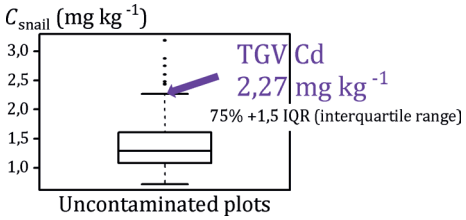

Figure 2 — Example of calculation of the TGV for Cadmium

8.2.2 Calculation of the sum of the excess of transfer of metal(loid)s: SET index

To identify the metal transfer from the environment to snails, the median of the snail's viscera concentration is compared to the TGV. If the median concentration in the snail exposed to the plot under investigation is higher than the TGV, then the soil presents an abnormal metal transfer to snail.

8.2.2.1 Calculation of the Accumulation Quotient

AQ = [Csnail-28d]/TGV for each metal(loid)s

With [Csnail-28d] = median concentration of the metal(loid) in the viscera of the 6 snails exposed on the studied plot.

An AQ > 1 identifies an excess of transfer.

8.2.2.2 Calculation of the sum of the excess of transfer of metal(loid)s: SET plot and SET site

SETplot = Σ(QA-1) and

SETsite = Σ(QA-1) / nplot

8.2.2.3 If the TGV is not available for a studied metal(loid)s, C snail-28d can be compared either to

1. the Csnail-28d of snails caged on a control site (*i.e.* uncontaminated site)
2. or to the Csnail-28d of snails reared in the laboratory during the exposure of snails on site (e.g. if it is not possible to find a plot on an uncontaminated site to serve as control)
3. or at least to the Csnail-t0

8.3 For other chemicals

For PAH, PCB, pesticides or any other chemicals for which no *in situ* TGV are available, Csnail-28d shall be compared to guide value as described in 8.2.2.3.