INTERNATIONAL STANDARD

ISO 24032

First edition
2021-12

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

Reference number
ISO 24032:2021(E)

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Published in Switzerland

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of 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 www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological characterization,* in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 444, *Environmental characterization of solid matrices,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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, and crawl on the ground where they lay their eggs. Therefore, snails 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[1)] (synonyms: *Helix aspersa aspersa*, *Cornu aspersum*) also known as common garden snail, brown garden snail, garden snail, land snail, nicked name in French "Petit-Gris" (see Annex A, Figure A.2). This species is a stylommatophoran pulmonate gastropod molluscs of the Helicidae family, widely distributed across the world[9],[28]. 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[1] 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[3] (relative to the TRIAD approach) which 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 ISO 19204:2017, Annex A, 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[2]).

Since farming is possible (see ISO 15952:2018, Annex B), 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 [10],[12],[13],[15],[19],[22],[23],[27],[29],[30] or in the laboratory [14],[18],[20],[21] 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[2)] 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 Reference [16]). The visceral mass is the main site of contaminant accumulation in snails.

This document 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.

1) Available from: https://inpn.mnhn.fr/espece/cd_nom/199863/tab/taxo.

2) Available from: https://ecobiosoil.univ-rennes1.fr/ADEME-Bioindicateur/english/worksheet.php.

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 (see 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. polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (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 [13],[19],[22].

This document excludes analytical methods. Preparation (extraction and mineralization) of the samples and quantification of chemicals 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 [8],[10], preferably 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 (see Annex I), 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

There are no normative references in this document.

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 https://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 snail
snail 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 are 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 inactive in dry wooden boxes (round wooden boxes, approximately 12 cm in diameter and 4 cm in height; see Figure 1 and Figure B.2). They are woken from aestivation by spraying them with water a few hours before they are placed in the microcosms. Here, they are 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 h. During the starvation, faeces are removed every 24 h. Snails are then frozen at –80 °C. After thawing, the soft body is removed from the shell; the visceral mass and the foot (see 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 weeks to 12 weeks old, having a mean fresh mass of (5 ± 1) g.

NOTE 1 Optionally, the shell diameter can be measured (mean ± SD of 25 mm ± 5 mm; min/max of 20 mm/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 a growth period (3 weeks to 6 weeks followed by 4 weeks 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 shall be avoided), in a temperature-controlled room between 15 °C 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 ISO 15952:2018, Annex G).

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 (C snail-t0). 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 8.2.1); 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 8.2.2.4) among which are Csnail-t0.

The sub-adult snails used shall present usual concentrations in the visceral mass before caging (see Annex E). For PAH and PCB data, as extraction are often made on fresh tissues, the 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), the data are 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 cm or 1 cm mesh netting.

An example is presented in Figure 1 and in Annex F, Figure F.1.

NOTE 1 Other devices can 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 Reference [11] that used stainless steel cages of 25 cm × 25 cm × 15 cm (mesh size of grid: 1 cm) closed by a stainless steel grid of 30 cm × 30 cm (mesh size: 1 cm) held by four pickets (see Annex F, Figure F.2)].

NOTE 2 In some cases, it can be necessary to protect the microcosm from predators or cattle (see examples in Annex F, Figure F.3) or from the sun (see Annex F, Figure F.4).

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

5.2.3 Pickets, stainless steel picket (diameter 5 mm; length 46 cm 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 B.2 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, of purity at least deionized.

5.2.10 Feed, which 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, resealable bags.

6 Preparation of the organisms for the exposure

After the end of their growth (see 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 (5.2.5.). 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 Clause 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 are woken 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 necessary to wake them some hours before using in the field. They shall be sprayed with water in the wood box. 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 periods (>1 week but < 5 months) before exposure in the field, they should be awakened and fed with snail feed (5.2.10) for 2 days to 5 days in order they reach their initial mass. After being awakened by spraying water in the wood box, they are placed in cages or plastic box (see Figure C.2 in Annex C) for 2 days to 5 days and fed. Then again weighed and distributed in homogeneous batches (see example in Annex G, Table G.1 and Figure G.1) in the wood box in which they can be stored for a brief duration (0 to 1 week) before being again awakened and disposed in the microcosms.

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 G.1).

NOTE Optionally, the shell diameter can 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.

7 Exposure of the test organisms

7.1 General

The main steps of the bioassay are illustrated in Annex F, Figures F.5 and F.6 (an example of a table of data is given in Annex G, Table G.1).

7.2 Beginning of exposure

Three microcosms shall be placed at each plot. To consider soil heterogeneity in terms of intrinsic properties and contamination profiles, a minimum of 3 microcosms, per a certain plot area is used. Each microcosm should contain 15 snails that are exposed to soil, humus and vegetation under natural climatic conditions. This is the natural way of exposure of snails. Plants, humus that cover the soil (and also soil) are a source of feeding for snails. Pieces of tiles shall be placed in the cage to provide a shelter and a bonding surface to snails.

The snails shall be carefully removed from the wooden box, without pulling too hard to avoid braking the shell; they shall 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 or in the frame of a preliminary study.

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 snails 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 (see Figure 1). Finally, cover the microcosms with the netting and fix the netting with the pickets. About 20 min are required for this step.

a) Sub-adult snail, total fresh mass 4 g to 6 g

b) Open microcosm

c) Microcosms covered by a stainless steel netting (mesh size: 10 mm) securely fitted over the top of the microcosm by 4 pickets

d) Microcosms on site

Figure 1 — In situ exposure: Active biomonitoring using microcosms where snails are exposed

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

Snails are then frozen at –80 °C. They can be frozen in resealable bags or any other container that can be effectively closed.

Optionally a –20 °C freezer can be used if no –80 °C freezer is available. A -80 °C freezer allows to kill the snails by freeze drying more rapidly. It is also required for appropriate conservation before additional biomarkers analysis.

7.4 Sampling and preparation after exposure

For preparation of the visceral mass, the snails shall 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 (see step 3 in Figure B.3).

The removal of the visceral mass requires about 10 min for unskilled operator and 2 min 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)] can be stored frozen for further analysis. It provides a safety margin in case of mortality, and also allows to obtain enough biological material if analysis of other pollutants [polychlorinated dibenzo-p-dioxins and polychlorinated dibenzo-p-furans (PCDD/Fs), rare earths, polybrominated compounds, etc.] or biomarkers are needed.

NOTE 1 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 can be pooled to reach the required mass of sample for analysis.

NOTE 2 If only one microcosm is used on one plot (e.g. in a preliminary study), six snails are sampled in the microcosm.

8 Calculation and expression

8.1 General

Two ways are currently possible: one for metal(loid)s for which guide values are available, and other chemicals for which no guide value is available at the time of publication.

8.2 For metal(loid)s

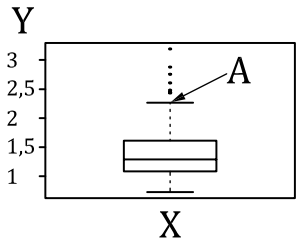
8.2.1 Threshold guide value

For 14 metal(loid)s threshold guide value [(TGV) previously named internal concentrations of reference (CIRef)[22]] 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[18],[23],[24],[25],[26].

Table 1 — Threshold guide value (TGV) of metal(loid)s in the viscera of snails after 1 month exposure on uncontaminated sites[23]

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	1 490
NOTE TGV are median value (see Figure 2).														



Key

X uncontaminated plots

Y C_{snail} ($mg.kg^{-1}$)

A TGV Cd 2,27 $mg.kg^{-1}$

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

8.2.2.1 General

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.

8.2.2.2 Calculation of the accumulation quotient (AQ)

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.

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

SETplot = Σ(AQ-1) and

SETsite = Σ(AQ-1) / nplot

8.2.2.4 If the TGV is not available for a studied metal(loid)s

C snail-28d can be compared either to:

a) the Csnail-28d of snails caged on a control site (i.e. uncontaminated site);

b) 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);

c) or at least to the initial concentration of snails (i.e. before exposure): 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 8.2.2.4.

9 Validity of the experiment

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

— the percentage of the mortality observed in the control containers (see 8.2.2.4) is less than or equal to 30 % at the end of the test.

10 Test report

The test report shall refer to this document and shall include the following information:

a) a reference to this document, i.e. ISO 24032:2021;

b) the description of the site and plot(s) of the site under study;

c) pictures of the studied site/plots;

d) environmental information (rainfall, min and max temperature measured or on the basis of a meteorological station near the studied zone) during exposure;

e) data available on the soil, the site (physico-chemical data);

f) geographical location of the microcosms (postal code, municipality, exact GPS coordinates (decimal degree or DMS: degree minute second and/or WGS world geodetic system);

g) description of the vegetal and the humus cover;

h) mass of the snails (total fresh mass) at the start of the test (when placed in the wooden box for transport) and after exposure (as stated in Clause 7);

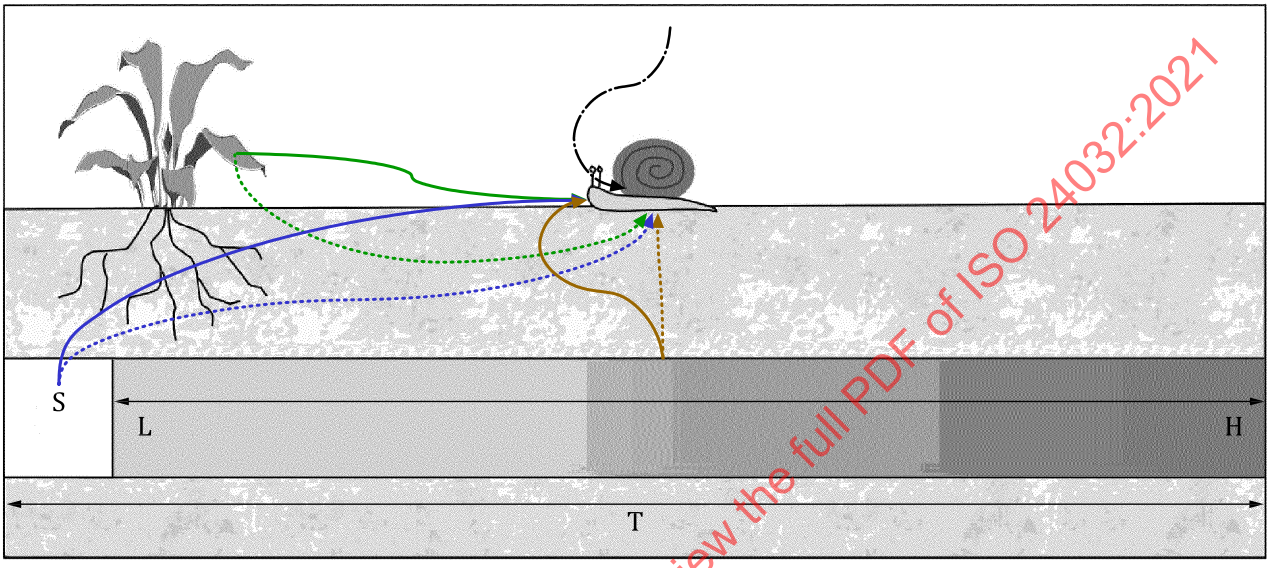
i) percentage of the survival in each microcosm, and the mean (±standard deviation) for the 3 microcosms per studied zone on the site;

j) the description of the obvious or pathological symptoms (e.g. snails producing a liquid, or showing a swelling shiny foot), or of the noticeable modifications in behaviour (e.g. sign of lethargy not withdrawing in the snail when handled), observed on the testing organisms;

k) any other manipulation not specified in this document and any events likely to have influenced the results.

Annex A
(informative)

Sources and routes of exposure of snails to contaminants in the field



Key

S	soil solution
T	total quantity of pollutants in the soil
L	pollutants poorly linked to the solid phase
H	pollutants strongly linked to the solid phase

Exposure route		**Exposure source**	
———	digestive	———	plant
·····	dermal	———	solid phase of the soil
—·—·—	respiratory	———	liquid phase of the soil
		—·—·—	air

NOTE Adapted from Reference [31].

Figure A.1 — Sources and routes of exposure of snails to contaminants in the field

Figure A.2 — Landsnail *Cantareus aspersus* — Sub-adult

Annex B
(informative)

Main steps of the bioassay in situ

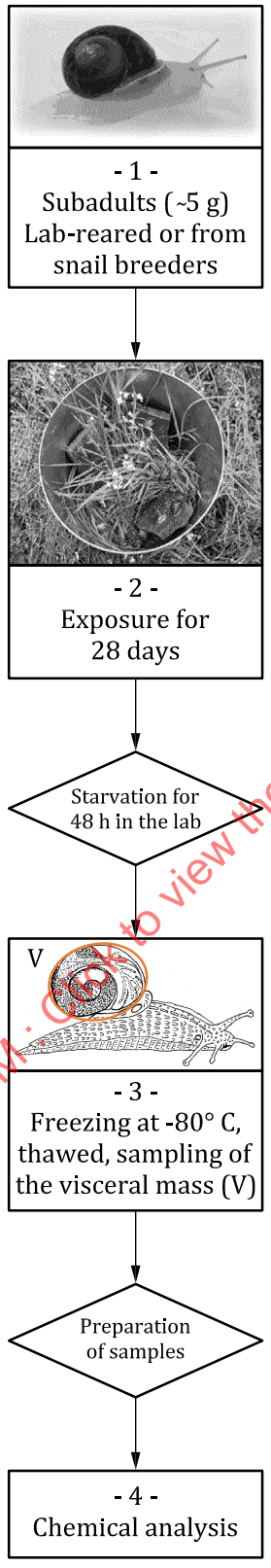
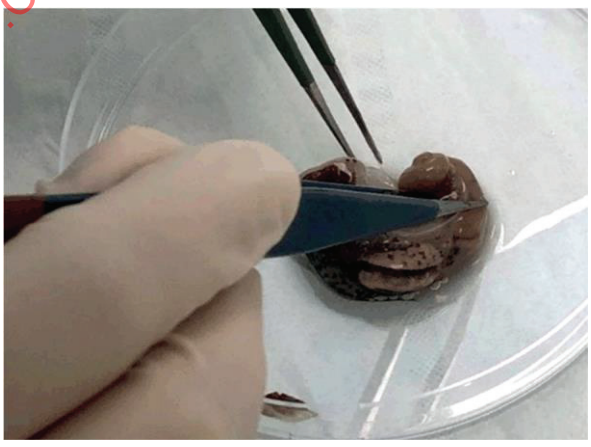


Figure B.1 — Main steps of the bioassay

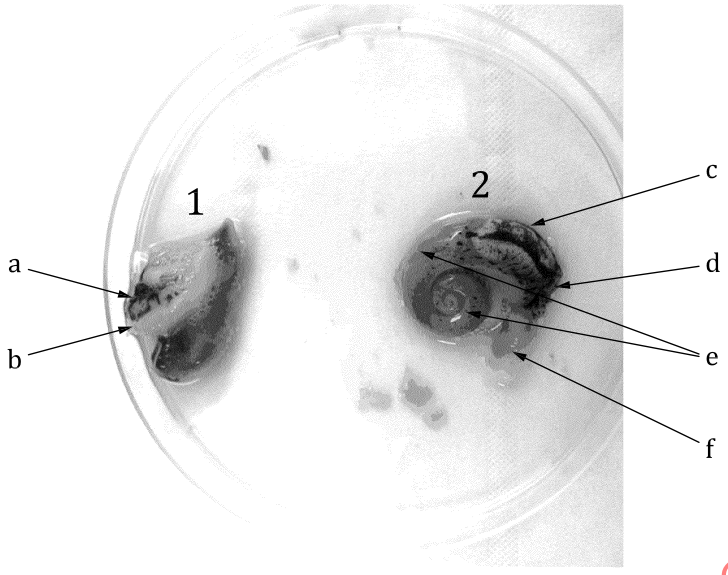
NOTE Transport to the site and back to the laboratory.

Figure B.2 — Wood bow for snail storage and transport

a) Removal of the soft body from the shell (pull the white muscle at the base of the columella)



b) Separation of the organs constituting the viscera from the "foot"



c)

Key

1	foot	c	kidney
2	viscera	d	part 2 lung
a	part 1 lung	e	hepatopancreas (large and small lobes) = liver
b	mantle edge	f	anterior part of the digestive tract

Figure B.3 — Preparation of the viscera after thawing

Annex C
(informative)

Breeding technique for snails

C.1 General

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

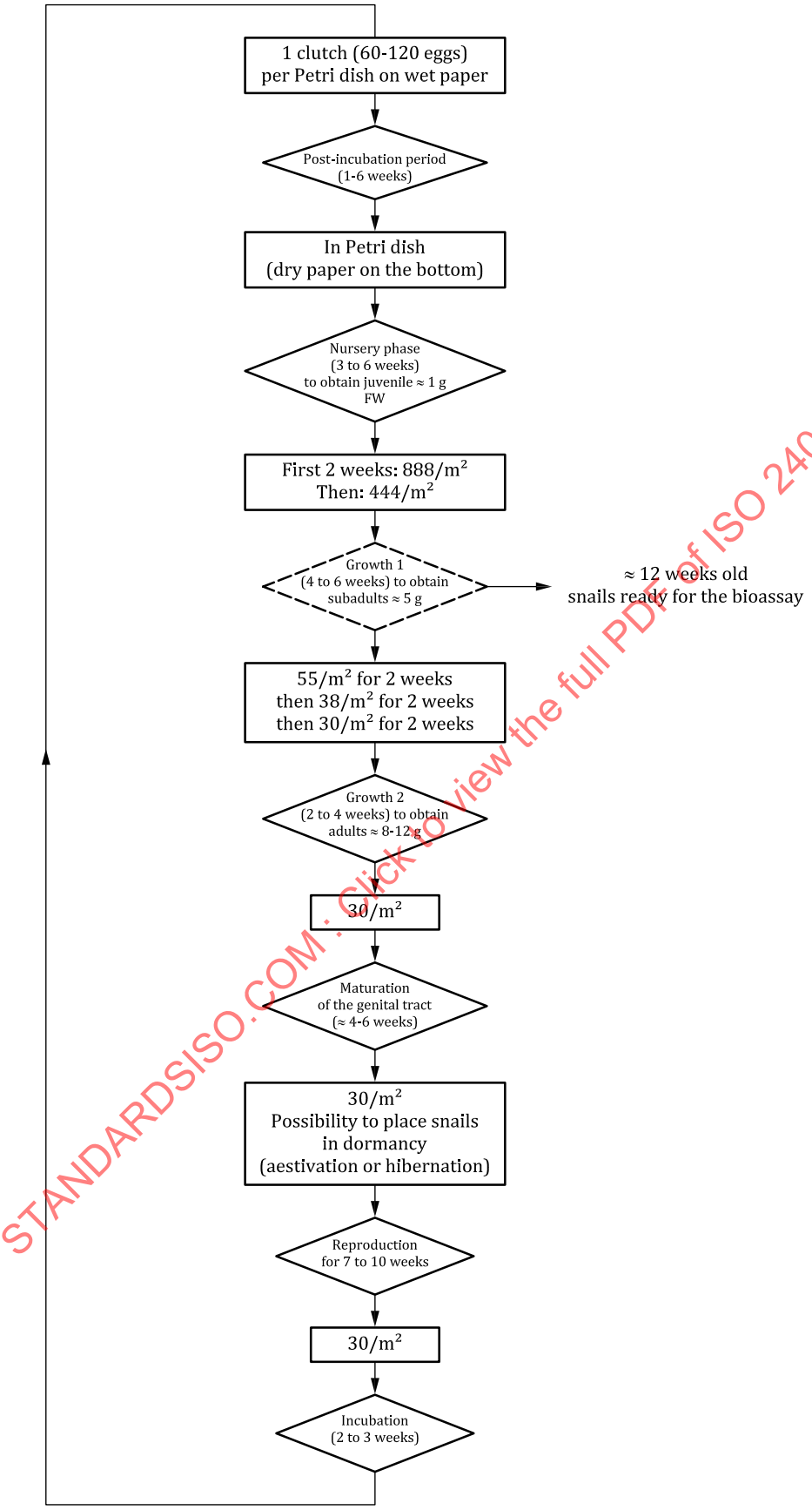


Figure C.1 — Breeding cycle under controlled conditions — Example for *Cantareus aspersus*

As from the beginning of the nursery phase, three to four 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 additional month is then necessary (i.e. Maturation step on Figure C.1) for these lipped shell snails to be capable of reproducing (four to five months after starting the nursery phase). This time corresponds to the maturation of the genital tract. Reproduction is monitored during 7 to 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 clutches and the number of breeders at the start of reproduction period). Subadult snails used for the in situ caging are sampled after the first period of growth (Growth 1, Figure C.1.); the second period of growth (Growth 2, Figure C.1.) provide shell lipped snails[14].

To have homogeneous and synchronous batches of young snails and in order to avoid the maintenance 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 indoor 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 5 °C to 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 5 °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. For longer duration of dormancy, it is recommended to place snails in hibernation.

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

The species mainly used for laboratory toxicity testing is currently *Cantareus aspersus* Müller (syn. *Helix aspersa aspersa or Cornu aspersum*, brown garden snail, Petit-Gris) of which the adult mass is comprised between 8 g to 12 g.

C.2 Environment parameters

C.2.1 Light cycle

Reproduction, incubation, nursery phase and growth take place under a long photoperiod of 18 h light and 6 h darkness. Artificial light 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 $W.m^{-2}$ to 15 $W.m^{-2}$ are required. The quantity of light measured in the breeding devices is 50 lx to 100 lx.

C.2.2 Temperature

All the breeding stages are conducted at (20 ± 2) °C.

C.2.3 Hygrometry

The relative humidity is 80 % to 95 %.

If disposable plastic container (PCs) are used, moisture is ensured by wetting the absorbent paper laid on 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 used, a humidifier ensures the humidification of the premises where the cages are located (one moistening apparatus for approximately 150 m^3).

C.2.4 Feed

During the nursery period (corresponding to the initial three weeks to five weeks of breeding), the growth and reproduction phases use a specific feed (flour for snails, see Annex D).

NOTE With an adapted feed, the weight of the snails shall increase from 0,03 g at hatching to 1 g after 3 to 5 weeks of nursery (see Figure C.1).

C.2.5 Density of organisms

For reproduction in cages [see Figure C.2 f)], use 45 breeders for three connected cages and approximately a number of 30 snails/m^2.

For reproduction in disposable plastic containers (PCs) [see Figures C.2 a) to C.2 e)], use four breeders per double container, equalling 30 snails/m^2.

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

For growth in PCs (e.g. disposable mouse boxes), use:

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

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

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

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

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

C.3 Breeding equipment (see Figure C.2)

C.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 [Figure C.2 f)].

This equipment can be used for the three breeding stages: nursery, growth and reproduction.

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 $l.min^{-1}$ (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 (see C.4.1). The cages of a same row can intercommunicate depending on whether growth or reproduction is being conducted. For reproduction, three cages are connected; in the middle cage, the spraying is eliminated and a laying recipient (transparent polystyrene plastic box) is installed.

C.3.2 Egg-laying containers

If reproduction is carried out directly in PCs (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.

If reproduction is carried out in cages (BCS) (C.3.1.), use transparent polystyrene plastic boxes (PCs) (5.2.6.), having a volume equal to 1 600 cm^3, filled with compost (universal horticultural uncontaminated compost). Glass pots (same as those used to receive the clutches with reproducers in PCs) can also be used.

C.3.3 Incubation containers

For the incubation of several clutches 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 premoistened absorbent paper in order to avoid dehydration of the eggs. Deposit a maximum of 18 to 25 clutches per container, i.e. 90 g to 150 g of eggs.

For the incubation of individual clutches, use Petri dishes (90 mm diameter × 15 mm high). Place one clutch (i.e. 60 eggs to 120 eggs) per dish, always on moistened absorbent paper. Spread the eggs on the paper in order to be able to close the lid without crushing the eggs, if necessary.

C.4 Maintenance of the breeding

C.4.1 Cleaning and food

Whether in BCSs (C.3.1) or in PCs (5.2.6), 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 the rhythm of activity and feeding. No 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; as snails stay in the cage during cleaning, the force of the water jet shall be adapted. Leave the cages to drip and dry for around 15 min to 30 min, then deposit the feed on the bottom of the cages.

In the PCs (5.2.6), after having cleaned the walls of the box, place clean absorbent paper on the bottom and moisten it. During the cleaning the snails are dispose in one of the two PCs (C.2.5) Then, deposit in the box a Petri dish containing fresh feed.

C.4.2 Reproduction

In BCSs, install the egg-laying containers (C.3.2) 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 breeding cage. The clutches are collected and deposited in the incubation container.

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

C.4.3 Incubation/hatching

During this period, the moisture content of the containers (C.3.3) should be monitored and it should be ensured that the absorbent paper is neither to dry (the eggs dehydrate) nor too moist (the eggs burst and/or become mouldy).

C.4.4 Nursery

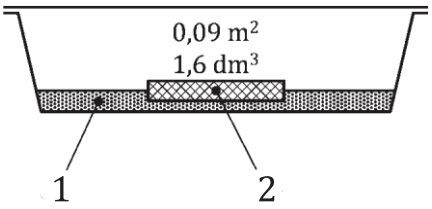
The hatching sessions begin 12 d to 20 d after egg-laying. First, the newly hatched snails remain clustered together on the bottom of the container. Then, after a few days, they start to migrate towards the lid. During this migration, they eat the paper which serves as an incubation substrate. A few days after this migration (6 d to 15 d), the newly hatched 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 *C. aspersus* (whereas, for example, it is 25 mg to 50 mg for *Helix aspersa maxima*, another subspecies well adapted for snail breeding).

The nursery phase is realized in PCs [888/m^2, then 444/m^2, Figure C.1 and Figure C.2 a) to e)]; after three to six weeks of breeding, snails reaching 0,8 g to 1,3 g are selected for the next step of growth.

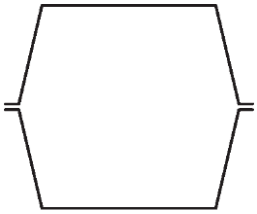
See C.2 for the environment parameters.

During the nursery phase, sort the snails having a mean mass between 0,8 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 (for ISO 15952) (an initial sorting operation is generally carried out after three weeks of breeding; next, sorting operations are conducted until six weeks), or for the continuation of growth (Growth 1 and 2, Figure C.1). This sorting operation is necessary because the population is relatively heterogeneous at the end of the nursery period; on average, 10 % to 30 % of the young snails stemming from a clutch remain “dwarfs” and are not kept for the remainder of the breeding process.

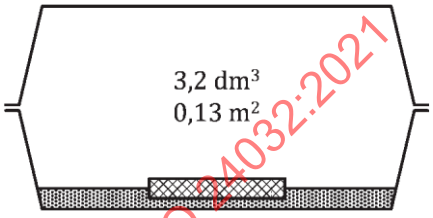
a) Single-use clear plastic box — Front view [a]



b) Single-use clear plastic box — Side view [a]



c) Single-use clear plastic box — Front view [b]



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

e) Single-use clear plastic 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 C.2 a) and C.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 dm^3.

b In Figures C.2 c) and C.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 plastic box which contains 5 snails for the test. the cover is held in place by two rubber bands.

Figure C.2 — Single-use clear plastic box and BCS specific breeding cages for snails

Annex D
(informative)

Example of composition of snail feed

Main constituents of the snail feed are for example wheat, wheat remoulding, oilcake feed soybean meal, oil cake sunflower extraction feed, oilseed rape feed, corn, corn distillery drills, $CaCO_3$, lysine, methionine, vitamins (A, D_3, E), trace elements (Cu, Fe, Mn, Zn, I, Se). Main analytical constituents are (g/100 g):

— proteins 15;

— crude fat 2 to 2,5;

— total cellulose 2,5 to 4;

— total ash 37;

— Ca 12 to 13.

Annex E
(informative)

Usual concentrations in the viscera of sub-adult snails before caging

Table E.1 — Usual concentrations in the viscera of subadult snails before caging

	Metal(loid)s - median value mg.kg^{-1} dw (visceral mass)																	
Research program	As	Cd	Co	Cr	Cu	Hg	Mn	Mo	Ni	Pb	Sb	Se	Sn	Sr	Tl	Zn	133Cs	238U
µscope 2015	0,172	0,706	1,240	1,250	78,6	0,082		2,11	4,570	0,367	0,036		0,015	29,3	0,064	550	0,074	0,49
µscope 2016	0,067	1,361	0,279	0,060	129,3	0,025		1,22	0,517	0,407	0,029		0,123	44,2	0,006	393	0,009	0,039
	0,127	1,53	1,19	0,825	52,3	0,017	256	2,59	4,24	0,510	0,043	1,57	0,032	28,0	0,022	512		
	PAH - mean ±SD - µg.kg^{-1} fm (visceral mass)																	
Combine	ACE[a]	ACY[a]	ANT[a]	BaAN-T[a]	BaPY-R[a]	BbFLT[a]	BghiP-L[a]	BkFLT[a]	CHY	dBa-hANT[a]	FLT	FLU[a]	IcdPYR[a]	NAP	PHE	PYR		
2018	<13,7	<16,9	<9,63	<1,44	<6,45	<2,28	<5,31	<2,01	3,99	<4,08	6,27	<6,45	<5,25	21	6,88	8,72		
									1,18		2,96			6,1	5,95	5,03		
	PCB µg.kg^{-1} fw (visceral mass)																	
	PCB28	PCB52	PCB101	PCB118	PCB138	PCB153	PCB180											
	<0,120	<0,180	<0,180	<0,240	<0,510	<0,360	<0,230											

[a] Values equaling the current quantification limit of Laboratoire Chrono-Environnement (LCE) (= detection limit x 3).

For metals and metalloids values are in mg.kg^{-1} dry weight of viscera. * For PAH and PCB data are in µg.kg^{-1} fresh weight and correspond to our current limit of quantification (DL *3).

Annex F
(informative)

Recommended test systems for in situ exposure to assess bioaccumulation of contaminants in snails

Figure F.1 — Microcosms (cylinder), covered by netting maintained by pickets

Figure F.2 — "Open" microcosms, covered by netting maintained by pickets[11]

a) In forest (with wood pickets around the microcosm to better see the microcosm)

b) In cultivated field

c) In a grassland (the wood device around the microcosm is to avoid that cattles knock over the cage)

Figure F.3 — Example of snail caging

Figure F.4 — Example of additional shade cloth

Key

1 snail, dry in the wood box used for transport
2 2 or 3 pieces of tiles (shelter for snails)
3 picked to maintain the steel netting (mesh size: 10 mm) over the top of the microcosm
4 microcosm closed by the steel netting
5 wood storage containing snails

Figure F.5 — Beginning of the exposure of snails in the field

Figure F.6 — End of the exposure of snails in the field

Annex G
(informative)

Example of mass of snails before exposure

Table G.1 — Example of table of data: mass of snails before exposure (Mic.: microcosm)

Mass of snails (g fw)																	
Microcosm n°	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	**Mean**	SD
A1	5,3	5,4	4,6	5,5	5,5	5	4,9	5	4,9	5	4,9	4,9	5,3	4,9	5,8	**5,1**	0,3
A2	5,2	5,2	4,4	5,6	5,5	5,4	5,1	5	4,9	4,7	5	4,8	5,6	5	6	**5,2**	0,4
A3	5	5,6	4,8	5,6	5,5	5,3	5,2	5,3	4,8	4,9	4,9	4,8	5,6	4,7	6,2	**5,2**	0,4
A4	5,2	5,2	4,5	6,1	5,5	5	5,5	5,3	4,7	4,9	5	5	5,2	4,6	6,1	**5,2**	0,5
B1	4,8	5,7	4,3	5,8	5,6	5,4	4,9	5	4,6	4,9	5	4,6	5,3	4,7	6	**5,1**	0,5
B2	5,1	5,6	4,6	6	5,7	5,1	5,3	5,2	4,7	4,8	5,2	4,5	5,1	5,1	6,1	**5,2**	0,5
B3	5,3	5,4	4,7	6	5,7	4,9	5,6	5,2	4,9	5	5,3	4,9	5,5	4,9	6,2	**5,3**	0,4
B4	5	5,7	4,6	5,7	5,8	5	5,2	5,2	5,1	5	5,4	4,5	5,5	4,8	5,8	**5,2**	0,4
C1	5,2	5,6	4,4	5,9	5,6	5,2	5,8	5,8	4,7	4,9	5,2	4,6	5,4	4,6	6,2	**5,3**	0,5
C2	5	5,6	4,4	6,1	5,7	5,2	5,1	5,1	5,4	5	5	4,5	5,5	4,6	6,1	**5,2**	0,5
C3	4,8	5,2	4,8	5,8	5,7	5,2	5	5,5	5	5,1	5,2	4,6	5,7	4,4	5,9	**5,2**	0,5
C4	5	5,8	4,7	5,7	5,6	5,3	5,2	5,2	5	4,8	4,8	4,6	5,6	4,8	5,8	**5,2**	0,4
D1	4,9	5,5	4,6	5,8	5,8	5	5,6	5,3	4,9	4,9	4,9	4,7	5,5	4,5	6	**5,2**	0,5
D2	5	5,4	4,5	5,9	5,7	5	5,1	5,2	5,5	5,1	5,3	4,6	5,5	4,5	6	**5,2**	0,5
D3	5,3	5,4	5,3	5,9	5,9	5,1	5,7	5,4	4,8	5,5	4,9	4,7	5,5	4,5	5,9	**5,3**	0,4
D4	4,9	5,5	5	6,2	5,9	5,2	5,9	5,4	5,1	5,2	4,9	4,5	5,5	4,5	5,9	**5,3**	0,5
E1	5,1	5,5	4,2	6	5,6	5,1	5	5,8	5	5,4	5	5	5,4	4,6	6,1	**5,3**	0,5
E2	5,1	5,5	4,3	5,7	5,6	5,2	5,3	5,3	5,3	5,4	5,2	4,7	5,7	4,5	6	**5,3**	0,5
E3	5	5	5,3	6,1	5,8	5,1	5,2	5,5	5,4	5,4	5	4,6	5,6	4,5	5,8	**5,3**	0,4
E4	5,1	5	5,2	5,8	5,6		5,6	5,5	5,2	5,3	5,4	4,6	5,4	4,6	5,8	**5,3**	0,4

Key

1 wood box for storage (snails inactive, estivating in dry box)

2 container for starvation or to prepare the distribution of snails in homogeneous batch

Figure G.1 — Distribution of the sub-adult snails in homogeneous batches

Annex H
(informative)

Results of the international ring test

H.1 General for in situ exposure

Six laboratories from five European countries [Czech Republic (CZ), two for France (FR), Switzerland (CH), Spain (SP), Portugal (PT)] participated in the ring test that took place from May 2019 to June 2019 in France.[18] The main objective was to handle all the steps of the method from the installation of the cages and the snails on the site of the ring test, to the snails sampling at the end of the exposure, the starvation and the tissue preparation.

Chemical analyses were done separately by 4 laboratories (CZ, FR, SP, PT) out of the 6 laboratories on snails they prepared themselves. The four laboratories (CZ, FR, PT, SP) were equipped to analyse metal(loid)s (ME) in snail tissues (visceral mass) and two for the analysis of PAHs (CZ, FR). The organizing laboratory (LCE: Laboratory Chrono-environment) did not analyse the samples for all the participants. One reason is that due to mass of tissue needed for chemical analysis, separating the visceral mass of one snail in 4 parts for the 4 laboratories would have given an insufficient mass for analysis. Another reason is the financial cost of these analysis (6 snails per plot × 5 plots × 4 participants for ME and 3 pools of 2 snails per plot for PAH) that could not be supported by the organizing laboratory. This experimental design implies several factors of variability between the results of the 4 laboratories but corresponds to a realistic situation (i.e. a company or a laboratory that wish to apply the snail caging method to assess contamination of a site).

To optimize the duration of the stay of the participants in France, the ring test was organized as follows: the participants started mid-June 2019 a test (named hereinafter ring test 2: RT2) and ended a test previously started mid-May 2019 (named hereinafter ring test 1: RT1) by the test organizing laboratory.

The schedule of the ring test was:

— Day 1-May 16-: The LCE started in situ exposure of RT1: snails distributed in microcosms

— Day 27 – June 11 evening: arrival of the participants

— Day 28 – June 12 - all the participants:

  — prepared the material (for snail caging, recipients for snail sampling, etc.)

  — travelled to the studied site (about 1h hour from the organizing laboratory)

  — sampled exposed snails after 28 days (RT1)

  — started a new exposure on site (RT2)

  — distribution of the sampled snails (RT1) for starvation in the laboratory

— Day 28+1- June 13:

  — in the morning first faeces removal after first hours of starvation of RT1

  — demonstration of freezing snails, defrosting and dissection of the visceral mass

  — demonstration of the ex situ method

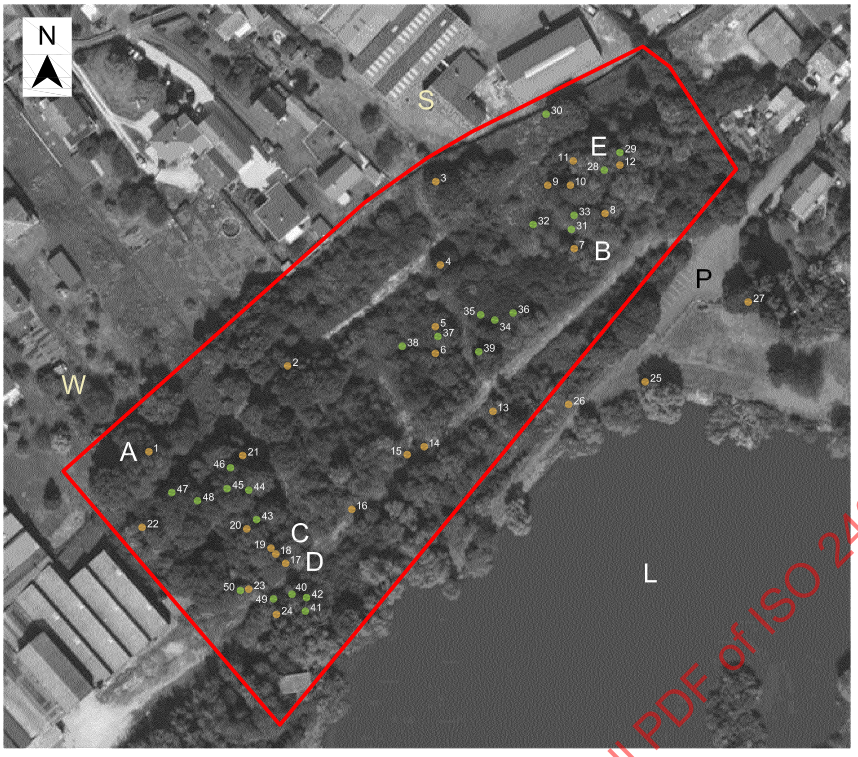
  — day end, second faeces removal after starvation

  - detailed explanation of the method
  - discussion on the draft standard (WD) based on the participants' comments
- Day 28+2- June 14: last faeces removal of starved snails, snails were then stored inactive in dry wood box.
- June 19: snails were sent to laboratories CZ, PT and SP.
- When all the laboratories have received the snails, each of them frozen the snails at –80 °C and after defrosting, prepared the visceral mass for the analysis of metal and metalloid (arsenic: As and antimony: Sb) and for PAH analysis (FR, CZ and SP).
- July 11: LCE, stop the second exposure on site (RT2).

The organizing laboratory gathered and analysed the data obtained by the 4 laboratories. They are presented in this document (for the ex situ only FR laboratory performed the assay, see Annex H).

H.2 Site and soils

The studied site (21 601 m^2) is located in France (near Vieux-Charmont, 47°31'16.37N; 6°50'24.54'E). This industrial wasteland presents heterogeneous contamination by metal(loid)s, PAH, (cyanides, HCT). Five plots (A, B, C, D, E) were selected for snail exposure on the site (see Figure H.1).



Key

A	plot A – tree 1 – Ctl
B	plot B – tree 7
C	plot C – tree 18 – Cr++
D	plot D – tree 17 – HAP++
E	plot E – tree 28 – MEM+++
L	pond
S	former pond of stabilization
P	parking
W	waste dumping
———	limit of the study site
●	sampling point (06/2018)
●	sampling point (07/2018)

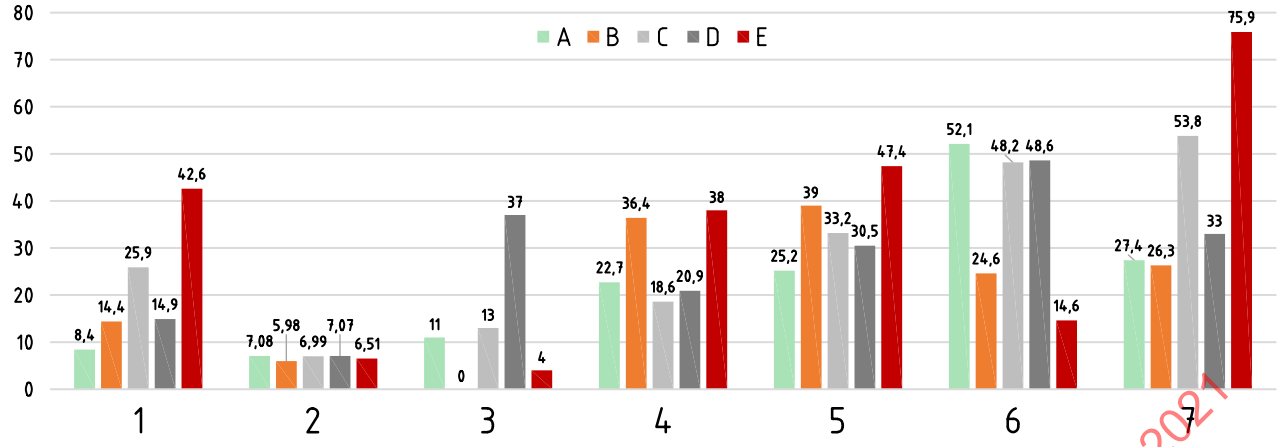
A numbered tree was present on each plot which made it easier to locate the cages.

Figure H.1 — Site located at Vieux-Charmont, France

The main characteristics of the soils are presented in Table H.1 and illustrated in Figure F.2. The concentrations of MEs and PAHs are presented in Table H.2 and Table H.3, respectively. As an indication of the level of contamination of the studied soils, soil guide values (SGV) of MEs are indicated in Table H.2 and Table H.3; these guide values are concentrations below which a soil can be considered as "unpolluted". As described in Reference [22], these concentrations were retained as threshold values of soil pollution for each metal based on (1) the highest limit of the range of "normal concentrations in French soil" proposed for the metals studied in the ASPITET program[5]; (2) the upper whisker of the soil concentrations of the RMQS program[32]; and (3) the BDETM program[6].

Table H.1 — Physico-chemical characteristics of the soils of the five plots of the site

Plot	A	B	C	D	E
LOI 550 °C (g/100 g)	11,9	20,2	32,6	20,4	53,6
C org g/100 g	4,85	8,31	15	8,6	24,6
N total g/100 g	0,396	0,622	1,07	0,566	1,64
$CACO_3$ total g/kg	11	<1	13	37	4
OM g/100 g	8,4	14,4	25,9	14,9	42,6
C/N	12,3	13,4	14	15,2	15
pH_{water}	7,08	5,98	6,99	7,07	6,51
pH_{CaCl2}	7,55	6,57	7,49	7,6	7,17
Clay (0-2 µm) %	22,7	36,4	18,6	20,9	38
Silt (2-63 µm) %	25,2	39	33,2	30,5	47,4
Sands (63-2 000 µm) %	52,1	24,6	48,2	48,6	14,6
CEC Hexamminecobalt(III) chloride cmol/kg	27,4	26,3	53,8	33	75,9
Ca exch $cmol.kg^{-1}$	25,7	20,4	44,4	30,8	51,8
Mg exch $cmol.kg^{-1}$	1,08	2,07	3,17	1,27	5,34
Na exch $cmol.kg^{-1}$	0,023 5	0,023 3	0,030 1	0,019 3	0,057 6
K exch $cmol.kg^{-1}$	1,03	0,988	0,805	0,392	1,04
Fe exch $cmol.kg^{-1}$	<0,005	0,008 7	0,025 6	0,012 4	0,020 1
Mn exch $cmol.kg^{-1}$	0,008	0,063 3	0,015 5	0,005 8	0,12
Al exch $cmol.kg^{-1}$	0,020 2	0,032 7	<0,02	<0,02	<0,02
Cd extr $CaCl_2$ $µg.kg^{-1}$	3,8	95,2	2,9	2,3	81,8
Cr extr $CaCl_2$ $µg.kg^{-1}$	<10	<10	26,8	23,1	<10
Cu extr $CaCl_2$ $µg.kg^{-1}$	259	167	510	282	204
Ni extr $CaCl_2$ $µg.kg^{-1}$	73,4	305	232	250	176
Pb extr $CaCl_2$ $µg.kg^{-1}$	<3	164	<3	<3	303
Zn extr $CaCl_2$ $µg.kg^{-1}$	52,6	121 000	275	163	105 000
Corg extr $CaCl_2$ $mg.kg^{-1}$	639	479	676	459	895
For each plot a composite sample made of 4 subsamples of topsoil (0 cm-10 cm) was collected next to the microcosm, samples were dried at 40 °C, crushed at < 250 µm, and analysed by the laboratory LAS Arras.					



Key

1 organic matter g/100 g
2 pH water
3 $CaCO_3$total g/kg
4 clay (0-2 µm) %
5 slit (2-63 µm) %
6 sands (63-2000 %m) %
7 CEC cobaltihexamine cmol/kg

Figure H.2 — Soil characteristics of plots A to E

Most of the soils present a pH near the neutrality. Soil E shows a high content of organic matter (42,6 g/100 g). This soil also presents high $CaCl_2$ - extractible concentrations of Zn, Pb, Cd as soil B. Soils C and D mainly differ for the $CaCl_2$-extractible Cr and for soil C especially Cu (see Table H.1).

Regarding the concentration of the 13 analysed ME in soils (see Table H.2), soil A was the less contaminated excepted for As. Soil B present high concentration mainly for As, Pb Sb, and Zn. Soils C and D are characterized by high contents of As, Co, Cr, Cu, Mo, Ni, Sr, whereas soil E present the highest concentrations of Cd, Mo, Pb, Sb and Zn.

All soils except A showed high concentration of Mo and Zn. Soils B and E present the highest concentrations of Pb and Zn whereas soils C and D are mainly contaminated with Cr and Mo.

Table H.2 — Concentrations of metal(loid)s in soils (mg.kg^{-1} dw, median of 3 samples collected near the microcosms for each plot)

Metal(loid)s	As	Cd	Co	Cr	Cu	Hg	Mo	Ni	Pb	Sb	Sr	Zn
Soil A	46,8	0,50	8,34	32,1	28,8	0,25	3,7	16,8	44,6	3,8	21,2	200
Soil B	47,9	3,84	14,82	122,2	84,4	0,82	147,0	42,7	1 978,6	19,0	20,3	5 303
Soil C	82,4	2,02	61,77	787,8	309,1	1,68	359,9	808,0	133,0	8,6	174,5	989
Soil D	95,8	2,23	63,96	914,8	312,4	3,38	565,8	796,0	148,2	10,0	75,7	1 014
Soil E	20,7	16,9	9,41	191,0	90,7	1,29	517,7	59,5	1 222 8,1	36,8	30,6	2 920 3
Soil guide value[a]	25,0	0,45	23,00	90,0	20,0	0,10	1,6	60,0	62,3	2,0	224,0	100

[a] Soil guide value (i.e., soil concentration below which a soil can be considered as uncontaminated) from Pauget et al. (2013).

For the PAHs, soils C and D show the highest concentrations of mainly PHE, FLT and PYR (see Table H.3).

Table H.3 — Concentrations of PAHs in soils (mg.kg^{-1} dw, analysis of a composite sample for each plot)

PAH soil	mg.kg^{-1}	LQ soil	A	B	C	D	E	Soil guide value[a]
NAP	naphtalene	0,000 1	0,034	0,064	0,194	0,215	0,220	0,11
ACY	acenaphthylene	0,000 2	0,061	0,038	0,121	0,163	0,059	0,23
ACE	acenaphthene	0,000 1	0,010	0,033	0,610	0,835	0,194	0,11
FLU	fluorene	0,000 1	0,006	0,029	0,523	0,618	0,098	0,11
PHE	phenanthrene	0,000 4	0,172	0,379	11,358	12,991	0,587	0,4
ANT	anthracene	0,000 6	0,073	0,086	2,566	2,961	0,118	0,6
FLT	fluoranthene	0,001 2	0,597	1,083	27,039	36,550	1,085	1,22
PYR	pyrene	0,001 0	0,415	0,672	18,262	25,760	0,834	1,02
BaANT	benzo[a]anthracene	0,000 6	0,218	0,206	7,725	9,484	0,135	0,63
CHY	chrysene	0,000 7	0,225	0,246	7,941	9,112	0,158	0,71
BbFLT	benzo[b]fluoranthene	0,000 9	0,368	0,277	8,853	11,239	0,162	0,91
BkFLT	benzo[k]fluoranthene	0,000 4	0,238	0,178	6,175	7,463	0,091	0,4
BaPYR	benzo[a]pyrene	0,000 8	0,283	0,111	7,607	9,332	0,148	0,75
IcdPYR	indeno[1,2,3-cd]pyrene	0,000 6	0,289	0,107	4,433	5,298	0,115	0,58
dBahANT	dibenzo[a,h]anthracene	0,000 4	0,057	0,006	1,438	1,580	0,017	0,35
BghiPL	benzo[g,h,i]perylene	0,000 6	0,272	0,091	4,769	5,985	0,109	0,56
	Sum (mg.kg^{-1})		**3,3**	**3,6**	**109,6**	**139,6**	**4,1**	

[a] Soil guide value from References [4] and [17]. LQ: limit of quantification. Naphthalene (NAP), acenaphthylene (ACY), acenaphthene (ACE), fluorene (FLU), phenanthrene (PHE), anthracene (ANT), fluoranthene (FLT), pyrene (PYR), benzo[a]anthracene (BaANT), chrysene (CHY), benzo[b]fluoranthene (BbFLT), benzo[k]fluoranthene (BkFLT), benzo[a]pyrene (BaPYR), indeno[1,2,3-cd]pyrene (IcdPYR), dibenzo[a,h]anthracene (dBahANT), and benzo[g,h,i]perylene (BghiPL).

H.3 Snails exposure, environmental parameters

On each plot, 4 microcosms (1 to 4) with 15 snails and 3 pieces of tile in each were placed for 28 days (see Figure H.3).

NOTE For MEs analysis, two snails from microcosms 1 to 3 were analysed (n = 6). To have a sufficient number of snails to perform likewise PAHs measurements, 4 microcosms per plot were used and snails from microcosms 1 to 4 were analysed (n = 3 pools of 2 snails per plot).

Key

E	plot E
µc E1	microcosm E1
µc E2	microcosm E2
µc E3	microcosm E3
µc E4	microcosm E4

Figure H.3 — Four microcosms (E1 to E4) located on plot E

One recorder (Hobo) was located on plot A to measure the temperature and brightness during the ring test 1. Rainfall was about 5 mm/day (min – max: 0-48 mm/day)[3)]. Data are presented in Table H.4.

Table H.4 — Environmental parameters (temperature, brightness) during the ring test (May 16 to June 12, 2019)

	Temperature (°C)	Brightness (Lx)
	hobo 1 RT1	hobo 1 RT1
Mean	16,4	2 139,8
Median	14,8	98,5
Min.	2,7	3,9
Max	33,3	29 938
Days > 30 °C, *n* =	3	
Days > 14 000 lx, *n* =		6

3) Available from: https://www.historique-meteo.net/france/franche-comte/montbeliard/2019/05/.

H.4 Results of the in situ exposure

H.4.1 Survival and mass loss

The mean fresh mass of the snails before exposure was (5,2 ± 0,4) g (see Annex G). Table H.5 presents the mass at the end of the test (here: after thawing and before sampling of the viscera).

Table H.5 — Mass loss and mortality of snails after 28 days caging on site

Plot	Mean mass at the start of the test (g)	SD	Mean mass after exposure (g)	SD	Mean mass loss per plot (%)	Min. mass loss per microcosm (%)	Max. mass loss per microcosm (%)	Mean mortality per plot (%)	SD	Min. mortality per microcosm (%)	Max. mortality per microcosm (%)
A	5,2	0,4	3,8	1,0	27	15,4	32,7	21,7	8,4	13,3	33,3
B	5,2	0,5	3,7	0,8	29	17,1	38	16,7	3,8	13,3	20
C	5,2	0,5	3,6	0,8	32	27	37	16,7	3,8	13	20
D	5,3	0,5	4,4	0,9	15	9	23,5	8,3	16,7	0	33,3
E	5,3	0,4	3,6	0,7	30	18,1	38	10	8,6	0	10

Mass loss and mortality did not vary according to the contamination of the plots. These data were obtained under the very high temperature recorded in 2019 (see Table H.4) and can be considered as extremes at least for the mass loss.

H.4.2 Concentrations of metal(loid)s (MEs) and polycyclic aromatic hydrocarbons (PAHs)

H.4.2.1 Before exposure

Table H.6 present the concentrations of MEs in the snails before exposure on site.

Table H.6 — Concentration of MEs in snails before exposure on site (n = 12) (mg.kg^{-1} dw).

Snail t0	As	Cd	Co	Cr	Cu	Hg	Mo	Ni	Pb	Sb	Sr	Zn
Median	0,06	1,07	1,04	0,68	91,55	0,28	2,28	3,51	0,28	0,03	8,81	404
Mean	0,10	1,11	1,10	0,69	90,51	0,27	2,45	3,41	0,29	0,02	12,24	463
SD	0,07	0,28	0,28	0,18	25,13	0,11	0,59	0,55	0,10	0,01	11,75	176
TGV	0,31	2,27	6,68	2,01	184,70	0,20	4,43	5,25	12,90	0,08	125,70	1 490
TGV = threshold guide value, equivalent of the CIRef value from Reference [23].												

Overall for MEs, all the concentration before exposure were in the same order as those usually found in sub-adult snails before exposure (see Annex E) and were lower than the TGV (except a slight excess for Hg) showing that snails were not contaminated before exposure on the studied site. Zn and Cu, both essential ME, were the most abundant in the snail tissues.

For PAH, the concentration before exposure were not available; thus results after exposure were compared to the TGV (see Table H.7).

Table H.7 — Concentration of PAHs in snails before exposure on site

PAH (µg.kg^{-1} fm)	ACE[a]	ACY[a]	ANT[a]	BaAN-T[a]	BaPY-R[a]	BbFL-T[a]	BghiP-L[a]	BkFLT[a]	CHY	dBa-hANT[a]	FLT	FLU[a]	IcdPYR[a]	NAP	PHE	PYR
TGV	13,7	16,9	9,63	1,44	6,45	2,28	5,31	2,01	1,47	4,08	1,92	6,45	5,25	26,1	4,2	1,83
LQ	13,7	16,9	9,63	1,44	6,45	2,28	5,31	2,01	1,47	4,08	1,92	6,45	5,25	4,56	4,2	1,83
LD	4,57	5,63	3,21	0,48	2,15	0,76	1,77	0,67	0,49	1,36	0,64	2,15	1,75	1,52	1,4	0,61

[a] Values equalling the current limit of quantification (LQ) of the LCE laboratory. TGV (= threshold guide value from Reference [17]) equal the current LQ except for NAP (LQ = 4,56). n = 3 pools of 2 snails.

H.4.2.2 Comparison of the recovery rates for ME analysis by the 4 laboratories

As each laboratory made his own analysis separately, a validation of the analytical method was involved in the frame of the ring test. To check the variability between laboratories, a certified reference material was provided to each laboratory. All analysed a standard biological reference material for which certified concentration of ME are known (TORT-3, lobster hepatopancreas; Institute for National Measurement Standards, National Research Council of Canada, Ottawa, ON, Canada). Although, the same reference material has been used by all the laboratories, the sample preparation methodologies (including mineralization) present some differences that can have influenced the interlaboratory comparison of concentrations measured: CZ: nitric acid and hydrogen peroxide microwave digestion then ICP-MS; FR: nitric acid digestion using DigiPREP MS then filtration at 1 µm and analyse with ICP-MS; PT: nitric acid digestion in Polytetrafluoroethylene (PTFE) bomb under pressure then ICP; SP: microwave acid and hydrogen peroxide digestion then filtration 0,45 µm then ICP-MS). Interlaboratory differences also result of inter-individual variations as all the analysis were performed on different snails exposed in the same microcosm.

Table H.8 presents the percentage of recovery for the 4 laboratories (CZ, FR, PT, SP).

For PT most of the values were high in the TORT (lobster hepatopancreas); for this reason, data (median internal concentration in snails) were corrected with the percentage of recovery (see Table H.9). For example, if the percentage of the recovery of the certified material (TORT) for cobalt (Co) is 222 % and the raw data in a snail sample is (1,41 mg.kg^{-1}), the corrected value will be (1,41 × 100/ 222 = 0,63 mg.kg^{-1}). For Pb, laboratory PT found high recovery possibly due to a contamination of the water or acid used for analysis (blanc); thus, another recovery rate was calculated by subtraction of the blanc value to the value read in mineralized samples of tissues (last line of Table H.8).

Table H.8 — Certified material for ME analysis: TORT (lobster hepatopancreas) and calculation of the percentage of recovery[a] of the laboratories

	As	Cd	Co	Cr	Cu	Hg	Mo	Ni	Pb	Sb	Sr	Zn
TORT-Certified values (mg.kg^{-1} dw)	**59,5**	**42,3**	**1,06**	**1,95**	**497**	**0,292**	**3,44**	**5,3**	**0,225**		**36,5**	**136**
±	3,8	1,8		0,24	22	0,022	0,12	0,24	0,018		1,6	6
Mean Tort-CZ	70,0	43,3	1,11	1,3	488	0,281	3,40	4,70	0,27	0,062	34,40	138
Mean Tort-FR	67,09	42,41	0,95	1,20	429	0,303	3,42	4,27	0,18	0,058	33,61	125
Mean Tort-PT	81,85	54,23	2,36	5,56	554	0,67	5,74	10,77	6,72	0,09	43,96	192
Mean Tort-SP	54,92	33,51	1,32	1,29	378	0,42		4,01	BDL	0,07	33,24	117
% recovery Tort-CZ	118	102	105	67	98	96	99	89	120		94	101
% recovery Tort-FR	113	100	90	62	86	104	99	81	80		92	92
% recovery Tort-PT	138	128	222	285	111	**230**	167	203	2985		120	141

Table H.8 *(continued)*

	As	Cd	Co	Cr	Cu	Hg	Mo	Ni	Pb	Sb	Sr	Zn
% recovery Tort-SP	92	79	124	66	76	143		76	<DL		91	86
Blank FR (µg.l^{-1})	<LQ	<LQ	<LQ	<LQ	0,480 35	<LQ	<LQ	0,0702	<LQ	<LQ	<LQ	2,716
Blank-CZ												
Blank-PT	0,842	0,013	0,204	39,950	2,198	0,580	1,083	2,694	5,110	0,019	0,089	3,793
Blank-SP	0,045	0,003	0,008	0,373	1,133	<0.3		0,173	0,069	0,020	0,160	23,702
Value of the blank PT substracted to Tort (µg.l^{-1})												
% recovery Tort1-blank	133	127	129	144	99	110	134	132	-658		116	131
% recovery Tort2-blank	137	129	231	160	104	-1	155	150	541		122	139
% recovery Tort3 - blank	138	128	247	203	106	-33	114	172	2063		122	144
Mean	**136**	**128**	**202**	**169**	**103**	25	**134**	**151**	**649**		**120**	**138**

NOTE (CZ, FR, PT, SP: the 4 laboratories. Blank = value in the solution of liquids used for the extraction and dilution, without any tissue: nitric acid, hydrogen peroxide if used, water; the blank is analysed to verify if liquids themselves do not contain impurities). For Sb, no certified value is available, thus no % recovery was calculated; for Co, indicative values are available.

[a] For PT the % of recovery used (last line of Table H.8) to revise the raw data was obtained by subtraction of the values in the blank to the value in µg.l^{-1} in the certified material (TORT), except for Hg because for this element negative values were obtained (thus the % recovery used was 230 %, i.e. uncorrected by the blank) and for Pb at the highest concentration (plots E).

H.4.2.3 After exposure for ME

Table H.9 present the concentrations of snails after exposure on site. Detailed data are presented in Table H.12 at the end of the report.

H.4.2.3.1 If results are interpreted as stated in 8.2.2.4. (i.e. considering the concentrations in samples without TGV), high concentration of Pb, Zn are found in snails of plots E and B which were also the most contaminated by these ME (see Table H.9). High contamination of soil D by Mo is reflected by the highest concentrations found in the snail visceral mass. Mo concentrations are much lower in snails from plot E, although the latter is as contaminated as soil D revealing a reduced bioavailability of Mo on this plot. For As, Co, Cr, Cu, Hg, Ni, Sr mainly present on plots C and D, the concentrations in the snail visceral mass were similar showing a reduce bioavailability of these ME for these plots.

Table H.9 — Median concentration of MEs (mg.kg^{-1} dw)

Plot	As	Cd	Co	Cr	Cu	Hg	Mo	Ni	Pb	Sb	Sr	Zn
A-CZ	0,24	1,49	1,10	1,99	119	0,32	9,20	5,93	1,41	0,04	31,88	605
A-FR	0,31	1,87	0,60	0,31	123	0,51	13,73	2,03	1,46	0,05	20,66	357
A-PT	4,57	1,80	0,69	1,66	207	1,49	10,56	4,73	0,99	0,11	26,07	559
A-SP	0,32	1,27	0,76	0,37	105	<0,3	3,45	1,67	1,10	0,05	54,70	393
B-CZ	0,36	2,53	0,87	15,31	114	0,61	62,58	4,55	12,67	0,08	22,89	996
B-FR	0,36	3,46	0,62	0,44	118	0,27	70,90	2,68	10,86	0,11	19,54	902
B-PT	0,81	3,39	0,62	1,80	200	0,39	60,74	4,85	3,29	0,16	25,66	1 031
B-SP	0,28	1,68	0,53	0,56	83	<0,3	18,35	1,92	7,62	0,09	35,96	859
C-CZ	0,18	2,08	0,90	1,03	141	0,39	34,51	4,95	2,33	0,04	46,98	522
C-FR	0,04	2,52	0,81	0,84	104	0,51	47,74	3,24	2,59	0,04	33,17	638
C-PT	0,31	2,87	0,82	1,97	226	0,34	44,83	5,97	1,38	0,06	47,60	654
C-SP	0,14	1,50	0,80	0,65	95	<0,3	24,22	3,31	1,87	0,04	60,12	477
D-CZ	0,19	2,11	1,26	1,50	117	0,28	199,55	4,88	2,91	0,05	27,14	392
D-FR	0,11	3,02	1,28	1,18	106	0,56	277,45	6,00	2,97	0,06	28,80	519
D-PT	0,26	1,98	1,58	2,50	208	0,23	210,56	7,10	1,39	0,09	39,17	386
D-SP	0,20	1,48	0,87	0,76	76	<0,3	136,95	3,44	1,99	0,06	39,11	321
E-CZ	0,15	3,16	0,58	0,78	116	0,32	28,17	2,59	123,87	0,09	18,01	2 430
E-FR	0,04	3,91	0,74	0,52	98	0,25	37,16	2,44	117,37	0,10	16,14	2 598
E-PT	0,24	4,08	0,84	1,67	204	0,21	33,04	4,36	114,95	0,13	27,53	2 115
E-SP	0,17	2,44	0,62	0,52	93	<0,3	20,48	1,75	110,72	0,10	51,66	1 679

For laboratory PT data were revised considering the % of recovery of the certified reference material (blanc subtracted excepted for Hg).

Coefficient of variation (CV = SD/mean) for each metal(oid) were calculated for the 4 laboratories): if the 4 laboratories are considered 45 % of the CV were lower than 30 % while without the PT laboratory, 73 % of CV were lower than 30 % (see Table H.13).

H.4.2.3.2 If results are interpreted as stated in 8.2.1. (i.e. considering the TGV value): Expressing the data on the basis of accumulation quotient (AQ = measured concentration in snails/TGV; Tables H.10) allows to take in consideration the usual internal concentration of unexposed snails and to calculate an index that synthetize the transfer of several ME.

On plot A, the least contaminated one, the SET value is quite low; Mo and Hg showed the highest AQ followed by As. For this metalloid, only laboratory PT found an anomalous transfer. The SET value is low, between 0 and 4 except for laboratory PT (22) in relation with higher AQ of As and Hg.

For plot B, Mo, Hg and to a lower extent As and Sb present the highest AQ. Laboratory CZ found a higher SET mainly due to the AQ for Cr which was not found by the 3 other laboratories. For SP the SET is the lowest, possibly due to the generally lower % of recovery of most of the ME for this laboratory (data not revised with the % of recovery notably because for Mo no data were available). For 3 on the 4 laboratories the SET value is between 16 and 22.

For plots C and D, Mo mainly drives the SET value.

Plot D showed the highest value of AQ for Mo. It is the first time that such AQ values were found in snails. All the laboratories found the highest SET value on this plot.

Plot E mainly causes high AQ values for Pb and Mo, and to a lower extent for Cd, Sb and Zn.

Table H.10 — Comparison of the AQ (accumulation quotient) and SET (Sum of the excess of transfer) between plots and laboratories

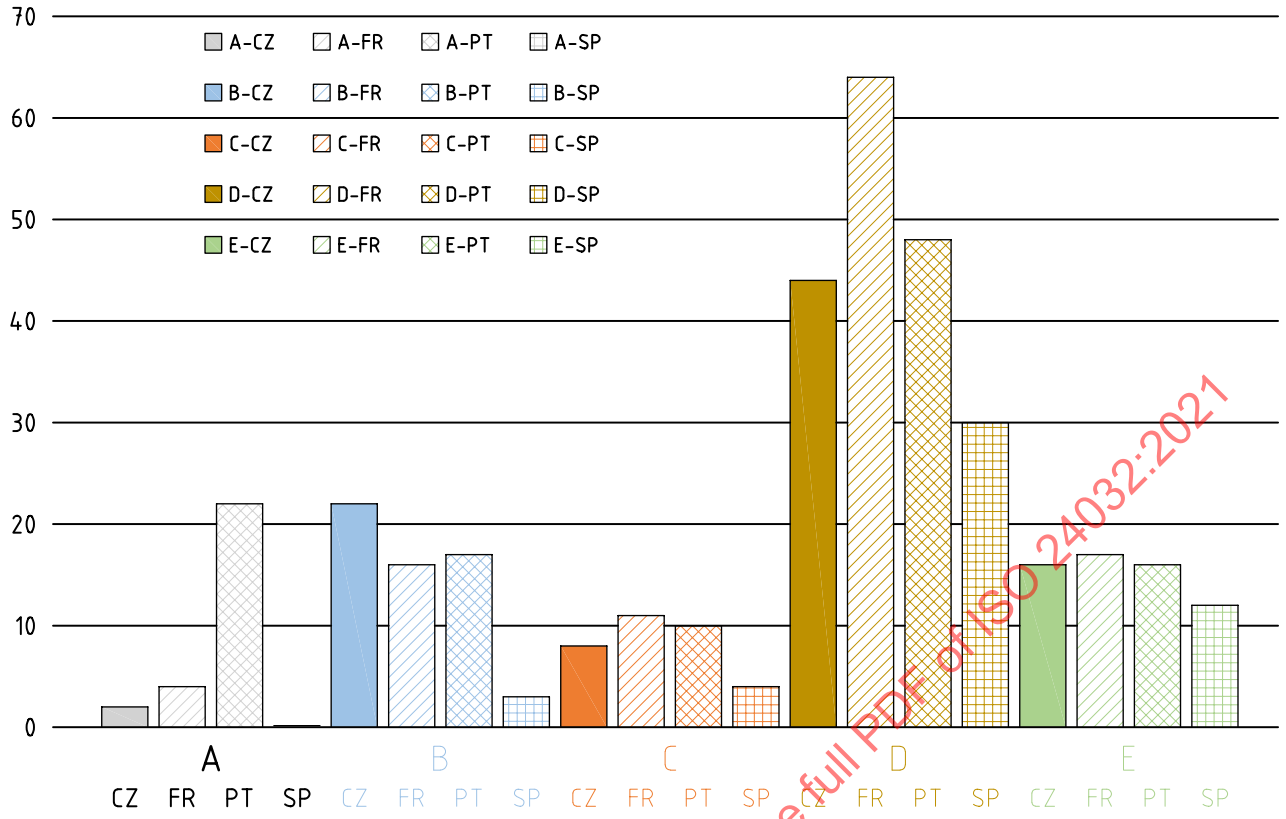
AQ<1=1	As	Cd	Co	Cr	Cu	Hg	Mo	Ni	Pb	Sb	Sr	Zn	SET
A-CZ	1,00	1,00	1,00	1,00	1,00	1,58	2,08	1,13	1,00	1,00	1,00	1,00	2
A-FR	1,02	1,00	1,00	1,00	1,00	2,55	3,10	1,00	1,00	1,00	1,00	1,00	4
A-PT	14,88	1,00	1,00	1,00	1,12	7,45	2,38	1,00	1,00	1,38	1,00	1,00	22
A-SP	1,05	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	0
B-CZ	1,18	1,11	1,00	7,62	1,00	3,07	14,13	1,00	1,00	1,00	1,00	1,00	22
B-FR	1,17	1,52	1,00	1,00	1,00	1,36	16,00	1,00	1,00	1,32	1,00	1,00	16
B-PT	2,64	1,49	1,00	1,00	1,08	1,95	13,71	1,00	1,00	2,00	1,00	1,00	17
B-SP	1,00	1,00	1,00	1,00	1,00	1,00	4,14	1,00	1,00	1,14	1,00	1,00	3
C-CZ	1,00	1,00	1,00	1,00	1,00	1,96	7,79	1,00	1,00	1,00	1,00	1,00	8
C-FR	1,00	1,11	1,00	1,00	1,00	2,54	10,78	1,00	1,00	1,00	1,00	1,00	11
C-PT	1,00	1,26	1,00	1,00	1,23	1,70	10,12	1,14	1,00	1,00	1,00	1,00	10
C-SP	1,00	1,00	1,00	1,00	1,00	1,00	5,47	1,00	1,00	1,00	1,00	1,00	4
D-CZ	1,00	1,00	1,00	1,00	1,00	1,38	45,05	1,00	1,00	1,00	1,00	1,00	44
D-FR	1,00	1,33	1,00	1,00	1,00	2,80	62,63	1,14	1,00	1,00	1,00	1,00	64
D-PT	1,00	1,00	1,00	1,24	1,13	1,15	47,53	1,35	1,00	1,13	1,00	1,00	48
D-SP	1,00	1,00	1,00	1,00	1,00	1,00	30,92	1,00	1,00	1,00	1,00	1,00	30
E-CZ	1,00	1,39	1,00	1,00	1,00	1,60	6,36	1,00	9,60	1,14	1,00	1,63	16
E-FR	1,00	1,72	1,00	1,00	1,00	1,27	8,39	1,00	9,10	1,27	1,00	1,74	17
E-PT	1,00	1,80	1,00	1,00	1,11	1,05	7,46	1,00	8,91	1,63	1,00	1,42	16
E-SP	1,00	1,08	1,00	1,00	1,00	1,00	4,62	1,00	8,58	1,20	1,00	1,13	12

H.4.2.3.3 Main conclusion for metal(loid)s

All the laboratories detect ME concentrations in snails, showing their transfer from the environment (soil, humus, vegetation, air) to the snails, mainly for plots D, B-E and C, if the SET values are considered (see Figure H.4). Plot A, the less contaminated, causes the lowest SET (except for one laboratory).

The snails reveal low transfer of Cr, Cu, Ni from soil to their tissues although some plots were contaminated (mainly C and D). $CaCl_2$-extractable fractions (see Table H.1) of these metals is not correlated with the transfer to snails; the same trend is found for Zn in soils B and E that present close $CaCl_2$-extractable values (see Table H.1) but reached internal concentrations in snails (see Table H.9) two times higher for soil E.

It is also shown that the bioavailability of Mo is quite different between soils D and E: indeed, these plots present similar Mo concentration (about 500 $mg.kg^{-1}$) but probably due to its high content of OM, Mo is much less bioavailable to snails in soil E than in soil D (concentration in visceral mass: 30 $mg.kg^{-1}$ vs 200 $mg.kg^{-1}$).



Key

A plot A
B plot B
C plot C
D plot D
E plot E

Figure H.4 — Sum of the excess of transfer (Set index) for the 4 laboratories and the 5 studied plots

H.4.2.4 After exposure for PAHs

Two laboratories (CZ, FR) analysed PAH concentrations in snails. For each plot, 3 pools of 2 snails' visceral masses were analysed.

The CZ-lab detected fluorene (FLU) and phenanthrene (PHE) (see Table H.11). For the FR-lab, only fluoranthene (FLT) was quantifiable in snails of plot A1. PYR, ANT, ACY were detected in snails but the measured concentrations do not reflect those found in the soils (see Table H.3). It is found that the PAH mainly present in soils C and D (see Table H.3) were not transferred to snail's viscera. This is probably due to a low bioavailability of PAHs to snails in these soils rather than to a biotransformation of PAHs by snails. Indeed, it has been shown that snails have high capacity to accumulate high concentration of PAHs, for example, when exposed to soils polluted by coal tar[7].

Table H.11 — Mean concentration of PAH in snails for laboratory CZ and FR

FR			A			B			C			D			E		
	LD	LQ	A1	A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	D3	E1	E2	E3
mass(g)			5,25	3,62	2,76	1,82	2,93	3,06	4,61	4,34	3,41	3,03	4,32	5,41	3,55	2,3	2,73
NAP	1,52	4,56	ND	ND	ND	ND	ND	ND	ND	ND	0,50	1,50	ND	0,89	0,41	ND	ND
ACY	5,63	16,9	0,16	0,37	0,06	0,23	0,40	0,08	0,55	0,30	0,17	0,78	0,32	0,65	0,14	0,37	0,13
ACE	4,57	13,7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2,17	ND	ND
FLU	2,15	6,45	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0,01	0,78	ND	ND
PHE	1,4	4,2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ANT	3,21	9,63	0,45	0,37	0,29	0,66	1,01	0,45	0,55	0,24	0,40	0,64	0,43	0,02	ND	1,29	0,77
FLT	0,64	1,92	3,47	0,32	0,33	0,47	ND	ND	0,45	1,20	0,33	1,56	0,38	0,39	0,26	ND	ND
PYR	0,61	1,83	1,51	0,28	0,11	ND	ND	ND	0,12	0,45	0,58	1,04	0,35	0,48	0,61	ND	ND
BaANT	0,48	1,44	0,17	ND	ND	ND	ND	ND	ND	ND	ND	0,04	ND	ND	ND	ND	ND
CHY	0,49	1,47	1,42	ND	ND	ND	ND	ND	0,00	0,49	ND	0,36	0,03	ND	ND	ND	ND
BbFLT	0,76	2,28	0,74	ND	ND	ND	ND	ND	ND	ND	ND	0,17	ND	ND	ND	ND	ND
BkFLT	0,67	2,01	0,30	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
BaPYR	2,15	6,45	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IcdPYR	1,75	5,25	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
dBahANT	1,36	4,08	0,28	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
BghiPL	1,77	5,31	0,27	ND	ND	ND	ND	ND	ND	ND	ND	0,80	0,69	0,04	ND	ND	ND
Sum (µg.kg^{-1} fw)			**8,77**	**1,34**	**0,78**	**1,36**	**1,42**	**0,53**	**1,68**	**2,68**	**1,98**	**6,88**	**2,21**	**2,48**	**4,37**	**1,66**	**0,90**
Mean (µg.kg-1 fw)			3,6			1,1			2,1			3,9			2,3		
SD			4,5			0,5			0,5			2,6			1,8		
Mean (µg.kg^{-1} dw)			23,6			7,2			13,7			25,0			15,0		
CZ			**A**			**B**			**C**			**D**			E		
		LQ	A1 A4	A2 A4	A3 A4	B1	B2 B4	B3 B4	C1 C4	C3 C4		D1 D4	D2 D4	D3 D4	E1 E4	E2 E4	E3 E4
FLU		0,22	ND	0,62	1,50	2,24	0,42	0,30	ND	ND		ND	ND	ND	ND	ND	ND
PHE		0,31	2,61	1,46	8,65	3,69	1,26	0,44	ND	0,60		0,09	0,29	0,05	0,44	ND	0,03
Sum (µg.kg^{-1} fw)			**2,61**	**2,08**	**10,15**	**5,93**	**1,68**	**0,74**	**ND**	**0,60**		**0,09**	**0,29**	**0,05**	**0,44**	**ND**	**0,03**
Mean (µg.kg^{-1} fw)			4,94			2,78			0,34			0,14			0,23		
SD			4,51			2,77			0,36			0,13			0,29		
Mean (µg.kg^{-1} dw)			36,90			20,78			,56			1,08			1,74		

NOTE Calculation of the fresh weight (fw) to dry weight (dw) based on 15,4 % and 13,4 % of dw in the visceral mass respectively for CZ and FR. The line "Mass" shows the mass in of the fresh viscera (g) used to performed analytical measurements.

H.5 Conclusions of the ring test on in situ caging of snails

All the participants to the ring test easily handled snails at the different steps. Separation of the visceral mass after defrosting snails was performed with no particular difficulty by the 4 laboratories even for

PT that could not be present in France for the demonstration (a short movie was send to this laboratory to show how to proceed).

Despite the extremely high temperature variation (from 2,7 °C up to 33,3 °C) observed during the ring test in the field, survival was acceptable and homogeneous among plots.

Overall, the conclusion of the participants that perform chemical analysis are consistent and allow evidencing the current bioavailability to snails of metal(loid)s on site.

Due to some differences in the preparation of samples for chemical analysis and for the equipment used, some discrepancies (Tables H.8 and H.13) appeared between laboratories highlighting the necessity to share a certified reference material. Such discrepancy between chemical analysis are often encountered. However, this was not the main scope of the ring test, which aimed mainly to evaluate the reproducibility of the method of in situ caging of the snails and the subsequent preparation of the snails to assess bioaccumulation of contaminants. Different snails were analysed by the 4 laboratories as it was not possible (due to limited mass) to share the tissues for such purpose; thus, the differences can be the result of inter-individual variability itself originating from individual differences on a genetical and behavioural level (feeding, movement, etc.) on site. Such an experimental allowed to run the method from start to the final step (chemical analysis) as any potential user will do to obtain a diagnostic on a contaminated site.

All the laboratories evidenced that plot D which was not the most contaminated presents the highest transfer of MEs to snails (mainly Mo and Pb) whereas contaminants of the most polluted plot (plot E) were less bioavailable to snails.

According to the data of the 2 laboratories analysing the PAH, a low in situ bioavailability of these compounds was demonstrated.

Table H.12 — Median, mean, SD of the concentrations of metal(loid)s measured in snails after exposure by the 4 laboratories (CZ, FR, PT, SP) on plots A to E (mg.kg^{-1} dw)

µc		As	Cd	Co	Cr	Cu	Hg	Mo	Ni	Pb	Sb	Sr	Zn
A-CZ	MEDIAN	0,24	1,49	1,10	1,99	119	0,32	9,20	5,93	1,41	0,04	31,88	605
	MEAN	0,29	1,50	1,02	2,95	121	0,62	9,50	6,96	1,74	0,04	34,89	586
	SD	0,16	0,16	0,29	2,44	17	0,69	2,32	3,09	1,07	0,01	15,96	127
B-CZ	MEDIAN	0,36	2,53	0,87	15,31	114	0,61	62,58	4,55	12,67	0,08	22,89	996
	MEAN	0,40	2,57	0,93	15,31	114	0,58	66,94	8,49	47,33	0,09	27,61	1344
	SD	0,17	0,64	0,32	19,46	21	0,25	49,33	10,57	64,09	0,03	16,94	823
C-CZ	MEDIAN	0,18	2,08	0,90	1,03	141	0,39	34,51	4,95	2,33	0,04	46,98	522
	MEAN	0,19	2,37	1,30	1,08	156	0,40	53,22	5,40	4,88	0,05	48,95	689
	SD	0,10	1,00	0,82	0,23	63	0,17	42,23	1,25	5,32	0,03	14,30	291
D-CZ	MEDIAN	0,19	2,11	1,26	1,50	117	0,28	199,55	4,88	2,91	0,05	27,14	392
	MEAN	0,17	2,02	1,09	1,44	125	0,28	206,56	4,61	2,99	0,05	28,49	425
	SD	0,09	0,37	0,46	0,68	25	0,09	68,77	2,38	1,30	0,01	4,20	180
E-CZ	MEDIAN	0,15	3,16	0,58	0,78	116	0,32	28,17	2,59	123,87	0,09	18,01	2430
	MEAN	0,16	3,50	0,64	0,81	116	0,39	30,53	2,58	112,89	0,08	18,55	2373
	SD	0,09	1,09	0,27	0,19	20	0,20	16,85	0,43	65,30	0,03	3,93	476
Tort-CZ	MEAN	70,0	43,3	1,11	1,3	488	0,281	3,4	4,7	0,27	0,062	34,4	138
	SD	1,3	0,5	0,01	0,1	10	0,002	0,1	0,5	0,10		0,7	2
µc		**As**	**Cd**	**Co**	**Cr**	**Cu**	**Hg**	**Mo**	**Ni**	**Pb**	**Sb**	**Sr**	**Zn**
A-FR	MEDIAN	0,31	1,87	0,60	0,31	123	0,51	13,73	2,03	1,46	0,05	20,66	357
	MEAN	0,28	1,87	0,68	0,35	119	0,64	13,77	2,06	1,42	0,05	22,62	410
	SD	0,16	0,14	0,34	0,15	25	0,36	3,19	0,29	0,31	0,01	7,66	133
B-FR	MEDIAN	0,36	3,46	0,62	0,44	118	0,27	70,90	2,68	10,86	0,11	19,54	902
	MEAN	0,36	3,62	0,64	0,54	111	0,32	96,20	2,63	42,59	0,10	24,81	1320
	SD	0,18	1,11	0,24	0,19	15	0,09	72,17	0,44	51,40	0,03	14,25	780
C-FR	MEDIAN	0,04	2,52	0,81	0,84	104	0,51	47,74	3,24	2,59	0,04	33,17	638
	MEAN	0,08	2,55	1,36	1,00	102	0,48	69,26	4,16	4,07	0,05	41,94	756
	SD	0,06	0,55	0,98	0,54	11	0,10	51,41	2,19	3,29	0,03	24,79	314
D-FR	MEDIAN	0,11	3,02	1,28	1,18	106	0,56	277,45	6,00	2,97	0,06	28,80	519
	MEAN	0,16	3,17	1,38	1,27	115	0,56	356,41	6,05	3,09	0,07	30,64	510
	SD	0,14	0,69	0,86	0,70	44	0,23	226,84	3,59	1,06	0,01	8,75	106
E-FR	MEDIAN	0,04	3,91	0,74	0,52	98	0,25	37,16	2,44	117,37	0,10	16,14	2598
	MEAN	0,10	4,01	0,80	0,50	103	0,25	35,47	2,48	110,49	0,10	17,64	2404
	SD	0,10	1,26	0,23	0,08	20	0,05	11,80	0,74	42,54	0,03	5,82	522
Tort-FR	MEDIAN	68,08	42,66	0,95	1,23	434,10	0,30	3,45	4,22	0,18	0,06	33,48	125
	MEAN	67,09	42,41	0,95	1,20	429,43	0,30	3,42	4,27	0,18	0,06	33,61	125
	SD	1,83	1,22	0,05	0,06	13,33	0,01	0,11	0,15	0,01	0,004	1,12	1,7
		As	**Cd**	**Co**	**Cr**	**Cu**	**Hg**	**Mo**	**Ni**	**Pb**	**Sb**	**Sr**	**Zn**
A-PT	MEDIAN	4,57	1,80	0,69	1,66	207	1,49	10,56	4,73	0,99	0,108	26,07	559
	MEAN	7,74	1,83	0,67	1,72	211	2,43	11,29	4,49	1,12	0,12	25,78	533
	SD	7,94	0,25	0,33	0,49	10	2,61	3,28	0,85	0,40	0,054	3,72	217

Table H.12 *(continued)*

µc		As	Cd	Co	Cr	Cu	Hg	Mo	Ni	Pb	Sb	Sr	Zn
B-PT	MEDIAN	0,81	3,39	0,62	1,80	200	0,39	60,74	4,85	3,29	0,158	25,66	1031
	MEAN	0,96	3,40	0,68	1,76	200	0,39	74,03	5,17	8,62	0,15	26,87	1134
	SD	0,32	0,62	0,25	0,20	15	0,08	44,43	1,31	9,35	0,041	6,64	449
C-PT	MEDIAN	0,31	2,87	0,82	1,97	226	0,34	44,83	5,97	1,38	0,061	47,60	654
	MEAN	0,33	2,93	0,95	1,99	239	0,33	83,84	5,95	1,70	0,08	54,07	616
	SD	0,13	0,74	0,46	0,55	58	0,06	80,52	1,55	1,03	0,041	21,95	114
D-PT	MEDIAN	0,26	1,98	1,58	2,50	208	0,23	210,56	7,10	1,39	0,091	39,17	386
	MEAN	0,27	2,04	1,41	2,60	204	0,22	206,09	6,91	1,38	0,10	41,04	422
	SD	0,10	0,47	0,63	0,66	14	0,05	79,29	3,30	0,19	0,030	5,55	143
E-PT	MEDIAN	0,24	4,08	0,84	1,67	204	0,21	33,04	4,36	17,72	0,129	27,53	2115
	MEAN	0,27	4,07	0,81	2,21	199	0,22	31,71	4,55	21,83	0,14	37,09	2304
	SD	0,15	1,00	0,18	1,15	27	0,07	10,27	1,06	14,28	0,062	18,81	826
Tort-PT	MEDIAN	60,66	42,37	1,32	3,21	543	0,26	4,28	7,11	1,01	0,09	37,16	140
revised according to the % recovery	MEAN	60,14	42,31	1,16	3,29	537	0,29	4,27	7,14	1,04	0,09	36,58	139
	SD	1,34	0,36	0,33	0,29	15,95	0,11	0,55	0,61	0,44	0,01	1,04	6,47
Plot		**As**	**Cd**	**Co**	**Cr**	**Cu**	**Hg**	**Mo**	**Ni**	**Pb**	**Sb**	**Sr**	**Zn**
A-SP	MEDIAN	0,32	1,27	0,76	0,37	105	<0,3	3,45	1,67	1,10	0,05	54,70	393
	MEAN	0,31	1,16	0,78	0,41	107	<0,3	3,36	1,77	1,32	0,05	51,96	439
	SD	0,10	0,31	0,17	0,14	36		1,00	0,75	0,61	0,01	28,44	146
B-SP	MEDIAN	0,28	1,68	0,53	0,56	83	<0,3	18,35	1,92	7,62	0,09	35,96	859
	MEAN	0,28	1,97	0,63	0,61	75	<0,3	50,84	2,05	37,29	0,11	45,42	1058
	SD	0,08	0,72	0,33	0,27	19		48,69	0,62	48,72	0,07	20,86	609
C-SP	MEDIAN	0,14	1,50	0,80	0,65	95	<0,3	24,22	3,31	1,87	0,04	60,12	477
	MEAN	0,13	1,63	1,06	0,69	107	<0,3	45,23	3,58	3,01	0,06	69,30	515
	SD	0,04	0,54	0,57	0,17	52		48,69	0,80	2,51	0,03	35,12	161
D-SP	MEDIAN	0,20	1,48	0,87	0,76	76	<0,3	136,95	3,44	1,99	0,06	39,11	321
	MEAN	0,20	1,52	1,00	0,83	80	<0,3	182,46	3,39	2,22	0,06	43,26	342
	SD	0,08	0,34	0,61	0,25	11		116,91	1,52	1,09	0,01	10,61	92
E-SP	MEDIAN	0,17	2,44	0,62	0,52	93	<0,3	20,48	1,75	110,72	0,10	51,66	1679
	MEAN	0,18	2,59	0,56	0,55	99	<0,3	19,33	1,79	95,44	0,09	46,00	1818
	SD	0,09	0,84	0,13	0,16	18		8,23	0,55	50,01	0,04	19,03	733
TORT-SP	MEDIAN	55,32	33,79	1,21	1,16	379	0,36		3,95	BDL	0,07	33,33	120
	MEAN	54,92	33,51	1,32	1,29	378	0,42		4,01	BDL	0,07	33,24	117
	SD	0,86	0,63	0,22	0,27	7,82	0,20		0,25	BDL	0,02	0,38	5,40
TORT= reference material with certified concentrations of metal(oid)s (except for Co: indicative value and Sb)													