INTERNATIONAL STANDARD

ISO 16198

First edition
2015-01-15

Soil quality — Plant-based test to assess the environmental bioavailability of trace elements to plants

Qualité du sol — Test végétal pour l'évaluation de la biodisponibilité environnementale des éléments traces pour les végétaux

Reference number
ISO 16198:2015(E)

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Published in Switzerland

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Foreword

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The committee responsible for this document is ISO/TC 190, *Soil quality*, Subcommittee SC 7, *Soil and site assessment*.

Introduction

One of the main objectives of ISO 17402 is to define a conceptual framework of the bioavailability of contaminants in soils and soil materials, and to provide a guidance for the selection of methods able to be standardized for the measurement of bioavailability. Bioavailability was thus defined according to three successive steps:

a) “environmental availability”;

b) “environmental bioavailability”;

c) “toxicological bioavailability”.

The environmental bioavailability is consequently a prerequisite to the assessment of the toxicological bioavailability and is directly related to the impact of pollutants on major functions of soil in the ecosystem and more particularly to habitat and retention functions.

Environmental bioavailability can be estimated with either chemical or biological methods. In the case of trace elements, chemical methods are usually the cheapest, easy to perform, and some of them are already standardized at national or international level (e.g. ISO 19730). However, chemical methods which, strictly speaking, measure the environmental availability in soils have to be correlated with biological measurements before being used as indicators of environmental bioavailability. Whatever chemical methods are employed, none are designed per se to address the diversity of responses observed among different plant species or cultivars which can be attributed to a) the uptake behaviour of plants (i.e. sensitive, tolerant, accumulator, or hyper-accumulator of trace elements) and/or b) the ability of plants to alter the biological, physical and physical-chemical properties of their “bio-influenced zone” at the soil-root interface, i.e. the so-called rhizosphere. It could alternatively, be suggested to apply chemical methods directly to the rhizosphere but the sampling of the rhizosphere is definitely too tedious to be applied routinely.

For biological methods, four standardized biotests account for rhizosphere processes as they are based on soil-grown plants (ISO 11269-1, ISO 11269-2, ISO 17126, and ISO 22030). However, these were only designed to predict trace element phytotoxicity, i.e. the toxicological bioavailability. In these biotests, roots grow directly in the soil, therefore requiring a tedious washing procedure to reliably measure trace elements accumulated in the roots. Indeed, the amount of trace elements accumulated in shoots of non-accumulator plant species is not sufficiently sensitive to be used for assessing the environmental bioavailability of trace elements compared to the amount accumulated in the whole plant, roots included. Thus, there is still a need to develop biological methods accounting for rhizosphere processes and enabling to include the root compartment in order to properly estimate the environmental bioavailability of trace elements to plants.

Consequently, the present International Standard introduces a biotest based on the growing of roots in contact with the soil but without penetrating it. Although this experimental design is partly artificial, it enables a fair comparison of the bioavailability of trace elements between tested soils. In addition, the end point measured can be more directly related to the measurement of the environmental availability than any end point based on the measurement of toxicity.

Soil quality — Plant-based test to assess the environmental bioavailability of trace elements to plants

1 Scope

This International Standard specifies the plant-based test, hereafter called the biotest. It enables estimation of the environmental bioavailability of trace elements to plants either basically as the concentration in shoots and roots or in a more integrative way as the net uptake flux in plants. The biotest procedure includes two successive steps: (i) a pre-growth of plants in hydroponics and (ii) a growth of plants in contact with soil samples. The concentration in shoots and roots as well as the net uptake flux of trace elements in plants are determined at the end of the second step of the biotest procedure.

This biotest is applicable to the assessment of environmental bioavailability of trace elements to plants, more particularly to agricultural plants, in soils or soil materials under oxic conditions, considering that

— three plant species (cabbage, *Brassica oleracea*; tall fescue, *Festuca arundinacea*; tomato, *Lycopersicon esculentum*; 7.1) are suggested in the standardized biotest procedure, but additional target-plant species can also be used (see 7.1, Annex A), and

— the standardized biotest procedure is validated for a range of trace elements including arsenic (As), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), lead (Pb), nickel (Ni), and zinc (Zn), but additional trace elements can also be accounted for (see Annex A).

The biotest can be applied to soils and soil materials, including soils amended before or after field sampling with composts, sludges, wastewaters, and other (waste) materials.

NOTE 1 This biotest is not designed to assess the environmental bioavailability of trace elements that are prone to volatilisation or resulting from uptake occurring in plant leaves following, e.g. atmospheric fallout.

NOTE 2 This biotest is not designed to assess the environmental bioavailability to plants of organic contaminants. A similar experimental procedure could be used but the physical separation between plant roots and soil using a polyamide mesh needs to be adapted to avoid organic contaminant sorption on the mesh.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

ISO 10390, *Soil quality — Determination of pH*

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

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

ISO 11277, *Soil quality — Determination of particle size distribution in mineral soil material — Method by sieving and sedimentation*

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

3 Terms and definitions

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

3.1
contaminant
substance or agent present in the soil as a result of human activity

[SOURCE: ISO 11074:2005, 3.5.1]

Note 1 to entry: There is no assumption in this definition that harm results from the presence of the contaminant

3.2
environmental availability
fraction of contaminant physico-chemically driven by desorption processes potentially available to organisms

[SOURCE: ISO 17402:2008, 3.4]

3.3
environmental bioavailability
fraction of the environmentally available compound which an organism takes up through physiologically driven processes

[SOURCE: ISO 17402:2008, 3.5]

3.4
habitat function
ability of soil/soil materials to serve as a habitat for micro-organisms, plants, soil-living animals, and their interactions (biocenosis)

[SOURCE: ISO 11074:2005, 3.4.3]

3.5
trace element
chemical element in soil occurring at concentration generally less than 100 mg kg^{-1}

Note 1 to entry: Given according to Reference [16].

3.6
retention function
ability of soil/soil materials to adsorb pollutants in such a way that they cannot be mobilized via the water pathway and translocated into the terrestrial food chain

[SOURCE: ISO 11074:2005, 3.4.13]

3.7
rhizosphere
volume of soil around living roots that is influenced by root activities

Note 1 to entry: Given according to Reference [17].

3.8
soil
upper layer of the earth's crust transformed by weathering and physical/chemical and biological processes. It is composed of mineral particles, organic matter, water, air, and living organisms organized in genetic soil horizons

[SOURCE: ISO 11074:2005, 2.1.8]

3.9
soil material
material coming from soil and displaced and/or modified by human activity, including excavated soil, dredged materials, manufactured soils, and treated soils and fill materials

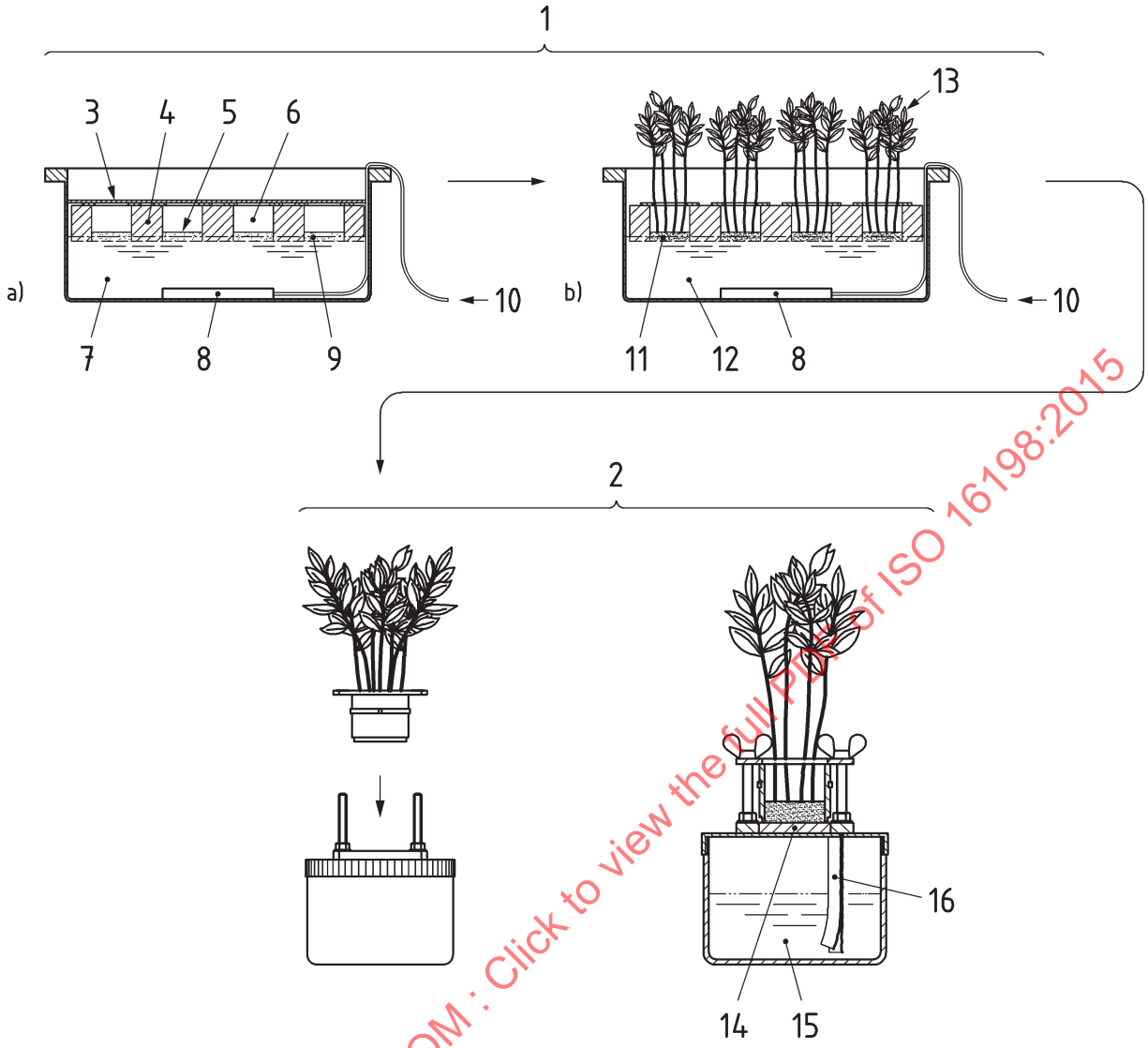
[SOURCE: ISO 17402:2008, 3.16]

3.10
toxicological bioavailability
internal concentration of pollutant accumulated and/or related to a toxic effect

[SOURCE: ISO 17402:2008, 3.18]

4 Principle

This International Standard describes the experimental procedure of the biotest developed initially by References [18], [19], and [20]. This biotest consists of two successive steps of plant growth (see Figure 1). During the first step (i.e. preculture period), plant seedlings are grown in hydroponics for 14 d to achieve an adequate plant biomass and a dense, planar root mat. During the second step (i.e. test culture period), the root mat of pre-grown plants is put in contact for 8 d with a 6 mm-thick layer of soil sample sieved to 2 mm.



Key

a	seed germination (7 d)	8	air diffuser
b	seedling pre-growth (7 d)	9	30-µm mesh
1	preculture period in hydroponics – 14 d	10	air
2	test culture period soil-plant contact – 8 d	11	root mat
3	aluminium foil	12	nutrient solution 2
4	floating platform	13	shoots
5	seeds	14	soil layer (6 mm thick)
6	plant pot	15	nutrient solution 3
7	nutrient solution 1	16	filter paper wicks

Figure 1 — The two-step procedure of the biotest

A set of control plants is harvested at the end of the preculture period in hydroponics to determine the pools of trace elements in plant shoots and roots before exposure to soil. Whole plants (shoots and roots) are then harvested at the end of the test culture period. Biomasses and trace element concentrations in shoots and roots are determined. The end points of the biotest are a) the concentration of trace elements in shoots and roots at the end of the test culture period and b) the net uptake flux of trace elements in the whole plants during the test culture period. If these end points are usually correlated,[21] the uptake flux is expected to be more representative of the trace element bioavailability to plants during the test

culture period (i.e. the exposure to tested soils) as, conversely to concentrations, the uptake flux does not include the portion of trace elements taken up during the preculture period (11.1).

As plant growth during the pre-culture period is usually sufficient to prevent the occurrence of phytotoxic symptoms induced by adverse soil chemical properties or excessive accumulation of trace elements in plant, the biotest enables a fair comparison of trace element bioavailability over a broad range of soils, including heavily contaminated soils.

5 Laboratory apparatus

The following equipment shall be used. All equipment that comes into contact with the sample (soils, plants, or reagents) shall not adsorb substantially trace elements and shall not contaminate the sample.

5.1 Sieving equipment, nominal screen size 2 mm.

5.2 Crushing equipment, jaw crusher and cutting device.

5.3 Balance, with an accuracy of at least 100 mg.

5.4 Balance, with an accuracy of at least 1 mg.

5.5 Growth chamber, suitable for maintaining specific climatic conditions as specified in 7.4.

5.6 Ventilated oven, suitable for drying soil or soil material at 25 °C and shoots and roots at 50 °C.

5.7 Scissors, with blades made of zirconium oxide.

5.8 Grinder and marbles, made of zirconium oxide.

6 Reagents

6.1 General

Use reagents of analytical grade purity with a concentration of investigated trace elements (e. g. As, Cd, Co, Cr, Cu, Ni, Pb, and Zn) lower or equal to 5 mg kg^{-1}. Water used shall comply with grade 3 according to ISO 3696.

6.2 Water, distilled or demineralized with a specific conductivity of at most 5 µS cm^{-1} at 25 °C and a pH within the range 5,0 to 7,5.

6.3 Hydrogen peroxide (H_2O_2, 34,01 g mol^{-1}).

6.4 Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$, 147,07 g mol^{-1}).

6.5 Boric acid (H_3BO_3, 61,83 g mol^{-1}).

6.6 Calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$, 236,15 g mol^{-1}).

6.7 Potassium nitrate (KNO_3, 101,1 g mol^{-1}).

6.8 Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$, 246,48 g mol^{-1}).

6.9 Potassium phosphate (KH_2PO_4, 136,09 g mol^{-1}).

6.10 Ethylenediaminetetraacetic acid iron(III) sodium (NaFe(III)EDTA, 367,05 g mol^{-1}).

6.11 Copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$, 170,48 g mol^{-1}).

6.12 Manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$, 197,91 g mol^{-1}).

6.13 Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$, 287,54 g mol^{-1}).

6.14 Sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$, 241,95 g mol^{-1}).

7 Biological and growing apparatus

7.1 Plant species

The three following species, i.e. cabbage (*B. oleracea*), tall fescue (*F. arundinacea*), and tomato (*L. esculentum*), are used during the biotest deployment. These three plant species were selected among non-accumulator, common agricultural species for their ability to collectively maximize the phytoavailability of trace elements (more specifically that of As, Cd, Cu, Pb, and Zn) in soils exhibiting a broad range of physical-chemical properties and origin of trace element.[21] For each of the three species, the following cultivars are recommended: castelard for *B. oleracea*, calina for *F. arundinacea*, and fline for *L. esculentum*. However, in the only case where recommended cultivars are not commercially available other cultivars may be used provided that they exhibit an adequate growth and homogeneous root mat during the biotest procedure for the different soils tested. Specify in the test report the reasons for selecting different cultivars than those recommended. For a given cultivar, seeds used for a single set of experiment shall come from the same batch.

Additional species can be selected, e.g. species with specific physiological characteristics or with ecological, agricultural or economic significance in certain regions of the world or for specific site assessment, provided that these species exhibit an adequate growth and homogeneous root mat during the biotest procedure for the different soils tested. A list of plant species adapted to be grown in the biotest, but only partly validated for the standardized procedure, is given in Annex A. Specify in the test report the reasons for selecting additional species.

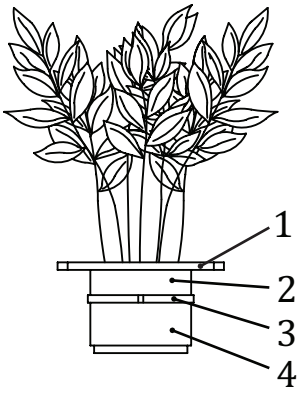
NOTE 1 The biotest procedure should be used with untreated seeds (i.e. not treated with any pesticide) as much as possible. If not feasible, specify it in the test report.

NOTE 2 If other cultivars than those recommended are used, it is to note that this can alter the biotest measurement in a similar extent than if different species were used.

7.2 Biotest apparatus

The following apparatus shall be used. Apparatus that comes into contact with the sample (soils, plants or reagents) shall not adsorb the component of interest and shall not contaminate the sample.

The plant-receiving pot (i.e. plant pot) is designed to contain the whole plants from the beginning of the preculture period to the end of the test culture period. The plant pot enables plants to develop a planar and dense root mat while maintaining a physical separation with the tested soil sample. The plant pot consists in a cylinder fitted to an upper plate at the top and closed at the bottom with a polyamide mesh (30 µm pore size) using an adjustable clamp (see Figure 2). The mesh shall be well tightened.

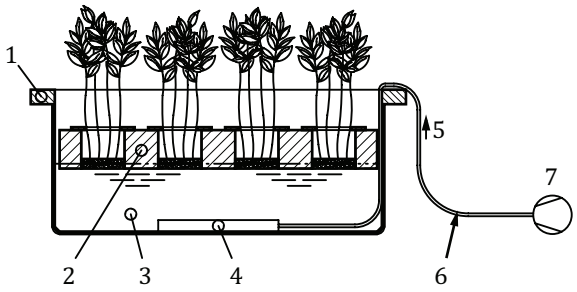


Key

1 upper plate
2 cylinder
3 adjustable clamp
4 30 µm polyamide mesh

Figure 2 — Plant-receiving pot assembly

The assembly used for the first step, i.e. the pre-culture period, is designed to enable seeds to germinate and for seedlings to develop a dense and planar mat of roots in hydroponics. This assembly enables a close contact between seeds or seedling roots and the nutrient solution. This assembly consists in a perforated platform floating over a tank-containing nutrient solution. Perforations passing through the floating platform enable to lodge plant pots. The nutrient solution is continuously aerated with a bubbling system composed of an air-pump, capillary tubes connected with derivations and diffusers diving into the nutrient solution (see Figure 3). The floatability of the platform is critical to ensure a homogeneous contact of all the plant pots in a tank with the aerated nutrient solution. This assembly also limits the exposure of the nutrient solution to light radiations, thereby avoiding algal development.

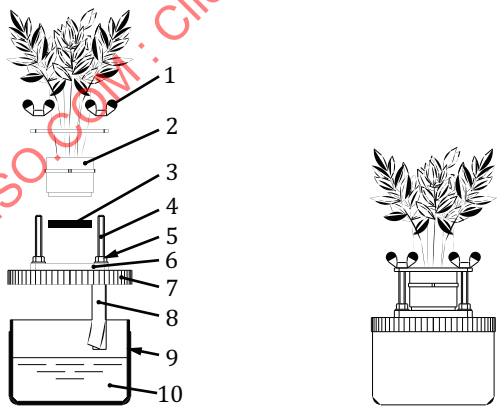


Key

1 tank
2 perforated floating platform
3 nutrient solution 1 or 2
4 air diffuser
5 air
6 tube
7 air-pump

Figure 3 — Assembly used for the preculture period

The assembly used for the second step, i.e. the test culture period, is designed to enable a close contact between the root mat and the soil layer. It is made of two parts and three filter paper wicks sandwiched in between: a) a contact assembly that firmly press the plant pot over the soil layer using fastenings and b) a 0,5 dm^3 screw-top jar filled with the nutrient solution 3 (see Figure 4). This assembly enables (i) the root mat to be maintained in contact with the whole surface area of the soil layer, and (ii) the filter paper wicks to remain fully moistened during the entire duration of the test culture period.



Key

1 wing nut
2 plant pot
3 soil layer (6 mm thick)
4 screw
5 screw nut
6 soil-receiving plate
7 screw-top
8 filter paper wicks
9 screw-top jar
10 nutrient solution 3

Figure 4 — Soil-plant contact assembly used for the test culture period

Except for the adjustable clamp, the filter paper wicks and the polyamide mesh, all the components of the plant-based test are reusable provided that they are subjected to a two-step washing, firstly, in hot water to remove adhering mucilage and microbial biofilms then secondly, in a volume fraction of 10 % HNO_3, followed by a thorough rinsing with distilled or demineralized water. A thorough list of the components along with specification is given in Table 1 for information and technical drawings are given in Annex B. Home-made apparatus may be built provided that the size of the different parts remains proportional and that component specification is similar.

Table 1 — Components of the biotest (informative)

Component		No.[a]	Quantity[b]	Specification
Plant pot	Cylinder	1	1	PVC, high density, high temperature, for food contact
	Upper plate	2	1	HDPE for food contact
	Polyamide mesh	/	1	100 × 100 mm, 30 µm pore size
	Adjustable clamp	/	1	180 mm × 2,4 mm
Preculture period	Tank[c]	3	1	12 dm^3, 400 mm×294 mm × 165 mm, Opaque HDPE for food contact
	Perforated floating platform	4	1	Extruded polystyrene platform
	Two outputs Air-pump	/	1	Air flow 100 - 200 $dm^3\ h^{-1}$ output^{-1}
	Capillary tube	/	2	PVC, int. diam. ca. 4 mm
	Derivation	/	1	If necessary
	Air-diffuser	/	2	Ceramic diffuser, 100 × 10 mm
Test culture period	Screwtop jar	5	1	Opaque and white PP for food contact
	Filter paper wick	/	3	Hardened ashless filter paper
	Soil-receiving plate	6	1	HDPE for food contact, 6 mm thickness
	Screw	/	4[d]	HDPE
	Screw nut	/	8[d]	HDPE
	Wing nut	/	4[d]	HDPE

[a] Component number as referenced in the technical drawings depicted in Annex B.

[b] For one item of each of the three biotest components (i.e. plant pot and apparatus for preculture and test culture periods).

[c] Adapted for 12 plant pots per tank filled with 6 dm^3 of nutrient solution.

[d] Only two screws can be used to reduce time-fitting, with only four screw nuts and two wing nuts.

7.3 Composition of the nutrient solutions

Three different nutrient solutions shall be prepared for the deployment of the biotest for (i) seed germination, (ii) seedling pre-growth in hydroponics [steps (i) and (ii) are included in the preculture period], and (iii) plant growth during the test culture period.

During seed germination (preculture period), the nutrient solution 1 shall be composed of 600 µmol·dm^{-3} $CaCl_2$ (6.4) and 2 µmol·dm^{-3} H_3BO_3 (6.5).

During seedling growth in hydroponics (preculture period), the nutrient solution 2 shall be prepared by adding the nutrients in the following order and concentration: 500 µmol·dm^{-3} KH_2PO_4 (6.9); 2 000 µmol·dm^{-3} KNO_3 (6.7); 2 000 µmol·dm^{-3} $Ca(NO_3)_2$ (6.6); 1 000 µmol·dm^{-3} $MgSO_4$ (6.8);

0,2 µmol·dm^{-3} $CuCl_2$ (6.11); 10 µmol·dm^{-3} H_3BO_3 (6.5); 2 µmol·dm^{-3} $MnCl_2$ (6.12); 1 µmol·dm^{-3} $ZnSO_4$ (6.13); 0,05 µmol·dm^{-3} Na_2MoO_4 (6.14); and 100 µmol·dm^{-3} NaFe(III)EDTA (6.10).

During plant growth in the test culture period, the nutrient solution 3 shall be prepared by adding the nutrients in the following order and concentration: 50 µmol·dm^{-3} KH_2PO_4 (6.9); 2 000 µmol·dm^{-3} KNO_3 (6.7); 2 000 µmol·dm^{-3} $Ca(NO_3)_2$ (6.6); and 1 000 µmol·dm^{-3} $MgSO_4$ (6.8).

Nutrient solutions shall be prepared with distilled or demineralized water (6.2).

7.4 Climatic conditions in the growth chamber

Plants can be grown in growth chamber, phytotron, or greenhouse provided that the climatic conditions comply with those listed in Table 2. Monitor and record the temperature and the relative humidity in the growth chamber in short intervals (<1 h). Measure and record the illumination at least at the beginning and at the end of the biotest procedure.

Table 2 — Climatic conditions for the deployment of the biotest (normative)

Climatic conditions	Day	Night
Photoperiod, in h	16	8
PAR[a], in µmol photons m^{-2} s^{-1}, at 400–700 nm	200 - 400[b]	/
Temperature, in °C	25 ± 3	20 ± 2
Relative humidity, in %	75 ± 5	70 ± 5

[a] Photosynthetically active radiation.

[b] At the canopy level.

8 Pre-treatment and analysis of soil or soil material sample

8.1 Sample size and particle size reduction

The test portion shall be prepared to have a grain size less than or equal to 2 mm. The material shall not be finely ground, which would likely alter the bioavailability. Material >2 mm of natural origin (e.g. stones, pebbles, twigs) shall be separated and discarded from the sample. If the laboratory sample cannot be crushed or sieved because of its water content, it is allowed, in this case only, to reduce the water content until the laboratory sample can be sieved. The drying temperature shall not exceed 25 °C.

NOTE Drying, even below 25 °C, of samples collected from volcanic soil exhibiting allophanic properties should be avoided. This kind of soil sample should be stored moist at 4 °C.

8.2 Analyses

Determine the following physical-chemical properties of soil or soil material:

— initial water content according to ISO 11465;

— water holding capacity according to ISO 11269-2;

— texture according to ISO 11277;

— pH in H_2O or $CaCl_2$ according to ISO 10390;

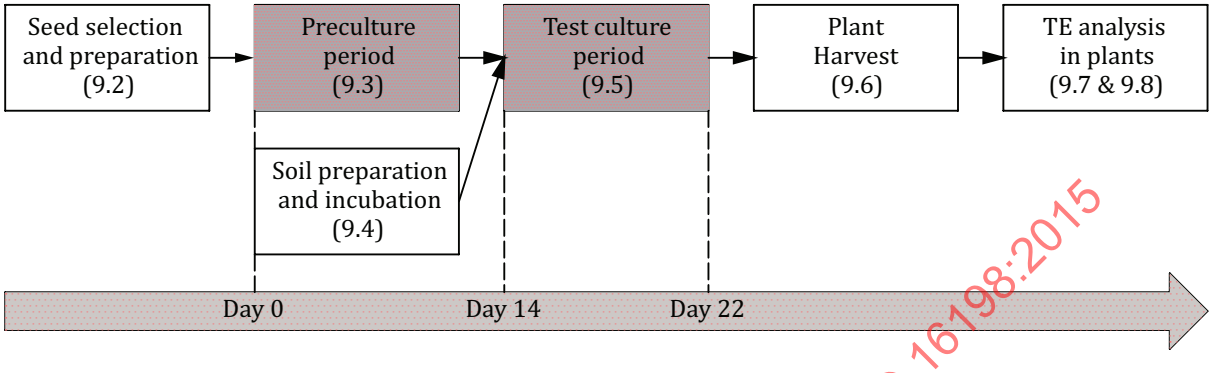
— organic carbon concentration according to ISO 10694.

NOTE The determination of texture, pH and organic carbon concentration is necessary for the interpretation of the results.

9 Experimental and analytical procedure

9.1 Overview of the procedure

Figure 5 introduces the different steps of the procedure described hereafter.



NOTE 1 Dark boxes stand for the two steps of the biotest procedure.

NOTE 2 TE means trace element.

Figure 5 — Sequential steps of the experimental and analytical procedures

9.2 Selection and preparation of seeds

Select the seeds to be used in the biotest (Annex C). Reject empty husks, damaged seeds, or unevenly shaped or sized seeds.

Store selected seeds in darkness and in dry conditions, ideally at 4 °C, to preserve germination capacity.

9.3 Preculture period: Germination and pre-growth in hydroponics

Before germination, sterilize the surface of the seeds by immersion for 10 min in a volume fraction of 6 % H_2O_2, then rinse the seeds three times with distilled or demineralized water.

Then put the required number of seeds at the surface of the polyamide mesh in the plant pot, according to the following density: 50, 80, and 40 seeds per plant pot for *B. Oleracea*, *F. arundinacea*, and *L. esculentum*, respectively. Additional seed density for a range of species tested with the standardized experimental procedure is given in Annex C.

Prepare for each soil or soil material tested a minimum of five replicates (i.e. plant pots) per plant species. In addition, prepare a minimum of five plant pots to serve as controls of trace element concentration in plants (particularly for the micronutrients Cu and Zn) upon completion of the preculture period. To obtain enough plant pots with a homogeneous plant biomass to serve as controls and for the test culture

period, multiply the number of initial plant pots to grow by a security factor of 1,2 at least. Calculate the total number of plant pots to prepare per plant species using Formula (1):

$$n_p = [(n_s \times n_r) + n_c] \times f \quad (1)$$

where:

n_p is the total number of plant pots to prepare per plant species;

n_s is the number of soil or soil material tested;

n_r is the number of replicates (minimum 5);

n_c is the number of plant pots which serve as control of the preculture period (minimum 5),

f is the security factor (minimum 1,2).

After sowing, plant pots are passed through the floating platform (12 plant pots per floating platform) placed at the top surface of 6 dm^3 of the nutrient solution 1 in the tank. Germinate the seeds for two to four days in darkness by covering the tank with an aluminium foil. When functional photosynthetic organs (green pigmentation on cotyledons or leaves) appear, the aluminium foil can be removed. Seedlings are then grown up to the end of the first week over the same nutrient solution. Nutrient solution 1 does not need to be renewed.

After the first week, seedlings are grown for one additional week at the top surface of 6 dm^3 of the nutrient solution 2. The nutrient solution 2 shall be renewed every third day. Randomize the plant pot position in the floating platform at each renewal. At each renewal, the preculture apparatus (tank and floating platform) shall be replaced and thoroughly washed in hot water and then in a volume fraction of 10 % HNO_3 followed by a thorough rinsing with distilled or demineralized water.

During these two weeks in hydroponics, nutrient solutions shall be aerated with a bubbling system.

9.4 Preparation and incubation of soil or soil material

In parallel with the preculture period, tested samples of soil or soil material are incubated for two weeks in darkness in similar climatic conditions as the plants (7.4). This incubation step should enable to reach chemical equilibrium in samples following the microbial flush due to moistening. After putting the adequate mass (as dry matter) of each sample in an adequate container, samples are packed to a soil density of ca. 1,2 g cm^{-3}, then moistened at 70 % of their water holding capacity with the nutrient solution 3. Determine the water holding capacity with a soil or soil material packed to a soil density similar to that of the soil laid in the soil-receiving plate (i. e. ca. 1,2 g cm^{-3}). An opening in each container should allow equilibrium with the ambient atmosphere. Moisture content shall be checked regularly and adjusted if necessary with the addition of the nutrient solution 3.

One day before the beginning of the test culture period, a fresh equivalent of 9 g of dry soil or soil material are laid down on each soil-receiving plate to obtain a soil layer of ca. 6 mm thickness and ca. 1,2 g cm^{-3} soil density. If a soil cannot be packed to a density of 1,2 g cm^{-3}, adjust the mass of soil to obtain in any case a soil layer thickness of ca. 6 mm and report this deviation in the test report (see Clause 13). Soil-receiving plates are then individually put over 0,5-dm^3 screwtop jars containing the nutrient solution 3 in which the filter paper wick is dipping. The water holding capacity of samples is subsequently increased up to 100 %. Soil-receiving plates shall be covered up to the beginning of the test culture period to maintain the soil samples in darkness and to limit evaporation.

9.5 Test culture period: Plant growth in contact with soil or soil material

At the end of the preculture period, visually select the replicates of plant pots showing homogenous plant biomasses, and discard the others. Thoroughly rinse the selected plant pots under a flow of distilled or demineralized water. Finally, put each plant pot in contact with a soil-receiving plate for 8 days using

the contact system. Nutrient solution 3 should be renewed every second day, excepted on the last day of the test culture period. Randomize soil-plant contact assembly at each renewal. At each renewal, replace over 0,5-dm^3 screw-top jars and thoroughly wash them in hot water, then in a volume fraction of 10 % HNO_3 followed by a thorough rinsing with distilled or demineralized water. During renewal, soil receiving systems should be handled cautiously to avoid any alteration of the wicks. The wicks should continuously be kept wet to ensure an adequate and continuous supply of water and nutrients to the roots. Any altered wick shall be reinforced by opening the soil receiving system and inserting a new wick.

9.6 Plant harvests

At the end of the preculture period, select visually and harvest the replicates of plant pots serving as control for each plant species. At the end of the test culture period, separate the plant pots from the soil receiving systems and harvest the plants.

Remove the polyamide mesh from the bottom of plant pots and rinse the plants (both shoots and roots) thoroughly under a flow of distilled or demineralized water. Thoroughly rinse the surface of the root mat that was previously in contact with soil or soil material, to avoid significant contamination by <30 µm soil particles (i.e. smaller than the pore diameter of polyamide mesh). Then remove the plants from the pots.

Depending on the scope of the investigation, two end points, i.e. trace elements concentration in shoots and roots and/or uptake flux in the whole plant, can be determined. If trace element concentrations in shoots and roots are considered as end points, shoots have to be separated from roots by cutting. Alternatively, if the flux of trace elements to plants is considered as the unique end point of interest, plants can be kept intact (i.e. shoots and roots pooled together). Seed husks should be removed and thrown out or pooled with roots for easiness provided they significantly modify neither the biomass, nor the trace element stock in root compartment.

Put the plant samples (i.e. shoots, roots or shoots, and roots pooled together) in adequate containers, then oven-dry at max. 50 °C. When steady biomass is achieved (usually after ca. 3 d), remove the plant samples from the oven and weigh with an accuracy of ±1 mg for biomass determination. Then conserve plant samples under dry conditions in their container until grinding.

To prevent any contamination, handle plant samples with laboratory gloves and separate shoots from roots with scissors made of zirconium oxide (5.7).

9.7 Grinding and digestion of shoots and roots

Grinding step is only necessary if a subsample is going to be digested. In that event, grind plant samples (i.e. shoots and roots individually or pooled together) with a grinder made of zirconium oxide (5.8). Alternatively, if digestion concerns the whole sample, cut the plant sample in small fragments with scissors made of zirconium oxide blades (5.7).

Use an adequate procedure to digest plant samples. The chosen procedure shall enable to thoroughly digest plant tissues including silicon dioxide (i.e. phytoliths) and other poorly soluble minerals. In case concentration of volatile trace elements (e.g. arsenic or selenium) has to be determined, adapt the procedure to avoid any loss of trace element by volatilisation during the digestion. An example of a digestion procedure is provided in Annex D.1.

Insert several blank samples during the digestion and the analytical procedures (9.8) to check that no contamination occurs and to account for background concentration of the matrix. Insert at least one standard reference plant material(s) to check the accuracy of the digestion and the analytical procedures (9.8). Select the adequate standard reference plant material(s) as regards to the list of trace elements investigated and the expected ranges of concentration.

9.8 Analytical determination

To determine the trace element concentration in plant samples, analyse the plant digests with a sufficiently sensitive analytical method capable to determine concentrations in the ng to mg g^{-1} (dry biomass) region. Use atomic absorption methods described in ISO 11047 and ISO 20280, inductively coupled plasma atomic emission spectrometry described in ISO 22036,or inductively coupled plasma mass spectrometry described in ISO 17294-2, or any other relevant technique. An example of the usual range of trace element concentration in the plant digests of a biotest experiment is given in Annex D.2.

10 Validity criteria

Meet all the following performance criteria during the test, otherwise discard the related plant pot.

— The seeds germinate homogeneously and do not exhibit mould during the first week of the preculture period.

— The seedlings exhibit a healthy root mat that covers the whole surface area of the bottom of the plant pot at the end of the preculture period.

— The seedlings exhibit homogeneous biomasses for a given species at the end of the preculture period.

— The shoots do not exhibit pest, disease, nutritional disorders, or phytotoxic symptoms (e.g. chlorosis, dessication, etiolation, scorching, necrosis, staining, or wilting) during the preculture and the test culture periods.

— The shoot and root biomasses significantly increase between the end of the preculture period and the end of the test culture period (12.2).

— The filter paper wicks are still fully moistened and are not torn at the end of the test culture period.

— The soil layer are still fully moistened at the end of the test culture period.

— The contact between the plant pot and the soil layer is maintained under steady pressure during the test culture period.

11 Assessment of the results

11.1 Determination of trace element concentrations and uptake flux in plants

Following the measurement of trace element concentration in plant digests, determine the trace element concentration in the plant sample (i. e. shoots and roots individually or pooled together) of each plant pot at the end of the preculture period (control plant pots) and the test culture period according to Formula (2):

$$C_p = C_d \times V_d / m_d \quad (2)$$

where

C_p is the trace element concentration in the plant sample, in micrograms per gram, µg g^{-1} (dry biomass);

C_d is the trace element concentration in the plant digest, in micrograms per cubic decimetre, µg dm^{-3};

V_d is the volume of the plant digest, in cubic decimetres, dm^3;

m_d is the dry mass of ground plant sample used for digestion, in grams, g.

The flux of trace element taken up in plants during the test culture period can be calculated according to Formulae (3) and (4) if shoots and roots are treated separately or Formula (5) if shoots and roots are pooled together:

$$F_p = Q_p / (A \times t) \quad (3)$$

$$Q_p = [(C_{f,s} \times m_{f,s} - C_{c,s} \times m_{c,s}) + (C_{f,r} \times m_{f,r} - C_{c,r} \times m_{c,r})] \times 1\,000 \quad (4)$$

or

$$Q_p = (C_{f,p} \times m_{f,p} - C_{c,p} \times m_{c,p}) \times 1\,000 \quad (5)$$

where

F_p is the flux of trace element to the plants during the test culture period, in nanograms per square meter per second (ng m^{-2} s^{-1});

Q_p is the quantity of trace element accumulated by plants during the test culture period, in nanograms (ng);

A is the surface area of the root mat in contact with soil which is equal to 12,6 cm^2, in square meters (m^2);

t is the duration of the test culture period (which is equal to 8 days), in seconds (s);

$C_{f,s}$ is the trace element concentration in shoots at the end of the test culture period, in micrograms per gram (µg g^{-1}) (dry biomass);

$m_{f,s}$ is the dry biomass of shoots at the end of the test culture period, in grams (g);

$C_{c,s}$ is the mean trace element concentration in shoots of control plant pots at the end of the pre culture period, in micrograms per gram (µg g^{-1}) (dry biomass);

$m_{c,s}$ is the mean dry biomass of shoots of control plant pots at the end of the preculture period, in grams (g);

$C_{f,r}$ is the trace element concentration in roots at the end of the test culture period, in micrograms per gram (µg g^{-1}) (dry biomass);

$m_{f,r}$ is the dry biomass of roots at the end of the test culture period, in grams (g);

$C_{c,r}$ is the mean trace element concentration in roots of control plant pots at the end of the preculture period, in micrograms per gram (µg g^{-1}) (dry biomass);

$m_{c,r}$ is the mean dry biomass of roots of control plant pots at the end of the preculture period, in grams (g);

$C_{f,p}$ is the trace element concentration in the plant (shoots and roots) at the end of the test culture period, in micrograms per gram (µg g^{-1}) (dry biomass);

$m_{f,p}$ is the dry biomass of plants (shoots and roots) at the end of the test culture period, in grams (g);

$C_{c,p}$ is the mean trace element concentration in the plants (shoots and roots) of control plant pots at the end of the pre culture period, in micrograms per gram (µg g^{-1}) (dry biomass);

$m_{c,p}$ is the mean dry biomass of the plants (shoots and roots) of control plant pots at the end of the preculture period, in grams (g).

11.2 Data presentation

Present the data per replicate in tabular form, recording the biomasses (shoots and roots individually or pooled together), the quantity of trace elements accumulated in the whole plants (shoots + roots), the concentration of trace elements in shoots and roots if determined individually and the uptake flux of trace elements in plants for plant pots harvested at the end of the preculture period (control) and at the end of the test culture period. Insert the results of statistical analysis in each table (see Clause 12).

11.3 Expression of the results

The data should be also presented in graphical form, for example as a bar chart of the mean values of biomass (i.e. shoots and roots individually or pooled together) including standard deviation. Depict side-by-side the results for a given species including control plant pots and each soil material tested. When shoots and roots were handled separately, a single graphic should be presented by depicting shoot biomasses above the x-axis and the root biomasses below the x-axis.

The mean values of trace element concentrations in shoots and roots (if measured individually) including standard deviation should be presented as bar charts. Depict side-by-side the results for a given soil including the different plant species. A single graphic per trace element should be presented by depicting shoot concentrations above the x-axis and root concentrations below the x-axis.

The mean values of trace element uptake fluxes in plants including standard deviation should be presented as bar charts. Depict side-by-side the results for a given soil including the different plant species. A single graphic per trace element should be presented.

The results of statistical analysis (12) should be inserted in each figure.

12 Statistical analysis

12.1 General

Significant differences in the bioavailability of each trace element between several field-collected soils, ex situ treatments of soils (e.g. addition of organic wastes) or different plant species are commonly assessed with an adequate statistical test (e.g. analysis of variance (ANOVA), involving multiple comparisons of end point data, $P \leq 0{,}05$). If the statistical approach is based on parametric analyses, it consequently assumes that data are normally distributed, that the treatments are independent, and that the variance is homogenous among the different treatments. First, test these assumptions. If the data satisfy these assumptions, analysis may proceed. If not, data may be transformed and tested again. As parametric tests are reasonably robust in the face of moderate deviations from normality and equality of variance, proceed with parametric analysis, even if moderate nonconformity continues after transformation. Otherwise, if large nonconformity continues after transformation, use non parametric methods to perform analysis.

12.2 Plant biomasses

The validity of the biotest notably depends on a significant increase in shoot and root biomasses between the end of the preculture period and the end of the test culture period. Test the significance of this increase in biomass for each plant species.

The biotest is designed to avoid any phytotoxic effect, thereby enabling a fair comparison of the effect of the different treatments on trace element bioavailability. Test for each plant species the occurrence of a

significant difference in shoot and root biomasses between the different soils or soil treatments at the end of the test culture period.

12.3 Bioavailability end points

If shoots and roots were treated individually, test for each plant species and trace element the occurrence of a significant difference in trace element concentration in each plant compartment between the different soils or soil treatments at the end of the test culture period.

Regardless whether shoots and roots were treated individually or pooled together, calculate the uptake flux of trace elements in plants (11.1). The quality of flux calculation is strongly related to the homogeneity of biomasses and trace element concentrations in control plants harvested at the end of the preculture period. If the determination of biomass is hardly an issue, the determination of trace element concentration is more prone to analytical artefacts and more particularly to sample contamination. If, in spite of particular cautions taken during this analytical step, sample contamination is suspected in control plants, a Grubbs' test (i.e. extreme Studentised Deviate Test) should be performed on the trace element quantity accumulated in the five replicates of plant pots to detect outliers. Comprehensive guidance to the Grubbs' test is given in Reference [22]. Report any deletion of replicate considered as outlier in the test report with adequate justifications (see Clause 13). Once flux calculation is performed satisfactorily, test for each plant species and trace element the occurrence of a significant difference in trace element uptake flux in plants between the different soils or soil treatments at the end of the test culture period.

13 Test report

The test report shall include the following information:

a) a reference to this International Standard (i.e. ISO 16198);

b) information about the test plant species (Linnaean classification, cultivar, and source);

c) description of the biotest apparatus, especially if it differs from that introduced in Table 1;

d) description of the climatic conditions monitored in the growth chamber (7.4);

e) description of the test soils and the soil treatments:

   1) sampling protocol and location;

   2) protocol used for the addition ex situ of any material (e.g. organic wastes) to the test soils before starting the experimental procedure;

   3) physical-chemical properties of soils and soil materials (8.2);

f) description of all operating details not specified in this International Standard and any occurrence liable to have affected the results, notably including the following:

   1) visual damage (photographs are acceptable);

   2) values of biomass and trace element quantity in plants at the end of the preculture period (i.e. control plant pots) that are outside the range depicted in Annex E (presentation of the data and explanatory statement necessary);

g) presentation of the experimental data, expression of the results and description of the statistical analysis:

   1) tables (11.2);

   2) figures (11.3);

3) statistical methods used for the analysis of biomass (12.2) and bioavailability end points (12.3);

h) discussion of the results.

Annex A
(informative)

Plant species adapted to the biotest procedure

A range of plant species has been grown with this biotest but with experimental procedures more or less altered in comparison with the experimental procedure depicted in this International Standard.

Table A.1 — Plant species adapted to the biotest procedure

Species	Cultivars	Trace elements	Experimental procedure[a]	Observations	Suitability[b]	Ref.
Brassica napus	Duo Goéland	As, Cd, Cu, Pb, Zn Cu ^{137}Cs, ^{63}Ni	2 3 4	Shoots are fragile when manipulating	+	[21] [23] [24] [28]
Brassica oleracea[c]	Castelard	As, Cd, Cu, Pb, Zn	1		++	[21]
Bromus mollis	Samson	^{137}Cs	4		++	[25]
Festuca arundinacea[c]	Calina	As, Cd, Cu, Pb, Zn	1		++	[21]
Festuca ovina	Spartan	^{137}Cs, ^{63}Ni	4		++	[24] [25]
Festuca rubra	Bastide	^{63}Ni	4		++	[24]
Hordeum vulgare	Campanile Vertige	As, Cd, Cu, Pb, Zn ^{137}Cs	2 4	Root mat too thick	+	[21] [25]
Lactuca sativa capitata	Carmen Sucrine	As, Cd, Cu, Pb, Zn ^{137}Cs	2 4	Shoots are fragile when manipulating	+	[21] [25]
Lolium perene	Oustal Aubisque	As, Cd, Cu, Pb, Zn ^{137}Cs, ^{63}Ni	2 4		++	[21] [24] [25]

[a] Difference with the standardized experimental procedure:

— Exp. Proc. 1: Fully similar;

— Exp. Proc. 2: Soil layer thickness of 1 mm to 2 mm (i.e. 3 g dry soil) and slightly different biotest apparatus;

— Exp. Proc. 3: Three-week preculture period, soil layer thickness of 1–2 mm (i.e. 3 g dry soil) and slightly different biotest apparatus;

— Exp. Proc. 4: Sixteen-day preculture period, 7 d test culture period, soil layer thickness of 3 mm and slightly different biotest apparatus;

— Exp. Proc. 5: Four-week preculture period (2 weeks in nutrient gel, then 2 weeks in hydroponics), soil layer thickness of 1 mm (i.e. 2 g dry soil) and slightly different biotest apparatus.

[b] Adaptation to the biotest procedure and apparatus.

[c] The three species selected in the standardized procedure.

[d] Three genotypes were used, either neutrally or genetically transformed (see Reference [27] for rationale).

Table A.1 *(continued)*

Species	Cultivars	Trace elements	Experimental procedure[a]	Observations	Suitability[b]	Ref.
Lycopersicon esculentum[c]	Fline Saint Pierre Saint Pierre	As, Cd, Cu, Pb, Zn Cu ^{137}Cs	1 3 4		++	[21] [26] [25]
Medicago sativa	Prunelle Maya	As, Cd, Cu, Pb, Zn ^{137}Cs	2 4	Difficulties with germination and growth of the root system	+/–	[21] [25]
Nicotiana tabacum	SR1 [d]	Zn	5		++	[27]
Raphanus sativus	Flamboyant	^{63}Ni	4	Difficulties with shoot and root growth	–	[24]
Sorghum bicolour	Arkanciel	As, Cd, Cu, Pb, Zn	2	Problem of lodging and root mat too thick	+	[21]
Trifolium pratense	Miskawi	^{137}Cs, ^{63}Ni	4		++	[24] [25]
Triticum aestivum	Premio Aroona Songlen Tremie	As, Cd, Cu, Pb, Zn Cu ^{137}Cs	2 3 4	Root mat too thick	+	[21] [28] [25]
Triticum turgidum durum	Acalou	Cu	3	Root mat too thick	+	[20]

[a] Difference with the standardized experimental procedure:

— Exp. Proc. 1: Fully similar;

— Exp. Proc. 2: Soil layer thickness of 1 mm to 2 mm (i.e. 3 g dry soil) and slightly different biotest apparatus;

— Exp. Proc. 3: Three-week preculture period, soil layer thickness of 1–2 mm (i.e. 3 g dry soil) and slightly different biotest apparatus;

— Exp. Proc. 4: Sixteen-day preculture period, 7 d test culture period, soil layer thickness of 3 mm and slightly different biotest apparatus;

— Exp. Proc. 5: Four-week preculture period (2 weeks in nutrient gel, then 2 weeks in hydroponics), soil layer thickness of 1 mm (i.e. 2 g dry soil) and slightly different biotest apparatus.

[b] Adaptation to the biotest procedure and apparatus.

[c] The three species selected in the standardized procedure.

[d] Three genotypes were used, either neutrally or genetically transformed (see Reference [27] for rationale).

Annex B
(informative)

Technical drawings of the different components of the biotest

B.1 Plant-receiving pot

Dimensions in millimetres

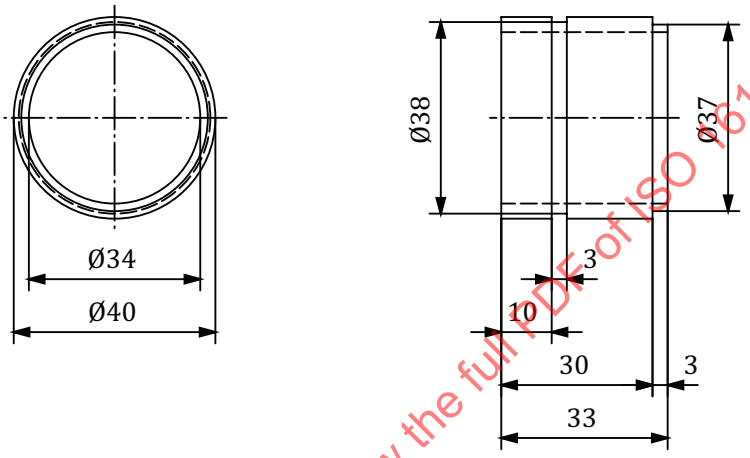


Figure B.1 — Component 1: Cylinder

Dimensions in millimetres

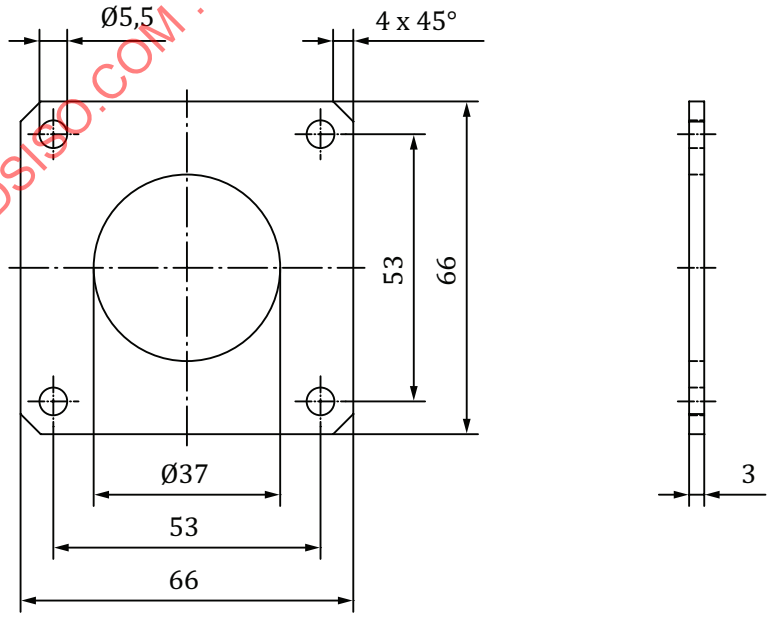


Figure B.2 — Component 2: Upper plate

B.2 Preculture period

Dimensions in millimetres

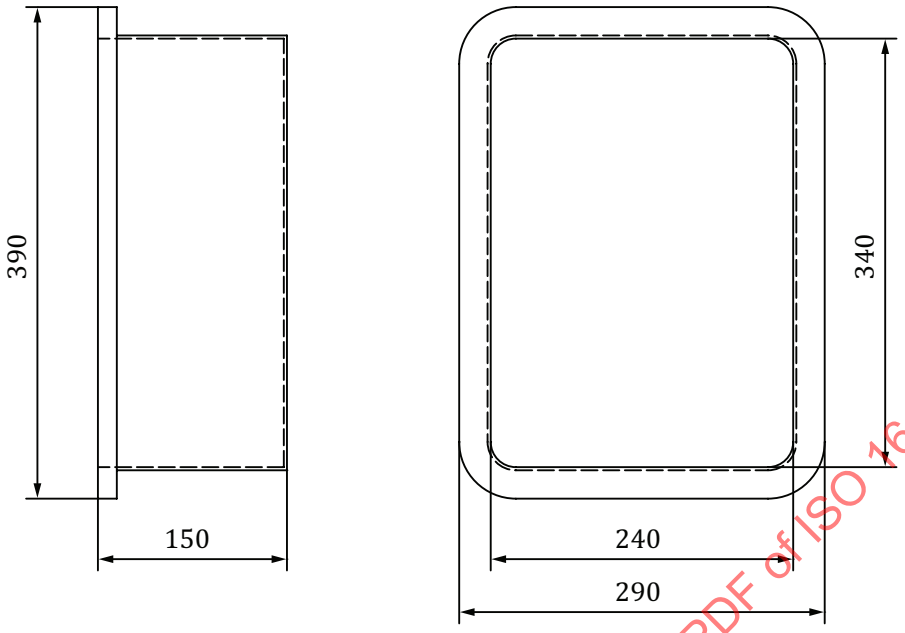


Figure B.3 — Component 3: Tank

Dimensions in millimetres

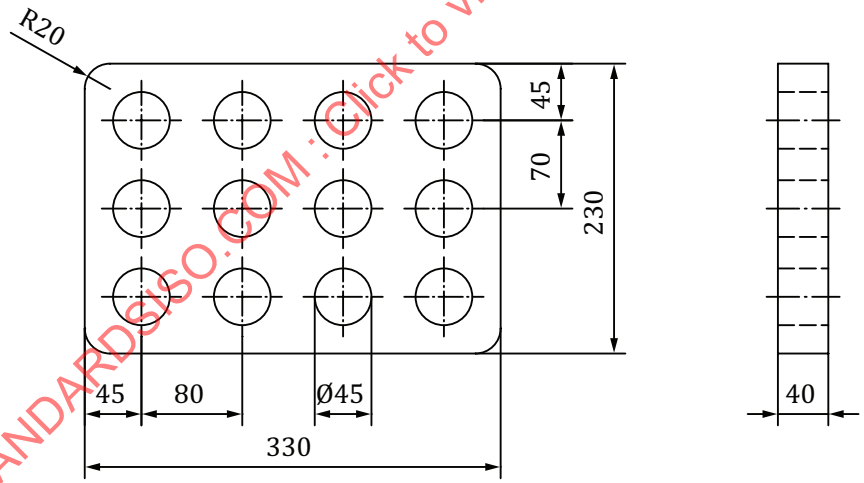


Figure B.4 — Component 4: Perforated floating platform

B.3 Test culture period

Dimensions in millimetres

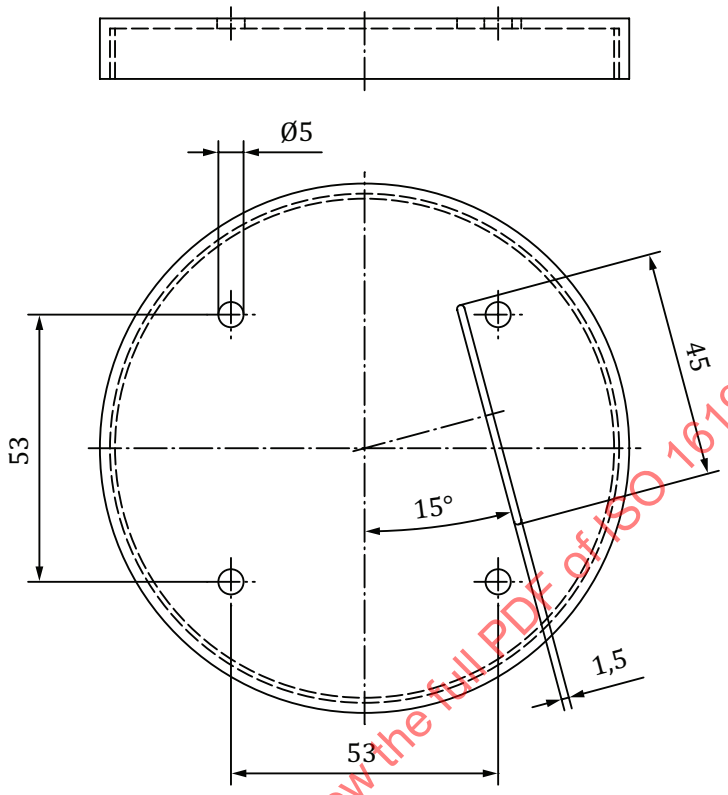


Figure B.5 — Component 5: Screwtop jar (only screwtop depicted)

Dimensions in millimetres

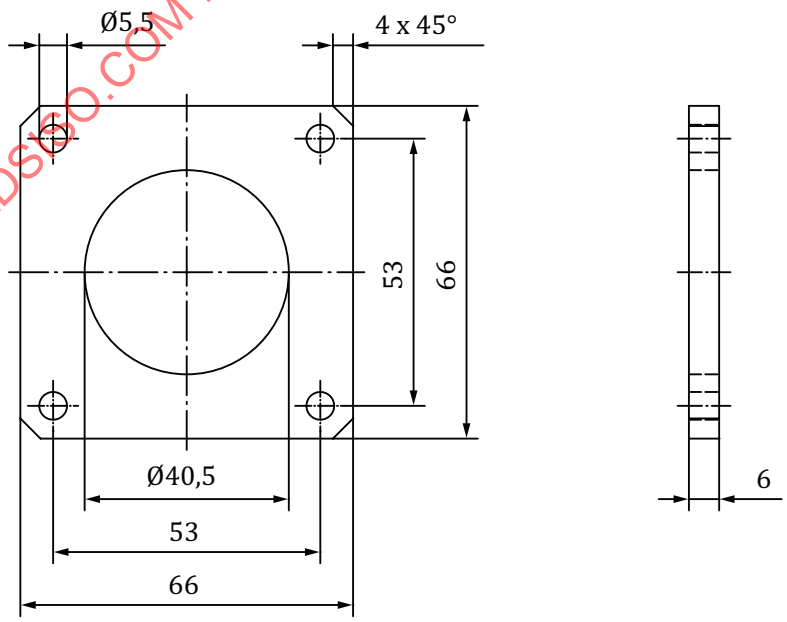
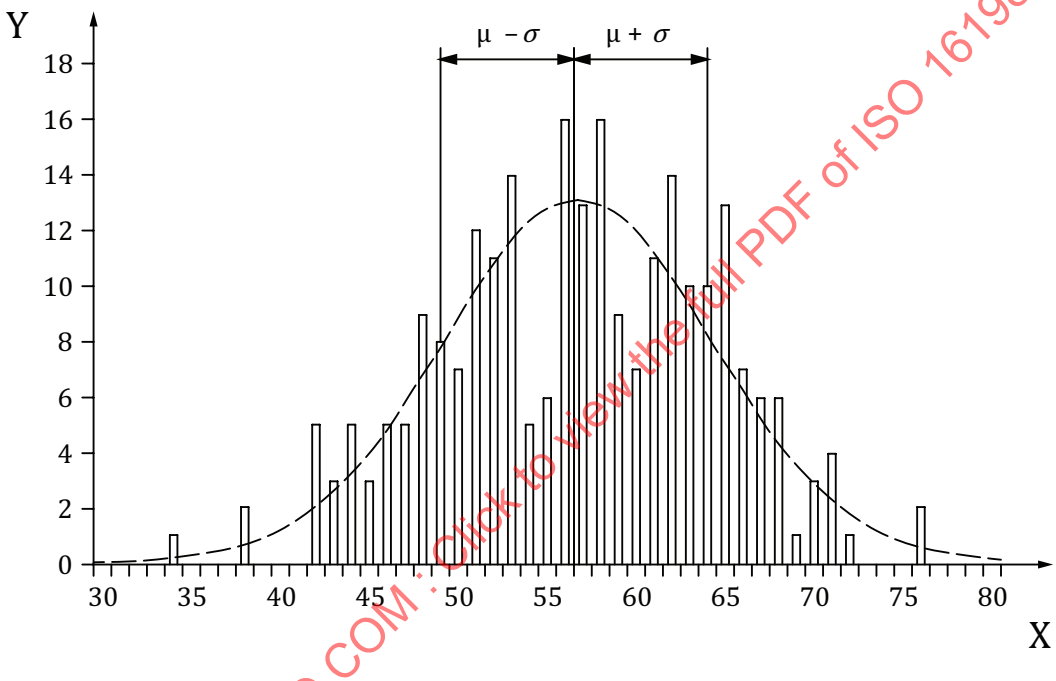


Figure B.6 — Component 6: Soil-receiving plate

Annex C
(informative)

Seed selection and seed density in plant pot for a range of species tested with the standardized experimental procedure

When the mass of a seed for a given species can be determined accurately with a balance (5.4) (i.e. ≥10 mg), a set of ca. 100 seeds are weighted one by one to draw a frequency chart and to select a mass range for acceptable seeds (see e.g. Figure C.1). Accordingly, the required number of seeds can be selected by weight.



Key

μ = 57 mg; σ = 8 mg; *n* = 250

X	frequency, number of seeds
Y	seed individual mass, mg
▭	experimental data
— — —	Gauss curve

Figure C.1 — Distribution of the mass of durum wheat (cv. Acalou) seeds

Acceptable seeds for biotest deployment can be selected in the interval (μ - σ; μ + σ) for example.

For seeds <10 mg per seed that cannot be manipulated and weighed one by one accurately, the specified number of seeds per plant pot integrate a significant part of the inherent mass variability (Table C.1). A seed counter can be used for easiness. Alternatively, primarily determine the mass of seeds that corresponds to the specified number of seeds per plant pot for each species. Then weigh the corresponding mass of seeds and put it in plant pots.

In any case, if the dimensions of the plant pot are modified in such way that the bottom section area changes, the number or mass of seeds shall be changed proportionally.

Table C.1 — Seed density per plant pot

Species[a]	Cultivars	Seed number per plant pot
Brassica napus	Duo	25
Brassica oleracea	Castelard	50
Festuca arundinacea	Calina	80
Hordeum vulgare	Campanile	5
Lactuca sativa capitata	Carmen	50
Lolium perenne	Oustal	40
Lycopersicon esculentum	Fline	40
Medicago sativa	Prunelle	60
Sorghum bicolour	Arkanciel	5
Triticum aestivum	Premio	2

[a] Seed density has been tested with an experimental procedure analog to that of this International Standard, i.e. with a 2-week preculture period.[24]

Annex D
(informative)

Digestion and analysis of plant samples

D.1 Example of procedure for the digestion of plant samples

The following digestion procedure was used during the different validation steps of the standardized biotest and is suitable for the digestion of a broad range of plant samples as well as for the analytical determination of Cd, Co, Cr, Cu, Ni, Pb, and Zn at least. Small but significant volatilisation of As can occur during this digestion procedure. Consequently, the method only gives an estimation for As and other volatile trace elements. Choose an alternative digestion procedure for an accurate determination of the concentration of volatile trace elements.

Step 1: Weigh 500 mg of plant sample previously oven-dried and ground (or cut in small pieces) and introduce the sample in a platinum cup. Burn off the sample in an oven at 500 °C for 2 h.

Step 2: After cooling, moisten the ashes with a few drops of ultra-pure water (18,2 MΩ ·cm), add 2 ml of 6 mol·dm^{-3} HCl, then boil on a hot plate until evaporation. Add 2 ml of 6 mol·dm^{-3} HCl and wait 10 min before filtering using an ashless filter paper into a 50 ml volumetric flask. Wash the digestion residue on the filter over the volumetric flask with ca. 25 ml of warm ultra-pure water.

Step 3: Put the filter-containing residue in a platinum cup and burn off it at 500 °C for 30 min to remove the residual organic matter and the filter. Add a few drops of ultra-pure water and 2 ml of concentrated HF on the ashes and boil the solution on a hot plate until evaporation. This step enables the removal of silica dioxide by dissolution and SiF_6 volatilisation.

Step 4: Add 1 ml of 6 mol dm^{-3} HCl to the residue, filtrate the solution using an ashless filter paper and finally rinse the filter with ultra-pure water over the volumetric flask used in the step 2. Bring up to volume with ultra-pure water. The plant digest is then ready for analytical determination.

D.2 Range of trace element concentrations in plant digests

Trace element concentrations in plant digests were determined during the ring-test experiment on plant samples harvested at the end of the preculture period (control plant pots) and at the end of the test culture period. Details about the experimental procedure of the ring test are provided in Annex F.1.

Table D.1 — Trace element concentrations in plant digests (µg·g^{-1} dry biomass)

Species		As	Cd	Co	Cr	Cu	Ni	Pb	Zn
Brassica oleracea	Shoots	0,007 to 0,3	0,02 to 61	0,02 to 1,5	0,2 to 13,4	2,2 to 33,2	0,4 to 11	0[a] to 212	18 to 348
	Roots	0,09 to 29	0,01 to 275	0,14 to 21,8	1,6 to 21	37 to 512	2 to 57	0[a] to 10 100	43 to 1 336
Festuca arundinacea	Shoots	0,003 to 0,4	0,01 to 30	0,01 to 1,6	0,19 to 4,5	2,8 to 22	0,45 to 14,3	0[a] to 141	11,5 to 247
	Roots	0,04 to 20	0,015 to 288	0,1 to 9	1,5 to 15,6	7 to 345	2,1 to 96	0[a] to 7 608	32 to 2 060

[a] Some of the lowest Pb concentrations measured in plant digests were smaller than the mean Pb concentration in blanks. Lead concentration was therefore considered equal to zero in these samples.

Table D.1 *(continued)*

Species		As	Cd	Co	Cr	Cu	Ni	Pb	Zn
Lycopersicon esculentum	Shoots	0,001 to 1,2	0,004 to 38	0,009 to 1,2	0,15 to 11,3	0,65 to 17,6	0,17 to 22	0[a] to 651	2,5 to 280
	Roots	0,15 to 21,7	0,05 to 500	0,13 to 20	1,4 to 22	9,2 to 367	2,4 to 105	0[a] to 9 885	5 to 2 270

[a] Some of the lowest Pb concentrations measured in plant digests were smaller than the mean Pb concentration in blanks. Lead concentration was therefore considered equal to zero in these samples.

Annex E
(informative)

Range of biomasses and trace element quantities in control plant pots

The following biomasses and trace element quantities in plants were determined during the ring test experiment on plants harvested at the end of the preculture period (i. e. control plant pots). Details about the experimental procedure of the ring test are provided in Annex F.1. These data were obtained using the apparatus described in the present standard (7.2 and Annex B). Modifications of some components in size can influence the measurements.

Table E.1 — Range of biomasses in control plant pots

Species, number (*n*) of samples	Range of biomasses in control plant pots mg		
	Shoots	Roots	Whole plants
Brassica oleracea, *n* = 35	69 to 441	16 to 112	120 to 553
Festuca arundinacea, *n* = 40	56 to 214	32 to 207	137 to 403
Lycopersicon esculentum, *n* = 40	79 to 558	14 to 106	120 to 639

Table E.2 — Range of trace element quantities accumulated in control plant pots (shoots + roots). The number of data point is detailed between brackets

Species	Trace element µg							
	As	Cd	Co	Cr	Cu	Ni	Pb	Zn
Brassica oleracea	0,01 to 0,09 (33)	0,007 to 0,229 (31)	0,03 to 0,15 (27)	0,5 to 1,0 (34)	3,5 to 22,8 (35)	0,5 to 1,8 (35)	0,02 to 3,56 (29)	9,8 to 33,4 (29)
Festuca arundinacea	0,006 to 0,069 (35)	0,004 to 0,371 (34)	0,02 to 0,13 (34)	0,48 to 0,95 (34)	2,0 to 8,3 (35)	0,5 to 1,7 (35)	0,02 to 12,12 (28)	6,0 to 24,7 (35)
Lycopersicon esculentum	0,01 to 0,11 (36)	0,01 to 0,43 (38)	0,02 to 0,12 (35)	0,5 to 1,1 (36)	2,5 to 7,5 (35)	0,4 to 1,5 (37)	0,02 to 9,03 (34)	9,2 to 35,8 (34)

Annex F
(informative)

Ring-test

F.1 Material and methodology of the ring-test experiment

A ring-test was carried out from February to May 2012 and involved the participation of eight laboratories among which, six had never used the biotest before: (i) CIRAD, UPR Recyclage et risque, France; (ii) INERIS, France; (iii) INRA, UMR Eco&Sols, France; (iv) Institute for ecological chemistry, plant analysis and stored product protection, Germany; (v) Eurofins IPL Est, Laboratoires Etudes et Expertises, Unité Ecotoxicologie, France; (vi) Free university of Bolzano, University of Udine, Italy; (vii) University of Liège-Gembloux Agro-Bio Tech, Unité de Science du sol, Belgium; (viii) University of natural resources and life sciences, Rhizosphere Ecology and Biogeochemistry Group, Austria. Before carrying out the ring-test experiment, each laboratory took a 3-day training course on the theoretical and practical aspects of the biotest apparatus and experimental procedure. Then, each laboratory received the same biotest apparatus along with seeds and soil samples necessary to perform the ring-test experiment.

The ring-test experiment, performed independently and concomitantly by each laboratory, consisted in growing the three recommended plant species, namely *B. oleracea*, *F. arundinacea*, and *L. esculentum*, on four different soil samples as described in ISO 16198. The four-soil samples exhibit a fairly large range for several physical-chemical properties and substantial contaminations in several trace elements (Table F.1). Soil 1 was collected in the upper layer of a calcareous, agricultural soil contaminated with Cd, Cu, Pb, and Zn by the repetitive application of wastewater from Paris (France) during several decades. Soil 2 was collected in the upper layer of a mildly acidic vineyard soil contaminated with Cu by the repetitive application of Cu fungicides. Soil 3 was collected in the upper layer of an acidic agricultural soil contaminated with Cd, Pb, and Zn by atmospheric fallouts from a smelting plant. Soil 4 was collected in the upper layer of soil highly contaminated with As, Cd, Cr, Cu, Ni, Zn, and Pb more particularly by atmospheric fallouts from a battery recycling plant.

Table F.1 — Main physical-chemical properties of soil samples used in the ring-test experiment

	Sand	Silt	Clay	pH	$CaCO_3$	C_{org}	As	Cd	Cr	Cu	Ni	Pb	Zn
Soil 1	740	102	158	7,7	66	13	14	1,7	90	98	32	163	432
Soil 2	561	322	117	6,2	n.d.	13	24	0,5	33	453	14	215	74
Soil 3	550	337	113	5,9	n.d.	25	10	4,2	38	24	12	590	429
Soil 4	527	311	162	6,7	6	41	203	100	109	388	131	131 340	1 172

Sand, Silt, Clay: Concentrations of sand, silt and clay in soils, g kg^{-1} (dry mass), according to AFNOR NF 31–107[15].

pH: pH measured in H_2O, according to ISO 10390.

$CaCO_3$: Total carbonate concentration, g kg^{-1} (dry mass), according to ISO 10693.

C_{org}: Total organic carbon concentration, g kg^{-1} (dry mass), according to ISO 10694.

As, Cd, Cr, Cu, Ni, Pb, Zn: Total concentration of As, Cd, Cr, Cu, Ni, Pb, and Zn in soils, mg kg^{-1} (dry mass), according to ISO 14869-1.

n.d.: Not determined.

During the ring-test experiment, a growing accident occurred in one laboratory, which was subsequently not able to provide the samples for *B. oleracea*. Consequently, the interlaboratory comparison was performed on seven laboratories for *B. oleracea* and on eight laboratories for *F. arundinacea* and *L. esculentum*.

Each laboratory oven-dried the plant samples (shoots and roots separately) by themselves before sending the samples to the CIRAD at Montpellier (France) for performing biomass determination and digestion of plant samples according to Annex D. Trace element concentrations in plant digests were subsequently determined by ICP-MS.

F.2 Ring-test results and interpretation

As shoots and roots were harvested distinctly, the statistical analyses of repeatability and reproducibility were performed on shoot concentrations, root concentrations and uptake fluxes determined according to Formulae (2) and (3) (see 11.1). The general mean, $\bar{X}$, as well as the repeatability and reproducibility variances, s_r^2 and s_R^2, were computed according to ISO 5725-2 for each level, i.e. each triplet of plant species, soil, and trace element. All the data are summarized in Tables F.2 to F.10.

Over the 3 680 values obtained for each bioavailability end point, the percentage of outliers reaches 5 %, 10 %, and 10 % for uptake fluxes, shoot concentrations and root concentrations, respectively. The percentage of outliers is twice lower for the uptake fluxes than for the concentrations in shoots and roots. In addition, these outliers are not strictly related to a particular laboratory, soil or trace element. These results concomitantly indicate that the repeatability and the reproducibility are rather good with few and not recurrent outliers.

The mean coefficients of variation for shoot concentrations, root concentrations, and uptake fluxes range between 22 % to 32 % for repeatability and 47 % to 61 % for reproducibility. The highest coefficients of variation were mainly observed for only one trace element, namely Pb. The higher variability of Pb measurements is likely related to an analytical issue as some of the lowest Pb concentrations in plant digests were lower than those in blanks (Annex D.2).

Finally, the classification of the four soils was determined for each laboratory by integrating the uptake fluxes of the eight trace elements in the three species (results not shown). Seven laboratories classified the soils in the same order: soil 4 > soil 3 > soil 1 > soil 2. One laboratory inverted the soils 1 and 2. This result exemplifies the ability of the biotest to discriminate trace element bioavailability to plants between the different soils tested.

Table F.2 — Repeatability and reproducibility of trace element concentration in the shoots of *Brassica oleracea*

Element	l	n	n_o	n_{op}	$\bar{X}$	s_r	$C_{V,r}$	s_R	$C_{V,R}$
Soil 1									
As	7	35	*		0,12	0,04	37	0,06	50
Cd	6	35	5	14	0,5	0,1	19	0,3	63
Co	5	35	10	29	0,06	0,02	32	0,02	40
Cr	6	35	5	14	0,5	0,1	21	0,2	36
Cu	7	35			8,0	2,6	33	3,6	45
Ni	7	35			1,3	0,5	34	0,8	61
Pb	6	35	5	14	1,9	0,9	44	2,1	109
Zn	6	35	5	14	36	4	11	8	22
Soil 2									
As	7	35			0,04	0,01	28	0,03	61
Cd	6	35	5	14	0,14	0,03	23	0,05	35
Co	6	35	5	14	0,4	0,2	52	0,2	62
Cr	6	35	5	14	0,47	0,09	20	0,13	27
Cu	7	35			23	3	14	8	33

Table F.2 *(continued)*

Element	l	n	n_o	n_{op}	$\bar{X}$	s_r	$C_{V,r}$	s_R	$C_{V,R}$
Ni	5	35	10	29	1,0	0,2	19	0,2	22
Pb	6	35	5	14	1,0	0,7	71	1,0	97
Zn	7	35			44	7	17	18	42
Soil 3									
As	6	35	5	14	0,04	0,01	25	0,02	49
Cd	7	35			2,1	0,3	15	0,7	34
Co	6	35	5	14	0,8	0,1	16	0,4	49
Cr	6	35	5	14	0,7	0,2	22	0,6	81
Cu	7	35			5,1	1,1	22	2,3	45
Ni	6	35	5	14	1,0	0,2	23	0,6	58
Pb	7	35			1,1	1,3	114	1,4	129
Zn	7	35			243	28	11	72	30
Soil 4									
As	7	35			0,06	0,04	66	0,05	88
Cd	6	35	5	14	40	6	14	10	24
Co	6	35	5	14	0,28	0,05	18	0,07	24
Cr	7	35			0,7	0,2	23	0,5	71
Cu	6	35	5	14	8	2	19	2	21
Ni	7	35			7	1	20	2	25
Pb	7	35			111	40	36	56	50
Zn	7	35			100	17	18	25	25

l number of laboratories after elimination of outliers
n number of single analysis data before elimination of outliers
n_o number of outliers
n_{op} percentage of outliers
$\bar{X}$ general mean value, in micrograms per gram ($\mu g \cdot g^{-1}$)
s_r standard deviation of the repeatability, in micrograms per gram ($\mu g \cdot g^{-1}$)
$C_{V,r}$ variation coefficient of the repeatability, in percent (%)
s_R standard deviation of the reproducibility, in micrograms per gram ($\mu g \cdot g^{-1}$)
$C_{V,R}$ variation coefficient of the reproducibility, in percent (%)
* empty cell means value equal to zero

Table F.3 — Repeatability and reproducibility of trace element concentration in the roots of *Brassica oleracea*

Element	l	n	n_o	n_{op}	$\bar{X}$	s_r	$C_{V,r}$	s_R	$C_{V,R}$
Soil 1									
As	7	35	*		5	1	23	1	27
Cd	6	35	5	14	1,4	0,3	19	0,7	51
Co	6	35	5	14	0,8	0,2	22	0,2	22
Cr	7	35			9	3	39	3	40

Table F.3 *(continued)*

Element	l	n	n_o	n_{op}	$\bar{X}$	s_r	$C_{V,r}$	s_R	$C_{V,R}$
Cu	7	35			117	38	32	58	49
Ni	7	35			7	2	29	2	32
Pb	7	35			31	11	36	20	65
Zn	7	35			112	26	24	32	29
Soil 2									
As	5	35	10	29	2,7	0,5	17	0,5	18
Cd	6	35	5	14	0,56	0,08	15	0,18	32
Co	7	35			4	2	40	3	59
Cr	6	35	5	14	4,2	0,8	19	1,8	43
Cu	6	35	5	14	237	32	13	81	34
Ni	7	35			6	1	19	2	32
Pb	7	35			14	7	46	7	48
Zn	7	35			83	21	26	26	32
Soil 3									
As	7	35			3,0	0,5	15	1,5	49
Cd	7	35			12	2	19	5	40
Co	7	35			9	2	17	5	53
Cr	7	35			8	1	18	2	23
Cu	5	35	10	29	255	61	24	383	150
Ni	7	35			5,7	0,8	14	3,1	54
Pb	7	35			27	9	32	11	40
Zn	7	35			746	110	15	256	34
Soil 4									
As	7	35			11	4	39	6	55
Cd	6	35	5	14	156	32	21	52	33
Co	6	35	5	14	3,4	0,5	14	0,9	26
Cr	7	35			6	2	41	3	59
Cu	6	35	5	14	101	15	15	22	22
Ni	6	35	5	14	41	6	15	9	21
Pb	7	35			6805	1538	23	2150	32

Table F.3 *(continued)*

Element	l	n	n_o	n_{op}	$\bar{X}$	s_r	$C_{V,r}$	s_R	$C_{V,R}$
Zn	6	35	5	14	386	48	12	72	19

l number of laboratories after elimination of outliers
n number of single analysis data before elimination of outliers
n_o number of outliers
n_{op} percentage of outliers
$\bar{X}$ general mean value, in micrograms per gram ($\mu g \cdot g^{-1}$)
s_r standard deviation of the repeatability, in micrograms per gram ($\mu g \cdot g^{-1}$)
$C_{V,r}$ variation coefficient of the repeatability, in percent (%)
s_R standard deviation of the reproducibility, in micrograms per gram ($\mu g \cdot g^{-1}$)
$C_{V,R}$ variation coefficient of the reproducibility, in percent (%)
* empty cell means value equal to zero

Table F.4 — Repeatability and reproducibility of trace element uptake fluxes in *Brassica oleracea*

Element	l	n	n_o	n_{op}	$\bar{X}$	s_r	$C_{V,r}$	s_R	$C_{V,R}$
Soil 1									
As	6	35	5	14	0,9	0,2	27	0,4	41
Cd	7	35	*		0,6	0,1	21	0,2	39
Co	5	35	10	29	0,16	0,06	35	0,08	49
Cr	7	35			1,5	0,9	61	1,2	75
Cu	7	35			14	5	36	8	57
Ni	7	35			1,5	0,6	43	0,8	57
Pb	5	35			5	2	39	5	95
Zn	7	35			32	12	37	17	52
Soil 2									
As	7	35			0,6	0,2	36	0,2	42
Cd	6	35	5	14	0,14	0,04	26	0,06	39
Co	7	35			1,1	0,5	43	0,8	70
Cr	7	35			0,5	0,3	52	0,3	64
Cu	6	35	5	14	41	6	16	11	27
Ni	6	35	5	14	1,1	0,4	31	0,5	47
Pb	5	35			2	1	71	2	104
Zn	7	35			26	8	32	11	43
Soil 3									
As	5	35	5	14	0,59	0,08	14	0,10	17
Cd	7	35			3,6	0,4	11	0,9	26
Co	7	35			2,1	0,5	23	1,0	47
Cr	7	35			1,1	0,4	32	0,6	52
Cu	6	35	5	14	7	2	26	4	59
Ni	7	35			1,1	0,3	25	0,4	36

Table F.4 *(continued)*

Element	l	n	n_o	n_{op}	$\bar{X}$	s_r	$C_{V,r}$	s_R	$C_{V,R}$
Pb	5	35			4	2	48	4	114
Zn	7	35			305	43	14	99	32
Soil 4									
As	7	35			2	1	43	2	64
Cd	6	35	5	14	60	9	15	20	32
Co	7	35			0,9	0,2	17	0,3	30
Cr	7	35			0,8	0,4	55	0,5	68
Cu	7	35			16	3	21	6	40
Ni	6	35	5	14	13	2	15	5	35
Pb	5	35			1178	331	28	1040	88
Zn	6	35	5	14	127	18	14	45	35

l number of laboratories after elimination of outliers

n number of single analysis data before elimination of outliers

n_o number of outliers

n_{op} percentage of outliers

$\bar{X}$ general mean value, in nanograms per square meter per second ($ng \cdot m^{-2} \cdot s^{-1}$)

s_r standard deviation of the repeatability, in nanograms per square meter per second ($ng \cdot m^{-2} \cdot s^{-1}$)

$C_{V,r}$ variation coefficient of the repeatability, in percent (%)

s_R standard deviation of the reproducibility, in nanograms per square meter per second ($ng \cdot m^{-2} \cdot s^{-1}$)

$C_{V,R}$ variation coefficient of the reproducibility, in percent (%)

* empty cell means value equal to zero

Table F.5 — Repeatability and reproducibility of trace element concentration in the shoots of *Festuca arundinacea*

Element	l	n	n_o	n_{op}	$\bar{X}$	s_r	$C_{V,r}$	s_R	$C_{V,R}$
Soil 1									
As	8	40	*		0,13	0,03	22	0,03	26
Cd	8	40			0,3	0,1	30	0,2	53
Co	7	40	5	13	0,05	0,02	33	0,02	49
Cr	8	40			0,9	0,2	20	0,5	59
Cu	7	40	5	13	10,2	0,6	6	3,4	33
Ni	6	40	10	25	1,6	0,2	12	0,9	57
Pb	7	40	5	13	1,2	1,1	95	1,4	118
Zn	8	40			60	6	9	22	37
Soil 2									
As	6	40	10	25	0,07	0,01	18	0,03	42
Cd	6	40	10	25	0,2	0,1	41	0,1	62
Co	6	40	10	25	0,4	0,1	35	0,2	51
Cr	6	40	10	25	1,0	0,2	21	0,7	64
Cu	7	40	5	13	13	1	10	5	35

Table F.5 *(continued)*

Element	l	n	n_o	n_{op}	$\bar{X}$	s_r	$C_{V,r}$	s_R	$C_{V,R}$
Ni	8	40			1,9	0,6	30	1,3	65
Pb	5	40	15	38	0,8	0,4	52	0,5	61
Zn	8	40			49	12	25	25	51
Soil 3									
As	6	40	10	25	0,07	0,01	17	0,02	33
Cd	8	40			1,2	0,2	15	0,7	57
Co	8	40			0,7	0,2	27	0,4	58
Cr	8	40			0,9	0,2	17	0,5	57
Cu	7	40	5	13	9	1	12	3	40
Ni	8	40			1,5	0,3	18	0,8	53
Pb	6	40	5	13	1,4	2,1	153	2,3	169
Zn	8	40			161	19	12	57	35
Soil 4									
As	7	40	5	13	0,07	0,03	35	0,03	44
Cd	7	40	5	13	14	1	9	5	34
Co	6	40	10	25	0,23	0,03	14	0,11	46
Cr	7	40	5	13	1,0	0,2	16	0,5	51
Cu	8	40			11,2	0,8	7	2,4	21
Ni	6	40	10	25	5,8	0,6	10	2,6	45
Pb	7	40	5	13	60	10	17	13	22
Zn	8	40			108	10	9	26	24

l number of laboratories after elimination of outliers
n number of single analysis data before elimination of outliers
n_o number of outliers
n_{op} percentage of outliers
$\bar{X}$ general mean value, in micrograms per gram ($\mu g \cdot g^{-1}$)
s_r standard deviation of the repeatability, in micrograms per gram ($\mu g \cdot g^{-1}$)
$C_{V,r}$ variation coefficient of the repeatability, in percent (%)
s_R standard deviation of the reproducibility, in micrograms per gram ($\mu g \cdot g^{-1}$)
$C_{V,R}$ variation coefficient of the reproducibility, in percent (%)
* empty cell means value equal to zero

Table F.6 — Repeatability and reproducibility of trace element concentration in the roots of *Festuca arundinacea*

Element	l	n	n_o	n_{op}	$\bar{X}$	s_r	$C_{V,r}$	s_R	$C_{V,R}$
Soil 1									
As	8	40	*		2,5	0,6	22	0,7	26
Cd	7	40	5	13	2,2	0,4	20	0,6	28
Co	8	40			0,5	0,2	29	0,2	34
Cr	8	40			8	2	26	2	32