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Soil quality — Biological methods — Chronic toxicity in higher plants

Qualité du sol — Méthodes biologiques — Toxicité chronique sur les plantes supérieures

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 22030 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

Introduction

This International Standard describes a procedure for evaluating the quality of soils of different origin carrying unknown contaminations. The method, slightly modified, can also be used to measure the toxicity of known chemicals incorporated into soil.

The evaluation of the inhibition and chronic toxicity is based on emergence, vegetative growth and reproductive capacity of at least two species of higher plants.

This International Standard is based on:

a) results of the research project “Development of a chronic bioassay using higher plants”, sponsored by the German Ministry for Education and Research (BMBF), Bonn [3], and

b) discussions within the joint project “Ecotoxicological Test Batteries” forming part of the BMBF Joint Research Group “Processes for the Bioremediation of Soil” [10].

Soil quality — Biological methods — Chronic toxicity in higher plants

WARNING — Contaminated soils can contain unknown mixtures of toxic, mutagenic or otherwise harmful chemicals or infectious microorganisms. Occupational health risks can arise from dust or evaporated chemicals during handling and incubation. Furthermore, test plants can absorb chemicals from the soil and safety measures should also be considered when handling these test plants.

1 Scope

This International Standard describes a method for determining the inhibition of the growth and reproductive capability of higher plants by soils under controlled conditions. Two species are recommended: a rapid-cycling variant of turnip rape (*Brassica rapa* CrGC syn. Rbr) and oat (*Avena sativa*). The duration of test should be sufficient to include chronic endpoints that demonstrate the reproductive capability of the test plants.

By using natural test soils, e.g. from contaminated sites or remediated soils, and by comparing the development of the test plants in these soils with reference or standard control soils, the test can be used to assess soil quality, especially the function of the soil as a habitat for plants.

Annex A describes modifications allowing use of the chronic plant assay for the testing of chemicals incorporated into soil. By preparing a dilution series of a test substance in standard control soils, the same endpoints can be measured to assess the chronic toxicity of chemicals. This method is not applicable to volatile substances, i.e. substances for which H (Henry's constant) or the air/water partition coefficient is greater than 1, or for which the vapour pressure exceeds 0,013 3 Pa at 25 °C.

2 Normative references

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

ISO 11268-1:1993, *Soil quality — Effects of pollutants on earthworms (Eisenia fetida) — Part 1: Determination of acute toxicity using artificial soil substrate*

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

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

ISO 15176:2002, *Soil quality — Characterization of excavated soil and other soil materials intended for re-use*

ISO 15799, *Soil quality — Guidance on the ecotoxicological characterization of soils and soil materials*

ASTM D1076:2002, *Standard Specification for Rubber-Concentrated, Ammonia Preserved, Creamed, and Centrifuged Natural Latex*

3 Terms and definitions

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

3.1
artificial soil
mixture of sand, kaolinite, peat and calcium carbonate

NOTE ISO 11268-1 describes such a soil for toxicity tests using earthworms. Pure quartz sand, mineral wool, vermiculite or other synthetic substrates should not be used.

3.2
biomass
total mass of shoots, flowers and seed pods

NOTE 1 Biomass is expressed as dry mass per plant or, if needed, as dry mass per pot.

NOTE 2 During the test period, some of the test plants can reach different growth stages and their water content can differ when the plants are harvested. Thus the dry mass better represents the biomass produced during the growth period.

3.3
concentration
mass of test substance per amount of soil

NOTE Concentration is expressed as a mass fraction, in milligrams per kilogram (mg/kg) of dry soil.

3.4
contaminant
substance or agent present in the soil as a result of human activity

[ISO 15176:2002]

3.5
control soil
uncontaminated substrate, used as a control and as medium for preparing dilution series with test soils or chemicals, that allows the growth of healthy plants

NOTE Either artificial or natural standard or reference soils can be used, if unhindered growth of the test plants in these soils can be expected. In any case, differences in nutrient levels between a test soil and a control soil can affect the dose-response pattern. For example, a control soil much richer in nutrients than a test soil can result in a false positive result (i.e. the test soil appears to have a “toxic” effect on the growth of the test plants). If a control soil is poorer in nutrients than a test soil, hormesis (see 3.9) can be expected at low soil-mixture ratios, or even an inverse dose response relationship, if nutrient supply becomes the main effect. This International Standard does not provide numerical values for the nutrients.

3.6
effect concentration
EC_x
concentration (mass fraction) of a test chemical or the percentage (mass fraction) of a test soil at which a given endpoint is inhibited by x % compared to the control

NOTE The effect concentration is expressed in milligrams per kilogram. When chemicals are tested, the EC_x is expressed as mass of the test substance per dry mass of soil; when soils are tested, the EC_x is expressed as a percentage of test soil dry mass per soil mixture dry mass.

3.7
emergence
development of a seedling contained within a seed, ending the latent period

NOTE It is expressed as the percentage of seedlings which emerge from test pots as compared with the control pots.

3.8
habitat function
ability of soils/soil materials to serve as a habitat for microorganisms, plants, soil-living animals and their interactions (biocenosis)

[ISO 15799]

3.9
hormesis
improvement of seedling emergence, growth or survival (or other response of the test plants) at low concentrations of chemicals or mixtures of soil that are toxic when applied at higher levels in comparison to the control [1]

3.10
lowest observed effect concentration
LOEC
lowest tested concentration (mass fraction) of a test substance in soil at which a statistically significant effect on a given endpoint ($p < 0{,}05$) compared with the control is observed

cf. **NOEC** (3.11)

NOTE Analogously, the term LOEC is used for the lowest tested mixture ratio of a test soil in a reference or a standard control soil at which a statistically significant effect is observed. The LOEC is expressed as mass of the test substance per mass of dry soil or, in the latter case, as percentage of test-soil dry mass per soil-mixture dry mass. All test concentrations above the LOEC have a harmful effect equal or greater than that observed at the LOEC. If this condition cannot be satisfied, an explanation should be given for how the LOEC and NOEC have been selected.

3.11
no observed effect concentration
NOEC
test substance concentration (mass fraction) or soil mixture ratio immediately below the LOEC, which when compared to the control has no statistically significant effect ($p < 0{,}05$)

cf. **LOEC** (3.10)

3.12
reference soil
uncontaminated site-specific soil (e.g. collected in the vicinity of a contaminated site) with properties (nutrient concentrations, pH, organic carbon content and texture) similar to the test soil

3.13
soil mixture ratio
ratio of the dry mass of test soil to the dry mass of reference/control soil

NOTE It is expressed as a percentage.

3.14
standard soil
field-collected soil or artificial soil whose main properties (e.g. pH, texture, organic matter content) are within a known range

EXAMPLES Euro soils, artificial soil.

NOTE The properties of standard soils may differ from those of the test soil.

4 Principle

This International Standard describes a plant test that includes both acute and chronic endpoints. The test measures emergence, early growth and reproduction of two terrestrial plant species (*Avena sativa* and a rapid-cycling variety of *Brassica rapa* are recommended). The test compares responses of plants in a test soil and/or a series of dilutions with a control soil. This test method may also be used for the testing of chemicals by applying various concentrations of the test substance to a standard control soil. Seeds of both plant species are planted in pots containing the soil/soil mixtures and in control pots containing a reference or standard soil. Pots are placed in a temperature- and light-controlled room or growth chamber. They are watered via wicks. After emergence of the plants, emergence rates are determined and plants are thinned out to a specified number. After two weeks, some of the plants are harvested to determine their biomass. After another period of three weeks to four weeks (rapid-cycling *Brassica rapa*) or five weeks to 6 weeks (*Avena sativa*), the remaining plants are harvested for measuring additional endpoints characterizing their reproductive potential. In all cases, the test duration should be sufficient to determine reproductive endpoints (e.g. number or biomass of flowers or seeds or fruit).

Typically, 10 seeds are sown in four replicate test pots each. Plants are thinned out to 8 per pot, and four plants each are harvested at day 14 and at the end of the test. If in any pot less than 8 plants have emerged, the number of plants harvested at day 14 shall be reduced such that four plants remain for the final harvest.

The relative inhibition in undiluted test soils is determined to assess the suitability of the soil for plants. In addition, based on a dilution series, NOEC, LOEC and EC_x values can be calculated from the dose response curves. The latter is required when chemicals are tested.

5 Materials

5.1 Test plants

One monocotyledonous and one dicotyledonous species are tested in parallel. Oat (*Avena sativa*) is recommended as the monocotyledonous and *Brassica rapa* as the dicotyledonous plant species.

To shorten the test period, a rapid-cycling variety of turnip rape (*Brassica rapa* CrGC syn. Rbr) is strongly recommended[1)]. Flowering starts after two weeks and seed production can be determined after approximately five weeks.

Other species may be selected, e.g. from the list given in ISO 11269-2 or plants with specific physiological characteristics such as C-4 plants (corn, sugar cane, millet), plants in symbiosis with nitrogen-fixing bacteria (e.g. *Fabaceae*) or plants with ecological or economic significance in certain regions of the world. These plants shall grow unhindered in control soil under the conditions specified. Only plants that tolerate the properties of the test soils and test conditions (including their chemical contamination) should be selected. For example, a species sensitive to low pH values should not be used for testing forest soils with low pH-values. Oat and rapid-cycling turnip rape grow in sandy as well as loamy soil with varying water content and a range of pH values from 5,0 to 7,5. Species that do not tolerate wet soils should not be used in combination with wick watering. Reasons for selecting species other than oat and turnip rape shall be justified in the test report.

5.2 Soil and soil storage

5.2.1 General

The description of methods for representative sampling of soils from contaminated sites is not within the Scope of this International Standard. A suitable sampling method is given in ISO 10381-6 [13].

1) Seeds and Wisconsin Fast Plants kits are suitable products supplied by the Carolina Biological Supply Company, Burlington, NC, USA. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

The chronic plant test can be used to assess the toxic potential of natural soils from a variety of contaminated sites. It can also be used to assess the quality of remediated soils. The selected soils should have pH values after sieving within a range that is not toxic to the test plants, e.g. between 5,0 and 7,5 for *Brassica rapa* and *Avena sativa*. Test soils shall be passed through a sieve of mesh 4 mm to 5 mm square to remove coarse fragments, and mixed thoroughly. If necessary, soil may be air-dried without heating before sieving. Storage of test soils should be as short as possible. Storage at approximately 4 °C using containers that minimize losses of soil contaminants by volatilization and sorption to the container walls is recommended.

5.2.2 Test soil

The water-holding capacity shall be measured for all soil mixtures used in the test. Additionally, test soils should be characterized by:

— texture (sand, silt, clay);

— pH value;

— salinity;

— organic carbon;

— total and water-soluble amounts of potassium, nitrogen and phosphorus.

Soil pH should not be corrected. Measurements of soil contaminants (heavy metals, hydrocarbons, pesticides, explosives, PCBs and others) are not mandatory.

It should be checked whether the test soil sucks water via wicks sufficiently (see 5.4). Water repellency or poor water transport can occur with very sandy soils, soils highly contaminated with hydrocarbons or even with soils of high clay content that tend to compact even when these soils have a high water-holding capacity (determined after initially submerging the soils). To assure functioning of the watering system, a pre-test including all soils selected for the test and replicated twice should be performed to decide whether wick watering is sufficient or manual watering is required.

NOTE For the time being, pH limits for plant species other than turnip rape and oat cannot be given. It is matter of future research to systematically test more plants on a variety of soils. Furthermore, tolerance limits for texture, salinity or other soil properties cannot yet be given for different plant species.

5.2.3 Control soil

Either artificial, reference or standard soils may be used as control substrate.

If reference soils from uncontaminated areas near a contaminated site are available, they should be treated and characterized like the test soils. In addition, to verify that a reference soil does not carry toxic contaminants, chemical analysis of the expected contaminants shall be carried out. If toxic contamination or unusual soil properties cannot be ruled out, standard control soils should be given preference.

Standard soils should be uncontaminated, nutrient-poor natural or artificial soils. If a natural soil is used, its organic matter content should not exceed 5 %. Fine particles (< 20 µm) should not exceed 20 %.

Alternatively, artificial soil in accordance with reference [6] and ISO 11268-2 may be used, regardless of its higher organic matter content. However, the organic matter contents of the test and control soil should be as close to each other as possible. The artificial soil consists of the following components (percentage based on dry mass):

— 10 % sphagnum peat [air-dried and finely ground (2 mm ± 1 mm)];

— 20 % kaolin clay (kaolinite content preferably above 30 %);

— approximately 69 % (depending on the amount of $CaCO_3$ needed) air-dried industrial quartz sand (predominantly fine sand with more than 50 % mass fraction of particle size 0,05 mm to 0,2 mm).

Approximately 0,3 % to 1,0 % calcium carbonate ($CaCO_3$, pulverized, analytical grade) is necessary to obtain a pH of 6,0 ± 0,5. The amount of calcium carbonate required can vary, depending on properties of the individual batch (mainly the peat) and should be determined by measuring sub-samples immediately before the test.

The artificial soil is prepared by mixing the dry constituents listed above thoroughly in a large-scale laboratory mixer approximately one week before starting the test.

The mixed artificial soil shall be stored at room temperature for at least two days to equilibrate acidity. To determine pH and the maximum water-holding capacity, the dry artificial soil is premoistened one or two days before starting the test by adding enough deionized water to obtain approximately half of the required final water content of 40 % to 60 % of the maximum water-holding capacity (corresponding to 50 % ± 10 % moisture dry mass). The pH value is measured by mixing the soil with 1 mol/l KCl in a ratio of 1 to 5 (in accordance with Annex C of ISO 11268-2:1998). If the measured pH is not within the required range, a sufficient amount of $CaCO_3$ shall be added or a new batch of artificial soil shall be prepared. Parallel to determining the pH, the maximum water-holding capacity of the artificial soil shall be determined.

Afterwards, the artificial soil is divided into as many batches as the number of concentrations plus controls that is used in the test. Evaporation from the test substrate shall be avoided until the start of the test. The final moisture content is reached by adding water together with, or in parallel to, the application of the test substance. The moisture contents at the beginning and end of the test are determined by drying small samples at 105 °C overnight and reweighing.

If the control soil is richer in nutrients than the test soil, growth inhibition may become apparent from the dose (mixture) response curves. It is recommended to use nutrient-poor control soils or to add nutrients in order to avoid false-positive test results.

Addition of nutrients can reduce the relative difference between control and test soils. Nevertheless, nutrient additions may result in

— slower ripening of the plants,

— strong vegetative growth (larger test containers needed),

— adverse effects on the test plants, when a test soil already has high levels of nutrients or salts.

None of the soils used in the chronic plant test needs to be sterilized.

To obtain a dilution series, the test soil is thoroughly mixed with the reference or standard control soil (either manually or by using a hand mixer). The homogeneity of the mixture is checked visually.

5.3 Reference substance

Testing a reference substance in parallel can demonstrate the uniformity of the laboratory test conditions and the sensitivity of the test plants. In order to do this, a reference substance shall be added to a control soil (positive control). Recommendations found in literature are: zinc sulfate [7], boric acid (ASTM D1076) or sodium trichloroacetate (ISO 11269-2). Choice of a suitable reference substance should be subject to further testing and validation.

6 Apparatus

In addition to normal laboratory apparatus, the following are required.

6.1 Phytotron, plant growth room or greenhouse, suitable for maintaining the specified conditions.

6.2 Balance, capable of weighing with an accuracy of ± 0,1 mg.

6.3 Balance for heavier loads, e.g. of capacity 10 kg, for preparation of soil mixtures.

6.4 Sieve, 4 mm to 5 mm square mesh.

6.5 Test vessels

Plastic vessels appropriate to be filled with approximately 400 g of soil and a soil surface area of 73,5 cm^2 have successfully been used [3] 2). The vessels are equipped with fibreglass wicks. However, wicks are not used if a pretest shows that the test soil does not absorb water by wicks. Fibreglass wicks (10 mm $\pm$ 2 mm) are inserted into holes (1 mm to 2 mm narrower than the wick diameter). Wicks should not project more than 1 cm into the soil to avoid excess water within the lower soil layers. However, longer wicks can improve watering in soils with poor water absorption.

If the test and control soils are fertilized, the size of the test vessels and the amount of test substrate should be enlarged as appropriate in order to allow normal growth of the plants.

The following basic requirements should be considered.

— A fibreglass wick shall be introduced through the bottom of the vessels. The wicks reach a water reservoir and ensure the water supply during the test. Therefore, at least one hole shall be prepared to hold the wick. Commercial plant pots often have more than one hole, which can result in reverse flow of water. In addition, roots can grow through open holes and circumvent the soil contaminants. A filter disk can prevent growth of roots through additional holes.

  In cases where pots are watered manually without using wicks, vessels should have no bottom holes.

— Wicks should be large enough to ensure watering throughout the entire incubation time. That is, small wicks with a diameter of only a few millimetres sufficient for watering at the beginning might in some cases be clogged after a few weeks by soil particles or roots, or lose contact with shrinking soil.

— Pots are recommended that allow the growth of 8 plants until day 14 and four plants afterwards (Figure 1). If smaller pots are used, the number of plants per pot shall be reduced and the number of pots increased.

— If transparent vessels are used, an opaque sleeve should be mounted unless the test vessels are arranged close to each other.

2) Appropriate pots are white polystyrene beakers (height: 10 cm and base of approximately 4,5 cm $\times$ 7 cm at the bottom and 7 cm $\times$ 10,5 cm at the top) are commercially available, e.g. Bellaplast No. 507, Bellaplast, 9450 Altstaetten, Switzerland. This information is given for the convenience of users of this International Standard, and does not constitute and endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

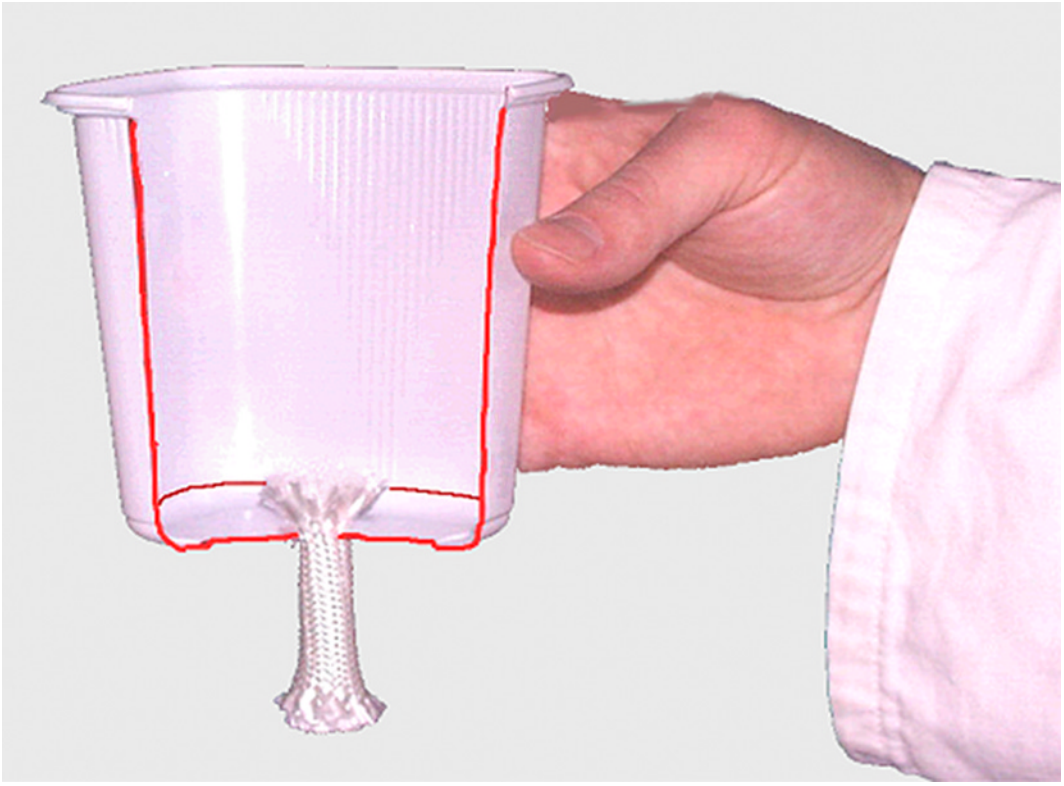

Figure 1 — Example of a test vessel with fibreglass wick

7 Methods

7.1 Experimental design

The applicability of wicks for watering of the test vessels should be assessed in a pre-test. A preliminary range finding test precedes the final test. Each final test consists of a series of soil mixtures (treatments). Each treatment is replicated at least four times, i.e. four test pots containing a number of test plants are used (see also 7.5).

7.2 Pre-test

Two pots equipped with wicks are prepared for each soil, i.e. the test soil, the dilutions of the test soil with control soil, the control soil and (if available) the reference soil. After filling with the sieved test soils and/or soil mixtures (approximately 400 g), the pots are installed above a water reservoir. The water should reach the soil surface via the wicks within 24 h. If this is the case, the soil is expected to be watered successfully by wicks. Otherwise, water should be added manually onto the soil surface until the soil is wet (but not highly soaked). In many cases, wick watering is successful after such an initial manual watering. In rare cases, the soils have to be watered manually throughout the entire test period.

7.3 Preliminary test (range-finding)

A preliminary test (range-finding) is mandatory when the toxicity of chemicals applied to soil is tested. When such soils are tested, a preliminary test to determine the range of mixture ratio affecting plant growth is optional. The test soil is mixed with the reference or a standard control soil by appropriate techniques. Mixture ratios of 0 %, 12,5 %, 25 %, 50 %, and 100 % test soil are suggested. The preliminary test can be much shorter than the chronic plant test and should be performed in accordance with ISO 11269-2. If toxic effects become evident after emergence, the test may be finished before the end of the growth period of two weeks.

7.4 Final test

At least five soil mixtures or test chemical concentrations are prepared. Therefore, a geometric series of mixture ratios or concentrations with a factor not exceeding two shall be selected, based on the preliminary test or the above soil mixtures shall be applied. At least four replicates of each treatment are prepared.

A limit test may be sufficient, if in a pre-test and/or a range finding test no toxic effect was observed. In the limit test, only the test soil without any dilution or one concentration of the test substance and the control (or the test soil vs. the control soil) shall be tested, with at least four replicates each.

7.5 Preparation of the pots

If soils or soil mixtures have been stored, they should be mixed a second time immediately before use. Fill pots, either with wicks or without wicks, with the soil mixtures to approximately 1 cm below the upper edge. All pots of each treatment should contain the same volume of soil. Most soils can be handled more easily when they are air-dried. Wet soils tend to compact strongly. In addition, seeds can stick to the tweezers when wet. Therefore, it may become necessary to partially air-dry the soils before filling the pots. The actual water content of each mixture should be known, in order to calculate the amount of water needed for initial watering at the start of the test.

The soil should not be compressed strongly. However, if the soil structure appears too loose or inhomogeneous, settling can be forced by dropping the vessels from a height of less than 5 cm onto a hard surface.

7.6 Preparation of the seeds

Plant 10 uniform undressed seeds of the selected species immediately after filling the pots. If pots other than those proposed (6.5) are used, the number of seeds may need to be corrected to make equivalent soil volumes and growth areas available to the plants. Prepare holes of a depth of either 5 mm to 10 mm for *Brassica rapa* or 10 mm to 15 mm for *Avena sativa*, put one seed into each hole and carefully smooth the soil surface. Alternatively, pick up seeds with the tip of tweezers and plant them directly at the required depth.

Seeds of oat can be selected by mass. Rejecting very light and heavy seeds can result in a slightly smaller variation in mass among plants. Seeds of *Brassica rapa* are too small for mass selection. There is no indication that seeds of varying colour, indicating different stages of maturity, develop differently. Unevenly shaped seeds should be rejected. If other test species have been chosen, other criteria for selecting seeds may be appropriate.

7.7 Growth conditions

Temperature, humidity and light conditions shall be suitable for normal growth of the test plants. Tests may be run in a phytotron, plant growth room or greenhouse. In addition to daylight (greenhouse), fluorescence tubes, gas-discharge, metal-halide, high-pressure mercury and high-pressure sodium lamps may be used. Lamps manufactured for plant growth should be chosen. The lamps should be powerful enough to be installed at least 1 m above the soil surface to allow handling of the plants during the test (rearrangement of pots, watering, pollination) and to avoid inhomogeneous temperature. In addition, the lighting level shall be essentially homogeneous across the area used in the test.

For *Avena sativa* and *Brassica rapa*, a 16 h illumination period at a light intensity of 13 000 lx ± 2 000 lx should be followed by 8 h of darkness.

A temperature of 23 °C ± 3 °C is appropriate for the two species. However, a wider range is acceptable as long as normal emergence and growth of the plants occur.

When testing contaminated soils, there should be sufficient ventilation to avoid cross-contamination of volatile toxicants between treatments and to prevent health hazards.

7.8 Start of the test

Immediately after planting the seeds, the soil shall be wetted as follows. Adjust the water content in each pot to 80 % for *Avena sativa* and 60 % for *Brassica rapa* (ISO 11269-2) of the water-holding capacity considering the mass of the plant pots, the soil mass and its actual water content. When doing this, the water-holding capacity of the control/test soil mixture should be considered. Carefully pour or spray demineralized water onto the soil surface until the calculated mass is reached. This method can be applied to test vessels both with and without wicks.

After wetting the soils, the pots shall be installed above water reservoirs. Only the wicks are allowed to be in contact with the water. Only pots from the same treatments may use the same reservoir. Since chemicals or nutrients can be washed out into the reservoirs, the water volume should be limited (e.g. $<$ 0,5 l per pot).

Individual pots or treatment groups should be placed randomly in the incubation area.

7.9 Handling during the test

7.9.1 Number of plants and thinning out

To compensate for non-germinating seeds, a higher number of seeds (typically 10) are planted in each pot than plants required for the growth test and the succeeding endpoints of the reproduction potential. Shortly after emergence, the number of plants should be reduced to 8 per pot (for *Avena sativa* and *Brassica rapa*, 7 days after sowing). Plants to be removed should be randomly selected and the remaining plants shall be evenly distributed. It is important that the density of plants in a test vessel does not limit normal growth. The number of 8 applies for *Avena sativa* and *Brassica rapa* and the pots specified above, and shall be adjusted if other species or differing sizes of pots have been used. To withdraw plants, they can be pulled out or, if the soil is very cohesive or plants grow very close to each other, cut off. When oat is cut off, a secondary shoot is sometimes produced, which has to be cut off again later.

Optionally, if the amount of test substrate and the available space is not limited, the plants to be harvested at day 14 can be grown in separate test vessels.

7.9.2 Watering

Demineralized water shall be used to fill the water reservoirs whenever needed. Ensure that the required soil moisture is maintained. Therefore, check regularly – for example visually or by carefully touching the soil surface – whether the surface is wet. If not, reweigh the pots and replenish the amount of water needed. If wick watering fails, carefully pour or spray the volume needed onto the soil surface regularly.

If no wicks are used, the soil moisture should be adjusted as described under 7.2. The mass of the test plants is small compared to the soil mass during the first two weeks and later, if the plants remain small. When plants grow strongly in soils with good nutrient supply, their mass becomes significant when calculating the soil moisture. During this period, the amount of added water is assessed by experience. The main criterion is to keep plants healthy (e.g. without wilting).

7.9.3 Rearrangement of test vessels

To prevent any effects of unequal lighting, temperature, humidity or ventilation on the growth of the test plants, the test vessels shall be rearranged randomly at regular intervals at least twice a week.

7.9.4 Pollination

The rapid-cycling variant CrGC, syn. Rbr of *Brassica rapa* requires pollination to produce fertile seeds and seed pods. Otherwise, seed pods are produced only occasionally. After approximately two weeks, when flowering starts, they should be pollinated manually using Q-tips, pipe cleaners, a soft paintbrush or “bee-sticks” (the hairy abdomen of a honeybee attached to a small stick) or the device supplied with the fast-growing variety. As long as significant numbers of flowers are produced, the procedure is repeated twice a week.

7.9.5 Reporting

The room temperature and humidity should be measured and recorded at short intervals (< 1 h) or continuously at the incubation area.

7.10 Endpoint measurements

7.10.1 Seedling emergence

The number of emerged seedling is counted for each pot. It is expressed as a percentage of the mean emergence in the control pots.

The day when 50 % of the seedlings in the control pots have emerged shall be determined.

7.10.2 Harvest at day 14

A portion of the test plants is randomly selected and cut at the soil surface on the 14th day after 50 % of the seedlings in the control pots have emerged. Four plants per pot shall be left for the final harvest. The following endpoints are measured:

— occurrence of visible flower buds per plant, i.e. determination by presence/absence (*Brassica rapa* only);

— number of flowers per plant (*Brassica rapa* only);

— fresh mass per plant, which should be measured immediately after cutting the plants;

— proportion of live plants (percentage of plants compared to the number after thinning out);

— number of damaged (yellowed, wilted, etc.) plants (qualitatively).

7.10.3 Final harvest

An exact date for the harvest of the remaining plants cannot be given. Oat should be harvested after the inflorescences in the control treatment have emerged (typically after 7 weeks to 8 weeks), and rapid-cycling turnip rape when seed pods have developed (typically after 5 weeks to 6 weeks) in the control treatment. Be aware that flowering and seed pod production can differ from these values (e.g. depending on nutrient levels, toxic substances and seed batches).

The plants are cut at the soil surface and the following endpoints are determined:

— growth stadium according to the BBCH scheme [5];

— total number of flowers per plant (*Avena sativa* only);

— number of seed pods carrying fertile seeds (visibly swollen) (*Brassica rapa* only);

— fresh mass of shoots[3)] (*A. sativa:* without inflorescences; *B. rapa:* without seed pods), which should be measured immediately after cutting the plants;

— fresh mass of inflorescences (*A. sativa*) or seed pods (*B. rapa*), which should be measured immediately after cutting the plants;

3) The dry mass can be calculated from the individual fresh mass and the water content of a representative subsample of plants of the respective treatment.

— water content of the shoots, inflorescences and seed pods of each pot (replicate), if a clear difference in the BBCH growth stage is found between control and test plants[4)];

— dry mass of shoots;

— dry mass of inflorescences (*Avena sativa*) or seed pods (*B. rapa*)[4)];

— proportion of dead plants (percentage of plants compared to the number after thinning out).

7.11 Summary and timetable of the test

Table 1

<table>
<tr><th>Period [a]</th><th colspan="2">Action (definitive test)</th></tr>
<tr><td>Pre-test activities
Step 1</td><td colspan="2">Preparation of test soil (air-drying, sieving, determination of soil properties).
Pre-test to check if soil takes up water by wicks.
Preparation of test vessels (labelling, mounting of wicks).</td></tr>
<tr><td>Pre-test activities
Step 2</td><td colspan="2">Preparation of test soil mixtures or application of test item.</td></tr>
<tr><td></td><td>Testing of chemicals:</td><td>Testing of soils:</td></tr>
<tr><td></td><td>Preparation of stock solution or emulsion and mixing into the soil or coating of quartz sand (if organic solvents are required). After evaporation of organic solvents, mixing of coated quartz sand into soil.</td><td>Mixing of test soil with control soil.
Filling of test vessels with test soils or soil mixtures.
Sowing of seeds.
Placing of test vessels in test area (random scheme).
Initial soil moistening.</td></tr>
<tr><td>Pre-test activities
Step 3</td><td colspan="2">If needed, refilling of water reservoirs or watering of test vessels from above.
Counting and thinning out of emerged seedlings.
Rearranging test vessels in test area (twice weekly).</td></tr>
<tr><td>Day 1</td><td colspan="2">50 % emergence in control pots</td></tr>
<tr><td>Day 14</td><td colspan="2">First harvest of test plants. Visual inspection and biomass determination of harvested plants.</td></tr>
<tr><td>Day 15 to 35</td><td colspan="2">If needed, refilling of water reservoirs or watering of test vessels from above.
Rearranging test vessels in test area (twice weekly).
Pollination of flowers of rapid-cycling Brassica rapa (twice weekly).</td></tr>
<tr><td>Day 35 [b]</td><td colspan="2">Final harvest of rapid-cycling Brassica rapa. Visual inspection and biomass determination of harvested shoots and seed pods.</td></tr>
<tr><td>Day 35 to 49</td><td colspan="2">If needed, refilling of water reservoirs or watering of test vessels from above.
Rearranging test vessels in test area (twice weekly).</td></tr>
<tr><td>Day 49 [b]</td><td colspan="2">Final harvest of Avena sativa. Visual inspection and biomass determination of harvested shoots and inflorescences.</td></tr>
<tr><td colspan="3">[a] The given timetable applies for tests with Avena sativa and rapid-cycling Brassica rapa. If other species are tested, plants might have to be harvested after different growth periods.
[b] Suggested dates. Depending on test conditions, the period until reproductive endpoints (seed pods of Brassica rapa or inflorescences of Avena sativa) can be determined may vary.</td></tr>
</table>

4) The water content of the shoots, seed pods, or flowers of each treatment is determined by drying a sample at 70 °C to 80 °C until a steady mass is reached. If the water content differs significantly between treatments, the dry mass of the shoots, seed pods or flowers can be calculated from the measured fresh mass and the water content, and used in subsequent assessments.

8 Validity criteria

The following criteria shall be fulfilled in the controls:

— emergence rate of the control plants of at least 75 % (mean value of all replicates);

— healthy plants develop: plants do not etiolate and flowers appear during the first three weeks (rapid-cycling turnip rape) or 8 weeks respectively (oat);

— not more than one emerged plant per pot has died during the test.

9 Assessment of the results

9.1 Presentation of measured data

Tables should be prepared that contain the following information:

— number of seeds per pot;

— number of emerged seedling per pot;

— number of live plants per pot at day 14;

— number of plants remaining per pot after cutting at day 14;

— shoot length of harvested plants at day 14;

— occurrence of flower buds at day 14 (*B. rapa*);

— number of flowers at day 14 (*B. rapa*);

— fresh mass of each harvested plant at day 14;

— number of plants per pot living at the end of the test;

— shoot length of harvested plants at the end of the test;

— number of flowers per plant at the end of the test (*A. sativa*);

— number of seed pods per plant at the end of the test (*B. rapa*);

— fresh mass of shoots per plant (*A. sativa*: without flowers; *B. rapa:* without seed pods);

— fresh mass of inflorescences per plant (*A. sativa*);

— fresh mass of seed pods (*B. rapa*);

— water content of the shoots, inflorescences and seed pods of each treatment;

— calculated dry mass of the shoots, inflorescences and seed pods (if water content differs significantly between treatments).

A graphical presentation of the mean values, including standard deviation of the measured values against the test chemicals concentration or soil mixture ratio, should be prepared. These curves give an impression of the quality of effects and their magnitudes and the occurrence of [1] (defined as an increase in the respective test parameter at low concentrations). Express the concentration or mixture ratio as based on soil dry mass.

9.2 Statistical analysis

9.2.1 Preliminary test

If a clear dose-response relation is obvious, EC_x values can be estimated by using regression techniques, such as logistic regression function or probit analysis. In other cases, the effect range should be determined by expert knowledge.

9.2.2 Final test

It should be kept in mind that the proposed statistical methods are not appropriate in the case of hormetic effects.

The data can be analysed in two ways: see 9.2.3 and 9.2.4.

9.2.3 NOEC (no observed effect concentration) approach

First, a statistical analysis of the homogeneity of the variances shall be made, e.g. by using Cochran´s test. With homogeneous data, an appropriate statistical analysis, e.g. a “One-Way Analysis of Variance (ANOVA)”, followed by a Dunnett test (a = 0,05), should be performed. Since at low concentrations an increase in the respective test parameter is often observed (= hormesis), Dunnett´s test should be performed two-sided. Alternatively, if one is only interested in a decrease in the respective parameter, Dunnett´s test should be performed one-sided in order to determine the NOEC.

If the homogeneity requirement is not fulfilled, it is recommended to evaluate whether an appropriate transformation of the data could solve the problem. Otherwise non-parametric methods, e.g. the Mann and Whitney U-test or the Bonferroni U-test can be used.

If a limit test has been performed and the pre-requisites (normality, homogeneity) of parametric test procedures are fulfilled, the Student t-test or the Mann-Whitney U-test procedure should be used.

9.2.4 EC_x (effect concentration) approach

The EC_x approach can only be used if a clear dose-response relationship is found. Problems may arise from hormesis effects. Wherever possible, R^2 should be 0,7 or higher ,and the test concentrations used should encompass 20 % to 80 % effects. If these requirements are not fulfilled, expert knowledge is necessary for the interpretation of the test results.

To compute an EC_x value, the treatment means are used for regression analysis after an appropriate dose-response function has been found (e.g. probit or logistic function). A desired EC_x is obtained by inserting a value corresponding to x % of the control mean into the equation found by regression analysis. Since EC_{50} values have smaller confidence limits compared with smaller effect concentrations (e.g. EC_{20}), it is recommended to determine EC_{50} values.

In any case, the results of the statistical evaluation should be biologically interpreted.

It is recommended that a statistician be involved in the analysis of the test, since in this International Standard guideline-specific guidance on statistical procedures is given only in limited detail.

10 Test report

The test report shall refer to the standard and shall contain a summary of the results obtained, the methods and parameters used during the study. The test report shall provide the following information:

a) a reference to this International Standard, i.e. ISO 22030:2005;

b) a full description of the experimental design and procedures;

c) test plant species (variety, source);

d) test soil (according to 5.2.2);

e) control soil (type, source);

f) pot size and material;

g) methods of soil pre-treatment, mixing or addition of test chemical (if applicable);

h) mass of soil per pot;

i) incubation conditions, including:

   1) type of environment (phytotron, laboratory, greenhouse, etc.);

   2) temperature;

   3) humidity;

   4) light conditions;

j) depth of sowing;

k) method of watering;

l) details on handling during the test, including pollination, harvest or other;

m) endpoints according to Clause 8, including statistical methods used;

n) qualitative description of visible damage;

o) discussion of the results.

Annex A
(informative)

Testing of chemicals in soil

For the assessment of the toxicity of chemicals in soil towards the growth and reproductive potential of higher plants, the chronic plant test can be used with the following modifications.

a) A well known, non-toxic natural or artificial standard soil, suitable for normal growth of the selected test plants is chosen. The soil should suck water sufficiently to allow watering by wicks. A reference soil is not needed.

b) The test chemical is added to the soil by appropriate methods to obtain a geometric series of at least five concentrations including the control. A range-finding test is mandatory to choose an appropriate range of concentrations. To introduce the test substance, use either method 1), 2) or 3), as appropriate.

   1) Water-soluble substances: immediately before starting the test, prepare a solution, emulsion or dispersion of the test substance in deionized water in a quantity sufficient for all replicates of one concentration (treatment). The amount of water should be small, since the soil should not be too moist before filling the test vessels. The amount of water should be the same for all treatments. Mix the emulsion or dispersion thoroughly with one batch of soil;

   2) Substances insoluble in water but soluble in organic solvents: prepare a solution of the test substance in an organic solvent (carrier). Spray the carrier onto or mix into a small amount of fine quartz sand. Then evaporate the carrier. It is important that the sand then be mixed thoroughly. Only solvents which readily volatilize can be used as carriers. Finally, mix the sand containing the test substance thoroughly with the soil. All treatments should receive the same amount of sand and solvent. Establish a second control with only sand and solvent.

      WARNING — Appropriate precautions should be taken when dealing with solvent vapour to avoid health risks from inhalation or explosion, and to avoid damage to extraction equipment, pumps, etc.

   3) Substances insoluble in water or organic solvent: prepare a mixture of 10 g of finely ground quartz sand and the quantity of the test substance required to obtain the desired concentration. Then mix this mixture thoroughly with the soil.

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