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ISO 11269-2

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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

Qualité du sol — Détermination des effets des polluants sur la flore du sol —

Partie 2: Effets des sols contaminés sur l'émergence et la croissance des végétaux supérieurs

Reference number
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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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

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

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

ISO 11269-2 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

This third edition cancels and replaces the second edition (ISO 11269-2:2005), which has been technically revised.

ISO 11269 consists of the following parts, under the general title *Soil quality — Determination of the effects of pollutants on soil flora*:

— *Part 1: Method for the measurement of inhibition of root growth*

— *Part 2: Effects of contaminated soil on the emergence and early growth of higher plants*

Introduction

This part of ISO 11269 describes a procedure for evaluating the quality of soils of different origin carrying unknown contaminations. The evaluation of the effects on plant growth is based on emergence and inhibitory effects on early growth of at least two species of higher plants. Guidance for assessing potential effects of substances on seedling emergence and growth is given in OECD Guideline 208[14].

This part of ISO 11269 refers closely to ISO 22030 and is based on:

a) results from the German research project "Entwicklung eines innovativen und technischen Instrumentariums zur Optimierung der ökotoxikologischen Bewertung von Böden im Hinblick auf Sanierungsziele und Schutzerfordernisse";

b) discussions within the joint project "Ecotoxicological Test Batteries" forming part of the BMBF Joint Research Group "Processes for the Bioremediation of Soil"[23];

c) results from the BMBF Joint Research Group ERNTE "Erprobung und Vorbereitung einer praktischen Nutzung ökotoxikologischer Testsysteme"[17];

d) ring-test results of "Ecotoxicological Characterisation of Waste — Results and Experiences from an International Ring Test"[8].

Plant growth can be influenced strongly by soil properties such as texture, pH or levels of nutrients. When testing natural soils either reference soils (uncontaminated soils with the same properties as the test soil) or standard soils are used as mixing and control substrate. In the latter case, variations in plant growth can result from either soil contaminants or differences in soil properties like nutrients and texture. Therefore, results from soil testing can less easily be interpreted than results from testing of chemicals .

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

WARNING — Contaminated soils may contain unknown mixtures of toxic, mutagenic, or otherwise harmful chemicals or infectious micro-organisms. Occupational health risks may arise from dust or evaporated chemicals during handling and incubation. Furthermore, test plants might take up chemicals from the soil and safety measures should also be considered when handling the test plants.

1 Scope

This part of ISO 11269 describes a method to assess the quality of an unknown soil and the soil habitat function by determining the emergence and early growth response of at least two terrestrial plant species compared to reference or standard control soils. It is applicable to soils of unknown quality, e.g. from contaminated sites, amended soils or soils after remediation.

2 Normative references

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

ISO 10381-6, *Soil quality — Sampling — Part 6: Guidance on the collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory*

ISO 10390, *Soil quality — Determination of pH*

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

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

ISO 11268-1, *Soil quality — Effects of pollutants on earthworms — Part 1: Determination of acute toxicity to Eisenia fetida/Eisenia andrei*

ISO 11268-2, *Soil quality — Effects of pollutants on earthworms — Part 2: Determination of effects on reproduction to Eisenia fetida/Eisenia andrei*

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*

ISO 22030, *Soil quality — Biological methods — Chronic toxicity in higher plants*

3 Terms and definitions

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

3.1
emergence
appearance of the coleoptile or cotyledon above the soil

3.2
contaminant
substance or agent present in the soil as a result of human activity

[ISO 15176:2002[28]]

3.3
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][2]

3.4
lowest observed effect rate or effect concentration
LOEC
lowest tested percentage of a test soil in a reference or a standard control soil or concentration of a substance at which a statistically significant effect is observed

NOTE The LOEC is expressed as a percentage of the test-soil dry mass per soil-mixture dry mass. All test mixtures above the LOEC have a harmful effect equal to 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 (3.5) have been selected.

3.5
no observed effect concentration
NOEC
test-soil percentage immediately below the LOEC, which when compared to the control has no statistically significant effect ($\rho < 0{,}05$)

3.6
***x* % effect concentration**
EC_x
***x* % effect rate**
ER_x
percentage of a test soil at which a given endpoint is inhibited by x % compared to the control

3.7
soil mixture ratio
ratio between the test soil and the reference/control soil in a soil mixture, expressed in percent based on soil dry mass

NOTE Different ratios may be applied in a dilution series to establish a dose-response relationship.

3.8
reference soil
uncontaminated site-specific soil (e.g. collected in the vicinity of a contaminated site) with similar properties (nutrient concentrations, pH, organic carbon content and texture) as the test soil

3.9
standard soil
field-collected soil or artificial soil whose main properties (e.g. pH, texture, organic matter content) are within a known range

EXAMPLE Euro-soils[11], artificial soil[14], LUFA soil.[1)]

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

3.10
control soil
reference or standard soil used as a control and as a medium for preparing dilution series with test soils or a reference substance

NOTE Both EC_{50} and NOEC are expressed in milligrams of test substance per kilogram (dry mass) of the test substrate. Soil mixtures are given in per cent based on soil dry mass.

4 Units

Emergence is expressed as the percentage of seedlings which emerge as compared with the control pots. The biomass of the shoots is expressed as dry mass per plant or, if needed, as dry mass per pot.

5 Principle

The test measures emergence and early growth of at least two terrestrial plant species (one monocotyledonous and one dicotyledonous). The test compares the development of plants in a test soil and/or a series of mixtures with a control soil. Seeds of the selected plant species are planted in pots containing the soil/soil mixtures and in control pots containing a reference or standard soil. Pots are kept under growth conditions for the test species selected. After 50 % of the seedlings in the control have emerged, emergence rates are determined and plants are thinned out to a specified number. After a period of two weeks to three weeks, the remaining plants are harvested to determine their biomass. The relative growth inhibition in undiluted test soil is determined to assess the function of the test soil as a habitat for plants. In addition, NOEC, LOEC or EC_x and ER_x values can be calculated from the dose response curve gained from mixtures of the test soil with control soil.

NOTE An early plant growth test may include additional testing endpoints, e.g. shoot length, root length and root dry mass. In many instances, root endpoints are more sensitive than shoot dry mass. In almost all cases, emergence is a less sensitive endpoint.

6 Test plants

One monocotyledonous and one dicotyledonous species are tested in parallel. Oat (*Avena sativa*) is recommended as the monocotyledonous and turnip rape (*Brassica rapa*) and/or wild turnip (*Brassica rapa* ssp. *rapa*) as the dicotyledonous plant species. Oat, turnip rape and wild turnip grow in sandy as well as in loamy soil with varying water content and a range of pH values from 5,0 to 7,5.

Other species might be selected, e.g. plants with specific physiological characteristics like C-4 plants (corn, sugar cane, millet), plants in symbiosis with nitrogen-fixing bacteria (e.g. Fabaceae) or plants with ecological or economical significance in certain regions of the world, provided that these species grow unhindered in control soil and fulfil the validity criteria of the test (Clause 11). Only plants that tolerate the properties of the test soils and test conditions (beside 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. Species that do not tolerate wet soils should not be used in combination with wick watering. Reasons for selecting species other than oat and wild turnip or turnip rape shall be justified in the test report.

NOTE Additional recommended species including validity criteria and reference toxicant test data for different endpoints are compiled in Annexes A and B.

1) Euro-soils, artificial soil and LUFA soil are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

7 Materials

7.1 Test vessels

The test vessels shall be non-porous plastics or glazed pots with a top internal diameter of between 85 mm and 95 mm, taking into account the size. It is recommended to use an automatic watering system, e.g. pots equipped with glass-fibre wicks, to avoid the time-consuming daily manual adjustment of soil moisture as proposed in ISO 22030. In this case, one or two glass-fibre wicks (∅ 1 mm) 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 fix the wick. Commercial plant pots often have more than one hole, what might result in flow back of water. In addition, roots might grow through open holes and circumvent the soil contaminants. A filter disk can prevent growth of roots through additional holes. Wicks are not used, when the test soil does not take up water by wicks as shown by a pretest (see 10.2).

7.2 Soil

7.2.1 General

Assessing the toxic potential of field soil from a contaminated site or that of remediated soil, 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*.

The soil pH should not be corrected. For the time being, pH limits for plant species other than turnip rape and oat cannot be stated. It is a 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 be given for different plant species so far.

When comparing soils of known and unknown quality, the control soil and field soil under test should be of the same textural class, and be as similar as practicable in all respects other than the presence of the chemical or contaminant being investigated. Indeed, significant differences in soil characteristics other than the presence of contaminants may lead to differences in plant growth and may induce false positive test results.

7.2.2 Test soil

The sample(s) of test soil might be field-collected soil from an industrial, agricultural or other site of concern, or waste materials (e.g., dredged material, municipal sludge from a sewage sludge treatment plant, composed material, or manure) under consideration for possible land disposal.

The soils used in the test shall be passed through a sieve of 4 mm square mesh to remove coarse fragments and thoroughly mixed. If necessary, soil may be air dried without heating before sieving. Storage of test soils should be as short as possible. The soil shall be stored in accordance with ISO 10381-6 using containers that minimize losses of soil contaminants by volatilization and sorption to the container walls. Soil pH should not be corrected as it may influence bioavailability of soil contaminants.

For interpretation of test results, the following characteristics should be determined for each soil sampled from a field site:

a) soil texture (sand, loam, silt) in accordance with ISO 11277,

b) pH (KCl) value in accordance with ISO 10390,

c) water content in accordance with ISO 11465,

d) water-holding capacity (Annex C),

e) cationic exchange capacity in accordance with ISO 11260,

f) organic matter content in accordance with ISO 10694,

g) total and water soluble amounts of potassium, nitrogen and phosphorous.

NOTE It is important to measure the water-holding capacity of all mixtures used in the test.

7.2.3 Control soil

Either reference or standard soils can be used as the control soil, 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 might 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 (3.3) can be expected at low soil mixture ratios or even an inverse dose response relationship if nutrient supply becomes the main effect. It is therefore recommended to add nutrients to test and control/reference soils in order to avoid false positive or negative test results (10.6.3).

7.2.3.1 Reference soils

If reference soils from uncontaminated areas near a contaminated site are available, they should be treated and characterized like the test soils. If a toxic contamination or unusual soil properties cannot be ruled out, standard control soils should be preferred.

7.2.3.2 Standard soils

Standard soils should be uncontaminated, nutrient-poor natural or artificial soil. If a natural soil is used, its organic matter content should not exceed 5 %. Fine particles (<20 µm according to ISO 11277) should not exceed 20 %. Alternatively, artificial soil according to ISO11268-1 and ISO 11268-2 may be used, regardless of its higher organic matter content. However, the organic matter content of the test and control soil should be as close to each other as possible.

The substrate called "artificial soil" has the following composition:

	Percentage expressed on dry-mass basis
— Sphagnum peat finely ground and with no visible plant remains	10 %
— Kaolinite clay containing not less than 30 % kaolinite	20 %
— Industrial quartz sand (dominant fine sand with more than 50 % of particle size 0,05 mm to 0,2 mm)	69 %

Approximately 0,3 % to 1,0 % calcium carbonate ($CaCO_3$, pulverized, analytical grade) is necessary to get a pH of 6,0 ± 0,5.

NOTE 1 Taking the properties of non-polar (log $P_{OW} > 2$) or ionizing substances into account, 5 % of peat have proven to be sufficient for maintaining the desired structure of the artificial soil. In this case, the respective percentages of the constituents are modified as follows: peat, 5 %; clay, 20 %; sand 75 %).

NOTE 2 pH (KCl) is measured in a mixed sample in a 1 M solution of potassium chloride (KCl) or a 0,01 M solution of calcium chloride ($CaCl_2$).

The artificial soil is prepared, at least three days prior to starting the test, by mixing the dry constituents listed above thoroughly in a large-scale laboratory mixer. A portion of the deionized water required to obtain half of the final water content of 40 % to 60 % of the maximum water-holding capacity is added while mixing is continued. The mixed artificial soil shall be stored at room temperature for at least two days to equilibrate acidity. The amount of calcium carbonate required might vary, depending on properties of the individual batch of sphagnum peat and should be determined by measuring the pH of subsamples immediately before the test. The total water-holding capacity is determined according to Annex C, the pH is determined according to ISO 10390.

Allowance should be made for any water that is to be used for introducing the test substance into the soil.

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

8 Equipment

Standard laboratory equipment including the following materials is required.

8.1 Controlled environmental chamber, phytotron, plant growth room or greenhouse suitable to maintain the specified conditions.

8.2 Balance (±0,1 mg).

8.3 Balance for heavier loads (e.g. 10 kg) for preparation of soil mixtures.

8.4 Sieve, stainless steel, with mesh size 4 mm.

8.5 Glass-fibre wicks (∅ 1 mm).

9 Reference substance

It is recommended that a reference substance be tested to demonstrate the uniformity of the laboratory test conditions and the response of the particular batch of seeds. Sodium trichloroacetate or boric acid is suggested as the reference substance. A reference test should be carried out regularly and if any major changes in operating procedures are introduced, for example, change of phytotron/growth room/greenhouse, change of soil or change of watering regime, etc. Examples of phytotoxic values for two reference compounds are given in Annex B.

10 Procedure

10.1 Experimental design

A sample of field-collected test soil may be tested at a single concentration (typically 100 %) or evaluated for toxicity in a multi-concentration test whereby a series of concentrations (dilutions) are prepared by mixing defined quantities with a control soil.

Depending on the knowledge of relevant response levels, a preliminary range-finding test may precede 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.

To avoid any masking of the phytotoxic effect expression by nutrient deficiency, all treatment groups including control soils are amended by fertilizers (10.6.3) after seedlings have emerged. It has been shown that effects of pollutants on plant growth are pronounced in soils with optimum nutrient supply[6][7][17][22][25].

To assure functioning of the watering system (see 10.6.2), it should be checked whether the test soil sucks water via wicks sufficiently. Water repellence or poor water transport can occur with very sandy soils, soils highly contaminated with hydrocarbons or even with soil of high clay content that tend to compact even when these soils have a high water-holding capacity (determined after initially submerging soils). For this reason, a pretest including all soils selected for the test and replicated twice should be performed to decide whether wick watering is applicable or manual watering is required.

10.1.1 Pretest

Two pots equipped with wicks are prepared for each soil, 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, the pots are installed above a water reservoir by using a suitable device that avoids direct contact of the pots with the water supply. The water should reach the soil surface 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 shall be watered manually throughout the whole test period.

10.1.2 Range-finding test

A preliminary test to find 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 rates of 0 %, 12,5 %, 25 %, 50 %, and 100 % test soil are suggested. If toxic effects become evident after emergence, the test may be finished before the end of the growth period of two weeks.

10.1.3 Definitive test

The design of the definitive test depends on the test objectives. Typically, the habitat properties of samples of a field-collected test soil are characterized by a comparison of the biological effects for the test soil(s) with the effects found in a reference soil or, if not available or not appropriate due to toxicity or atypical physicochemical characteristics, in a standard soil. Results for the standard soil assist in distinguishing contaminant effects from non-contaminant effects caused by soil physicochemical properties. Regardless of whether a reference soil or standard soil is used for the statistical comparisons, the results from standard soil shall be used to judge the validity and acceptability of the test (see Clause 11)[4].

If, for characterization purposes, a test design including a dilution series is required, the following three designs are possible (the concentrations shall be spaced by a factor not exceeding 2).

— For the NOEC approach, at least five concentrations in a geometric series should be used. Four replicates for each concentration plus eight controls are recommended.

— For the ER_x and EC_x approach, 12 concentrations should be used. Two replicates for each concentration plus six controls are recommended. The spacing factor may be variable; smaller at low concentrations, larger at high concentrations.

— In cases where both threshold levels are required for a mixed approach, 6 concentrations to 8 concentrations in a geometric series should be used. Four replicates for each concentration plus eight controls are recommended. This mixed approach allows a NOEC as well as an ER_x/EC_x evaluation.

A limit test may be sufficient if, in the range-finding test, no toxic effect was observed. In the limit test only the test soil without any dilution and the control shall be tested with at least four replicates each.

10.2 Preparation of the pots

If soils or soil mixtures have been stored, they should be mixed a second time immediately before use. Pots either equipped with wicks or without wicks are filled with the soil mixtures to approximately 1 cm below the upper edge. All the pots of each treatment should contain the same volume of soil. Most soils can be handled more easily if their water content equals 20 % to 40 % of their maximum water-holding capacity. Wet soils tend to compact strongly. In addition, seeds might stick to the tweezers when wet. Therefore, it may become necessary to partially air-dry or rewet the soils before filling the pots. The actual water content of each mixture should be known 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 incoherent, settling can be forced by dropping the vessels from a height of less than 5 cm onto a hard surface.

10.3 Preparation of the seeds

Plant 10 uniform undressed seeds of the selected species immediately after filling the pots. If pots other than the proposed ones are used, the number of seeds may need to be corrected to make equivalent soil volumes and growth areas available to the plants. Either prepare holes of a depth of 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 seeds with the tip of tweezers and plant them directly in the required depth.

Oat seeds can be selected by weight. Rejecting very light and heavy seeds can result in a slightly smaller variation in plant weight between plants. Seeds of *Brassica rapa* are too small for weight 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.

10.4 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 strong 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) and to avoid inhomogeneous temperature. In addition, the lighting rate shall be essentially homogenous across the area where pots are placed in the test.

For *Avena sativa* and *Brassica rapa*, a 16 h illumination period with an intensity of at least 7 000 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 are occurring.

There should be sufficient ventilation to avoid cross-contamination of volatile toxicants between treatments and to prevent health hazards.

10.5 Start of the test

In cases where sub-irrigation with glass-fibre wicks can be used (see 10.1), the pots may be installed above water reservoirs without any additional wetting of the moist soils (see 10.2). 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 might be washed out into the reservoirs, the water volume should be limited (e.g. <0,5 l per pot).

Soil moisture content shall not be adjusted through watering and reweighing but is kept constant at a percentage close to the maximum water-holding capacity ($C_{w,max}$). Pots shall be checked visually for sufficient water supply by the wick watering system to maintain good growing conditions.

If, according to the results of the pretest, wick watering is not appropriate, the soil shall be wetted to reach a water content that is equivalent to 60 % to 80 % of the $C_{w,max}$ immediately after placing the seeds into the moist soil (see 10.2).

Soil moisture content shall be adjusted daily to maintain a predetermined percentage of the water-holding capacity, e.g. 80 % for *Avena sativa* and 60 % for *Brassica rapa*. A sufficient check can be made by weighing several randomly selected pots daily. Anaerobic conditions should be avoided and noted in the test report.

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

10.6 Handling during the test

10.6.1 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 are required for the test. After the emergence assessment within each pot (for *Avena sativa* about three days to five days and for *Brassica rapa* about seven days to eight days after sowing), thin the seedlings to give a total of five evenly spaced representative specimens of the plants in the pots. It is important that the density of plants in a test vessel does not limit normal growth. The number of five specimens per pot 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, they can be cut off. When oat is cut, a secondary shoot is sometimes produced, which shall be cut again later. If necessary, water losses in the reservoirs are compensated with fertilizer solution (10.6.3). Terminate the test no sooner than 14 days and no later than 21 days after 50 % of the control seedlings have emerged.

10.6.2 Watering

Demineralized water shall be used to fill the water reservoirs whenever needed. Ensure that the required soil moisture is being 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 checked by weighing several randomly selected pots daily. If necessary the moisture content is adjusted to maintain the predetermined percentage of the water-holding capacity, e.g. 80 % for *Avena sativa* and 60 % for *Brassica rapa*.

10.6.3 Nutrient supply

After seedling emergence, the reservoirs are filled with a dilution of commercially available liquid fertilizer. Preferably, a weak nutrient solution, able to provide a minimal level of nutrient, should be used. The nutrient concentration of this solution (calculated from the information provided on the fertilizer package and the dilution factor) is recorded. Alternatively to wick-watering (time-consuming), manual watering with water or, later, with a diluted fertilizer solution should be performed. (Recommendations for nutrient solutions are given in Annex D.)

10.6.4 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.

10.6.5 Records

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

11 Validity criteria

For the test, the following performance criteria shall be met in the controls.

— emergence shall be sufficient to provide seven healthy seedlings of 10 per pot;

— the seedlings do not exhibit visible phytotoxic effects (e.g. chlorosis, necrosis, wilting, leaf and stem deformations) and the plants exhibit only normal variation in growth and morphology for that particular species;

— the mean survival of emerged seedlings is at least 90 % for the duration of the study.

12 Assessment of the results

12.1 Data presentation

Present the data in tabular form, recording the number of plants that emerge per replicate and the total dry mass of shoots of seedlings per replicate at harvest, after oven drying at 70 °C to 80 °C for 16 h.

12.2 Expression of the results

A graphical presentation of the mean values including standard deviation of the measured values against the soil mixture ratio should be prepared. This curve gives an impression of the quality of effects and their magnitudes and the occurrence of hormesis[2] (defined as an increase of the respective test parameter at low concentrations). Express the mixture ratio as based on soil dry weight.

13 Statistical analysis

13.1 General

Analyses of the results differ according to the purposes and particular designs of the test. Standard statistical procedures (e.g. analysis of variance and multiple comparisons) generally are sufficient for analysing the

results. Comprehensive guidance to select the appropriate statistical method for each data type (quantal or continous) is given in Reference [21].

13.2 Range-finding test

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

13.3 Final test

Test undiluted test soil by comparison with control soil(s).

Analysis of variance (ANOVA) involving multiple comparisons of endpoint data derived for undiluted test soils (single-concentration test) including field replicates of field-collected soil from more than one sampling location is commonly used for statistical interpretation of the significance of findings from soil toxicity tests. This is an hypothesis-testing approach, and is subject to appreciable weaknesses[4]. The parametric analyses (e.g. ANOVA and multiple comparisons) for such data assume that the data are normally distributed, that the treatments are independent, and that the variance is homogenous among the different treatments. These assumptions shall be tested. 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, parametric analysis should proceed, even if moderate nonconformity continues after transformation[4][5].

In cases where various dilutions (concentrations) of each sample of field-collected soil with negative control soil or clean reference soil are tested, data can be analysed in two ways.

a) NOEC (no-observed-effect-concentration) approach

If the NOEC is to be estimated, application of powerful statistical tests should be preferred and these should be selected on the basis of data distribution[21].

For the continous endpoint growth of the seedlings expressed in dry mass, the following recommendations may apply:

First of all, 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 ($\alpha = 0{,}05$), should be performed. Since, at small concentrations, an increase of 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 of the respective parameter, Dunnett´s test should be performed one-sided in order to determine the NOEC (no-observed-effect-concentration).

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 U-test by Mann & Whitney or the Bonferroni-U-Test could be used.

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

b) EC_x (effect concentration) or ER_x (effect rate) approach

The EC_x or ER_x approach can only be used if a clear dose response relationship is found. Problems may arise from hormesis effects. Wherever possible, R^2, where R is the rate, should be 0,7 or higher and the test mixtures used 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 or ER_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). For estimating ER_x and its confidence limits for emergence as quantal data, logit, probit, Weibull, Spearman-Karber, and trimmed Spearman-Karber methods, etc. could be appropriate. For the growth of the seedlings (dry mass), as continous endpoints, EC_x or ER_x and its confidence limits can be estimated by using appropriate regression analysis (e.g. Bruce-Versteeg non-linear regression analysis[1]). A desired EC_x or ER_x is obtained by inserting a value corresponding to x %

of the control mean into the equation found by regression analysis. Since EC_x or ER_x values have smaller confidence limits compared with smaller effect concentrations (e.g. EC_{20}, ER_{20}), it is recommended to determine EC_{50} or ER_{50} values.

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

The proposed statistical methods are not appropriate in the case of hormetic effects. It is recommended that a statistician be involved in the analysis of the test, since in this test guideline specific guidance on statistical procedures is given only in limited detail.

14 Test report

The test report shall include the following information:

a) a reference to this part of ISO 11269 (i.e. ISO 11269-2:2012);

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

c) description of the test conditions including

   1) pot size,

   2) mass of soil per pot,

   3) type of environment (greenhouse, etc.),

   4) temperature,

   5) humidity,

   6) lighting conditions,

   7) method of watering,

   8) general soil composition (details of additional nutrients, etc.),

   9) type and intensity of supplementary lighting, and

   10) justification of the selected concentrations of test substances;

d) all operating details not specified in this part of ISO 11269, and any occurrences liable to have affected the results;

e) method used to incorporate the chemical in the soil and the form of the substance dissolved, i.e. emulsion or suspension;

f) data concerning planting and harvest;

g) for each replicate:

   1) number of seeds emerging,

   2) number of plants remaining at harvest,

   3) total mass (fresh or dry) at harvest;

h) for each treatment, including the control:

   1) mean number of seeds emerging per replicate and standard deviation,

   2) mean number of plants per replicate at harvest,

   3) mean total mass (fresh or dry) per replicate at harvest and standard deviation,

4) mean mass (fresh or dry) at harvest per plant per replicate and standard deviation;

i) description of visual damage (photographs are acceptable);

j) table of percentage mean emergence and mass for each concentration;

k) depending on the statistical approach selected, list the lowest concentration causing significant effects (LOEC), the highest concentration causing no observed effects (NOEC), ER_{10} and ER_{50} for the growth reduction and the method used for calculation;

l) a test report on the performance of the reference compound shall be completed periodically and if the test conditions have changed.

Annex A
(informative)

Additional recommended plant species based on test results gained by applying Environment Canada Test Method: EPS 1/RM/45[4]

Table A.1 — Plant species

Latin name	Common name	Test duration (days)		No. of seeds	Comments
		Reference test [a]	Definitive test		
Festuca rubra	red fescue	10	21	5	compact soil gently after planting; delayed emergence; good growth; easy to work with, but roots are a bit fragile (extra caution required during processing)
Hordeum vulgare	barley	7	14	5	good emergence; good growth; very easy to work with; roots entangle, but are fairly hardy (i.e. not as fragile)
Medicago sativa	alfalfa	7	14	10	good emergence; good growth; easy to work with; similar to red clover for processing
Trifolium pratense	red clover	7	14	5	good emergence; good growth; very easy to work with; some shoot entanglement while processing; roots are less fragile

[a] Test using a reference toxicant performed in conjunction with the definitive test.

Table A.2 — Validity criteria based on testing using five field-collected soils, with no nutrient solution addition

Latin name	Common name	Test validity criteria [a]	
		Reference test	Definitive test
Festuca rubra	red fescue	Emergence: > 70 % Shoot length: > 40 mm	Emergence: > 70 % Shoot length: > 80 mm Root length: > 70 mm
Hordeum vulgare	barley	Emergence: > 80 % Shoot length: > 100 mm	Emergence: > 70% Shoot length: > 150 mm Root length: > 170 mm
Medicago sativa	alfalfa	Emergence: > 80 % Shoot length: > 20 mm	Emergence: > 70 % Shoot length: > 40 mm Root length: > 120 mm
Trifolium pratense	red clover	Emergence: > 70 % Shoot length: > 10 mm	Emergence: > 70 % Shoot length: > 30 mm Root length: > 40 mm

[a] Based on EPS 1/RM/45.[4]

Table A.3 — Selected reference toxicant (boric acid) test data (Nutrient solution was not added to any of the tests)

Species	Soil	Root/Shoot	Parameter	Statistical model	IC50	LCL	UCL	IC20	LCL	UCL
					mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Festuca rubra	AS	Shoot	Length	Logistic	797	672	945	442	364	536
		Shoot	Dry Mass	Logistic	531	376	749	329	188	574
		Root	Length	Logistic	498	407	609	283	205	390
		Root	Dry Mass	Logistic	738	336	1 617	446	184	1 082
Festuca rubra	AS	Shoot	Length	Gompertz	932	636	1 367	775	606	994
		Shoot	Dry Mass	Logistic	569	278	1 160	355	110	1 142
		Root	Length	Gompertz	497	360	686	367	231	585
		Root	Dry Mass	Logistic	890	403	1 976	434	133	1 405
Hordeum vulgare	AS	Shoot	Length	Logistic	1 053	1 005	1 102	578	528	627
		Shoot	Dry Mass	Gompertz	920	833	1 007	491	392	590
		Root	Length	Logistic	424	393	455	262	232	293
		Root	Dry Mass	Logistic	395	355	436	211	173	248
Hordeum	AS	Shoot	Length	Logistic	1 557	1 444	1 670	1 011	890	1 132
		Shoot	Dry Mass	Logistic	1 437	1 045	1 829	806	437	1 175
		Root	Length	Hormesis	670	628	711	333	291	375
		Root	Dry Mass	Hormesis	734	680	788	472	369	574
Medicago sativa	AS	Shoot	Length (day 7)	Gompertz	926	800	1053	393	269	517
		Shoot	Length	Hormesis	868	774	962	535	460	610
		Shoot	Dry Mass	Gompertz	514	425	604	223	138	309
		Root	Length	Hormesis	863	785	942	617	545	688
		Root	Dry Mass	Gompertz	516	438	593	294	201	387
Medicago sativa	AS	Shoot	Length	Hormesis	1 603	1 400	1 807	1 122	616	1 628
		Shoot	Dry Mass	Hormesis	1 174	903	1 445	711	496	926
		Root	Length	Hormesis	1 310	1 181	1 439	711	522	899
		Root	Dry Mass	Hormesis	918	645	1 192	513	288	739
Trifolium pratense	AS	Shoot	Length	Hormesis	1 370	711	2 029	830	675	985
		Shoot	Dry Mass	Hormesis	595	418	773	280	195	365
		Root	Length	Hormesis	752	617	888	578	507	650
		Root	Dry Mass	Hormesis	713	420	1 006	452	308	595
Trifolium pratense	AS	Shoot	Length	Gompertz	1 124	922	1 373	725	478	1 103
		Shoot	Dry Mass	ICPIN	754	129	992	66	3	1 513
		Root	Length	Logistic	713	589	862	364	265	499
		Root	Dry Mass	Logistic	493	339	720	258	141	474

LCL — lower 95 % confidence limit.

UCL — upper 95 % confidence limit.

NOTE Definitive test endpoints include shoot length and dry biomass, as well as root length and dry biomass.

Annex B
(informative)

Phytotoxic values for reference compounds: sodium trichloro-acetate and boric acid

Table B.1 — Phytotoxic values for reference compounds

Reference compound	Test species	Measurement endpoint	Phytotoxicity measure Range of EC_{50} mg/kg
Sodium trichloro-acetate	Barley *Hordeum vulgare*	Shoot biomass	6,8 to 13,5
	Lettuce *Lactuca sativa*	Shoot biomass	143 to 237
Boric acid	Oat *Avena sativa*	Shoot biomass	190 to 330 [a]
	Turnip *Brassica rapa*	Shoot biomass	80 to 240 [b]
Boric acid	Barley *Hordeum vulgare*	Shoot length	1 444 to 1 670
	Radish *Raphanus sativus*	Shoot length	1 236 to 1 665
	Tomato Lycopersicum esculentum	Shoot length	599 to 705
Boric acid	Barley *Hordeum vulgare*	Shoot length	1 444 to 1 670
	Tomato Lycopersicum esculentum	Shoot length	599 to 705

[a] Based on four tests.

[b] Based on five tests.

NOTE In a paper[18] covering different plant species, an EC_{50} (shoot biomass) range of 100 mg/kg to 400 mg/kg boric acid is defined which is very much in agreement with the data gained in the international waste ring test[8].

Annex C
(informative)

Recommended method for the measuring of the water-holding capacity of the soil

Fill a tube of known volume with the soil, with the base closed by a sheet of filter paper, and cap the tube. Submerge the tube in a water bath at room temperature (with the water level beneath the top of the tube) for 2 h. Then lower the tube below the water level for a further 1 h. Place the tube in a tray of wet, finely ground, quartz sand to drain for 2 h. Weigh the sample and dry it to constant mass at 105 °C.

The water-holding capacity, C_w, is calculated as a percentage of dry mass, with the help of the following equation:

$$C_w = \frac{m_s - m_t - m_d}{m_d} \times 100 \quad \text{(C.1)}$$

where

m_s is the water-saturated soil mass, plus the tube mass and the filter-paper mass;

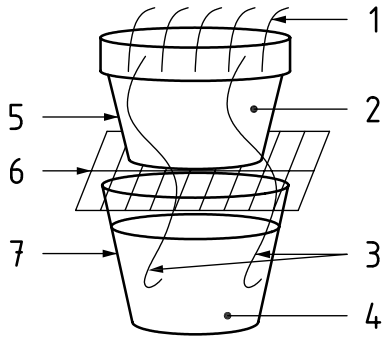
m_t is the tare (the tube mass and the filter-paper mass);

m_d is the dry mass of soil (the mass of the tube with dry soil and filter paper less the tare mass of the tube and filter paper).

Annex D
(informative)

Recommendations for nutrient supply of soils

Optional additional nutrient supply by using a commercially available nutrient solution for hydroponic culture e.g. Flory 9®[2] at 1 g of fertilizer per 1 l of water. The nutrient solution is added to the water supply of a self-watering system (Figure D.1)[5]. The nutrient content is given in Table D.1 according to Reference [14].



Key
1 plant seedings
2 soil
3 glass fibre
4 water or Flory 9®
5 plastic pot
6 wire gauze
7 glass pot

Figure D.1 — Self-watering system used for plant tests

Table D.1 — Ingredients of Flory 9®

Nutrient	Content mg/l
Nitrogen	150,00
Phosphorous	30,60
Potassium	220,00
Magnesium	60,00
Boron	0,30
Copper	0,02
Manganese	0,50
Molybdenum	0,05
Zinc	0,10
Cobalt	0,02

2) Flory 9 is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Bibliography

[1] BRUCE, R.D. and VERSTEEG, D.J. (1992): A Statistical Procedure for Modelling Continous Toxicity Data. *Environmental Toxicology and Chemistry* **11**, pp. 1485-1492

[2] CHAPMAN, P.M. (2002): Ecological risk assessment (ERA) and hormesis. *The Science of the Total Environment* **288**: pp. 131-140

[3] DECHEMA (1995): *Bioassays for soil*. Fourth report of the interdisciplinary DECHEMA Committee "Environmental Biotechnology — Soil", DECHEMA, Frankfurt am Main, Germany

[4] EC (Environment Canada) (2005): *Biological test Method: Test for Measuring Emergence and Growth of Terrestrial Plants Exposed to Contaminants in Soil*, Method Development and Applications Section, Environment Canada, Ottawa, ON, Report EPS 1/RM/45, 131 p.

[5] EC (Environment Canada) (2005): *Guidance Document on Statistical Methods for Environmental Toxicity Tests*, Method Development and Applications Section, Environment Canada, Ottawa, ON, Report EPS 1/RM/46, 241 p.

[6] GONG, P., WILKE, B.-M., STROZZI, E., FLEISCHMANN, S. (1998): *Higher plant seed germination and early seedling growth test: Evaluation and refinement of for the use in ecotoxicological assessment of soil*. Proceedings of the International Symposium on Soil, Human and Environment Interactions, May 4-11, 1997: Nanjing China

[7] GÜNTHER, P., RAHMANN, A., PESTEMER, W. (1989): Quantitative bioassay for determining residues and availability plants of sulfonurea Herbicides. *Weed Research* **29**: pp. 141-146

[8] HALLER, A., GOTH, M. & PATTARD, M. (2009): *Range of reference tests in terrestrial tests. In: Ecotoxicological characterisation of waste - Results and experiences from an International ring test*. Römbke, J., Becker, R. & Moser, H. (eds.). pp. 71-77, Springer Ltd., New York (ISBN 978-0-387-88958-0)

[9] KALSCH, W., RÖMBKE, J. & SCHALLNASS, H.-J. (2001): *Entwicklung eines chronischen Pflanzentests*. Kap. 9.3.7. Bericht für das BMBF, F+E-Vorhaben 1491077, 219 S. In: Leitfaden Biologische Verfahren zur Bodensanierung. Michels, J., Track, U., Gehrke, U. & Sell, D. (eds). Umweltbundesamt, Grün-Weisse Reihe des BMBF. Extended version only on CD-ROM

[10] KALSCH, W. & RÖMBKE, J. (2000): *Entwicklung eines chronischen Pflanzentests*. Final Report of a research and development project to the German Ministry of Education and Research (BMBF)

[11] KUHNT, G., MUNTAU, H. *EURO-Soils: Identification, Collection, Treatment, Characterization*. JRC Ispra, European Commission

[12] LANCASHIRE, P.D., BLEIHOLDER, H., BOOM, T.v.d., LANGELÜDDEKE, P., STRAUSS, R., & WITZENBERGER, E.W.A. (1991) A uniform decimal code for growth stages of crops and weeds. *Ann. Appl. Biol.*, **119**, pp. 561-601

[13] OECD Guidelines for the Testing of Chemicals 207 (1984): *Earthworm, Acute Toxicity Tests* (Original Guideline, adopted 4th April 1984). Organisation for Economic Cooperation and Development, Paris

[14] OECD Guidelines for the Testing of Chemicals 208 (2006): *Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test*

[15] OECD Series on Testing and Assessment (2005 draft). *Current Approaches in the statistical Analysis of Ecotoxicity Data: A Guidance to Application*, Organisation for Economic Cooperation and Development, Paris

[16] RIEPERT, F., FELGENTREU, D. (2002): Relevance of soil storage to biomass development, N-mineralisation and microbial activity using the higher plant growth test, ISO 11269-2, for testing of contaminated soils. *Applied Soil Ecology* **20**, pp. 57-68

[17] RÖMBKE, J., EISENTRÄGER, A., HUND-RINKE, K., JÄNSCH, S., NEUMANN-HENSEL, H., WEBER, G. (2006) *ERNTE Handlungsempfehlung für die ökotoxikologische Beurteilung von Böden*. Sidus-Verlag, 43 pages

[18] PESTEMER, W., PUCELIK-GÜNTHER, P. (1997) Standardized Bioassay for the determination of ED10 (NOEL) and ED50 –values for herbicides and selected following crops in soil. *Reports from the Federal Biological Research Centre for Agriculture and Forestry*, **29**, 26 pages

[19] SHEPPARD, S.C., EVENDEN, W.G., ABBOUD, S.A. und STEPHENSON, M. (1993): A plant Life-Cycle Bioassay for contaminated soil, with comparison to other bioassays: mercury and zinc. *Arch. Environ. Contam. Toxicol.* **25**, pp. 27-35

[20] SHEPPARD, S.C. (1994): *Developments of bioassay protocols for toxicants in soil*. Ontario Environment (Ed.). 97 pages

[21] STALDER, L. & PESTEMER, W. (1980): Availability to plants of herbicide residues in soil. Part I. A rapid method for estimating potentially residues of herbicides. *Weed Research* **20**: pp. 343-347

[22] STEPHENSON, G.L., SOLOMON, K.R., HALE, B., GREENBERG, B.M. & SCROGGINS, R.P. (1997): *Development of suitable test methods for evaluating the toxicity of contaminated soils to a battery of plant species relevant to soil environments in Canada*. In: Dwyer, F.J., Doane, T.T. & Hinman, M.L. (eds.). Environmental Toxicology and Risk Assessment: Modeling and Risk Assessment. Vol. 6. American Society for Testing and Materials, Philadelphia, PA, USA. pp. 474-489

[23] UBA (Federal Environmental Agency (Germany)) (1999): BMBF-Project Management Agency for Waste Management and Remediation of Hazardous Abandoned Sites (Section 3.6, PT-AWAS) (Ed.): *Processes for the Bioremediation of Soil* (Last Update: April 1999)

[24] WILKE, B.M., MAI, M., RIEPERT, F. (2004): *The use of liquid fertilizer in a higher plant growth test*. Poster presentation, SETAC Europe 2004

[25] ISO 11074, *Soil quality — Vocabulary*

[26] ISO 11259, *Soil quality — Simplified soil description*

[27] ISO 11274, *Soil quality — Determination of the water-retention characteristic — Laboratory methods*

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

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