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Soil quality — Test for estimating organic matter decomposition in contaminated soil

Qualité du sol — Essai d'estimation de la décomposition de la matière organique dans un sol contaminé

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Foreword

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

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

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

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

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

Introduction

Determining if the soil microbial community is healthy is a complex task and is influenced by the community's resistance and resilience to disturbances[1]. In its simplest terms, a healthy soil is one that functions in organic matter decomposition (OMD) and nutrient cycling. In fact, a suite of standardized test methods are needed to better understand the ecology of the soil microbial community and its role in soil function and structure[2]. However, one key soil microbiological function is organic matter decomposition. Unfortunately, there is a lack of standardized procedures for quantitatively measuring this important process. As such, the ability of soil microorganisms to decompose lignin cellulosic material provides evidence that the microbial population is active in OMD and carbon cycling. A standard field method currently available for assessing soil OMD inhibition from environmental contaminants involves using litter bags placed in experimental plots[3]; however, there is no standard method available for a laboratory-based assessment of organic matter decomposition. A laboratory-based method has been developed using the same principles as the litterbag method. In place of indigenous organic matter (i.e. tree leaves, crop material, etc.), the laboratory-based method uses readily accessible filter paper as a standard organic material for organic matter decomposition tests[4]. The laboratory-based method has been used and described in several research studies as part of a greater soil microbial health (SMH) assessment suite of tests[5],[6]. The studies evaluated the impact of contaminants in soil from brownfield sites or from testing of chemical-spiked control soil for risk assessment research.

This document outlines a procedure for determining the effects of contaminated soils on the decomposition of organic matter (lignin cellulosic filter paper) following a standardized methodology.

Soil quality — Test for estimating organic matter decomposition in contaminated soil

WARNING — Contaminated soils may contain unknown mixtures of toxic, radiotoxic, genotoxic, mutagenic, or otherwise harmful chemicals or infectious microorganisms. Occupational health risks may arise from dust or evaporated chemicals during handling and incubation. Precautions should be taken to avoid skin contact.

IMPORTANT — The electronic file of this document contains colours which are considered to be useful for the correct understanding of the document. Users should therefore consider printing this document using a colour printer.

1 Scope

This document specifies a test procedure for the evaluation of the habitat function of soils by determining effects of soil contaminants and substances on organic matter decomposition. This test is applicable to natural soils and soil materials of unknown quality (e.g. contaminated sites, amended soils, soils after remediation, agricultural or other sites under concern). This document also specifies how to use this method for testing substances under temperate conditions.

This document is not applicable to substances for which the air/soil partition coefficient is greater than 1. It is not applicable to substances with vapour pressure exceeding 300 Pa at 25 °C.

NOTE The stability of the test substance cannot be ensured over the test period. No provision is made in the test method for monitoring the persistence of the substance under test.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 10390, *Soil, treated biowaste and sludge – Determination of pH*

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

ISO 11265, *Soil quality — Determination of the specific electrical conductivity*

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

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

3 Terms and definitions

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

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

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

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

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

3.2
EC*x*
effective concentration
concentration (mass fraction) of a test sample or test substance that causes *x* % of an effect on a given endpoint within a given exposure period

Note 1 to entry: The EC*x* is expressed as a percentage of soil to be tested (dry mass) per soil mixture (dry mass). When substances are tested, the EC*x* is expressed as mass of the test substance per dry mass of soil in milligrams per kilogram. This can only be determined for chemically-spiked soil.

3.3
limit test
single concentration treatment consisting of at least five replicates for:

a) the *test soil* (3.8) or the highest concentration of test substance mixed into the *control soil* (3.7), and

b) the control soil

3.4
LOEC
lowest observed effect concentration
lowest test substance concentration that has a statistically significant effect (probability $p < 0,05$)

Note 1 to entry: In this test, the LOEC is expressed as a mass of test substance per dry mass of the soil to be tested. All test concentrations above the LOEC should usually show an effect that is statistically different from the control. This can only be determined for chemically-spiked soil.

3.5
NOEC
no observed effect concentration
highest test substance concentration immediately below the *LOEC* (3.4) at which no effect is observed

Note 1 to entry: In this test, the concentration corresponding to the NOEC, has no statistically significant effect (probability $p > 0,05$) within a given exposure period when compared with the control. This can only be determined for chemically-spiked soil.

3.6
reference soil
uncontaminated soil with comparable pedological properties (nutrient concentrations, pH, organic carbon content and texture) to the *test soil* (3.8) being studied

3.7
control soil
reference soil (3.6) used as a control against chemically-spiked *test soil* (3.8), which fulfils the validity criteria

Note 1 to entry: it is advisable that a control soil be proven to be suitable for use by demonstrating the ability of this soil to meet the standard's test validity criteria prior to definitive testing.

Note 2 to entry: Control soil cannot be artificial soil (AS) as it is known that this type of soil does not meet the validity criteria of the test.

3.8
test soil
sample of field-collected soil or chemically-spiked soil to be evaluated for toxicity to the soil microbial community

4 Principle

The ability of soil microorganisms to degrade cellulose filter paper (i.e. organic matter) in test soil (i.e. contaminated soil) is compared to the same cellulose filter paper in control or reference soil over an incubation period. In this method, the effects of individual substances can be assessed using standard natural soil used in chemical-spiking testing. For contaminated soils, the effects are determined in the test soil and in a control soil. This laboratory method uses sterilized filter paper disks as a source of organic matter. Organic matter decomposition is estimated based on the mass loss of filter paper disks placed between two layers of the test soil. If the contaminant in any way impairs the soil microorganism's ability to degrade cellulose filter paper through carbon cycle enzymes, a degradation rate difference is observed between the test soil and control treatments. Filter paper was chosen as the organic matter test material as it is universally available, is a more standardized media, allows for better inter-laboratory reproducibility, can be sterilized (e.g. autoclaved) and can be easily distinguished from native organic matter in the soil[4],[9].

The test involves pre-weighing filter paper disks, adding 10 g of soil on a dry mass basis to a 50 ml sterile centrifuge tube, placing the filter paper on top of the soil, adding an additional 10 g of soil (dry mass) on top of the filter paper, loosely capping the tube and incubating it at a constant temperature (e.g. 20 °C ± 2 °C).

For control versus contaminated soil design, the test is performed with a minimum of five replicates for each treatment with a minimum of five sampling time points. The incubation period depends on the degradation rate of filter paper, so the test length is tied to the extent of filter paper mass loss in the control treatment. A minimum mass loss in the control of 30 % is the earliest point for consideration of test completion but between 40 % and 70 % degradation is the ideal range. Beyond this range, it is more likely that the decomposed filter paper be difficult to clean and recover for mass loss measurements. The time interval between sampling time points is dependent on the degree of microbial activity in the control or reference soil.

In the case where soil is spiked with a contaminant, initial range-finding testing is advisable using a broad concentration range of the contaminant. For the definitive chemical dilution series, a minimum of 5 test concentrations is recommended. The duration of the test is influenced by the control and the time needed to observe a distinct concentration response. From experience, the test should not exceed 140 days.

5 Reagents and material

5.1 Reagents

5.1.1 Sterile deionized (or distilled) water.

5.2 Materials

5.2.1 50 ml plastic centrifuge tubes (sterile).

5.2.2 Cellulose filter paper; particle retention, ≥ 11 µm to 25 µm; thickness, ~180 µm; ash content, ~0,06 %; (sterile).

5.2.3 Hole punch (25 mm diameter).

5.2.4 Filter paper forceps (sterile).

5.2.5 Petri dishes (glass or plastic).

5.2.6 Small paint brushes (soft bristles).

5.2.7 Large plastic weigh-boats or alternate vessel.

5.2.8 Small aluminium weigh-boats or alternate vessel.

6 Soil

6.1 Field-collected soil

Field-collected soils, contaminated and control (i.e. reference), can be obtained from industrial, agricultural, boreal forest or other contaminated sites of concern. In the case of undisturbed soil, the organic horizon is used. The soil is shipped to the laboratory and stored at 4 °C.

All field-collected soils shall be passed through a 2 mm sieve. If required, soils may be slightly air-dried just to enable sieving; however, where possible, air-drying should be avoided. After sieving, the soil is homogenized and then stored again at 4 °C. Soil should be stored using containers that minimize losses of soil contaminants by volatilization and sorption to the container walls. Variable storage periods are possible for this test, so long as microbial activity is evident in the control or reference soil by meeting the validity criteria of the test. Soil pH, conductivity, moisture content and water holding capacity (WHC) are determined as per the methods below.

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

a) pH in accordance with ISO 10390;

b) texture (sand, loam, silt) in accordance with ISO 11277;

c) water content in accordance with ISO 11465;

d) organic carbon in accordance with ISO 10694;

e) specific electrical conductivity in accordance with ISO 11265;

f) water holding capacity in accordance with Annex A.

6.2 Control soil

The control soil can be the reference soil in the context of contaminated soil assessment. The reference soils from an uncontaminated area near a contaminated soil site should be handled and shipped, and characterized in a manner similar to the contaminated test soils. In the case of a chemically-spiked soil study, a known control soil is used.

7 Apparatus

Use laboratory equipment and the following.

7.1 Top-loading balance.

7.2 Apparatus to determine the dry mass of the substrate, in accordance with ISO 11465 (drying oven, desiccator, analytical balance).

7.3 Digital camera (optional).

7.4 Desiccant chamber.

7.5 pH meter.

7.6 Analytical balance, capable of weighing with an accuracy of ±0,000 1 g.

7.7 Drying oven, set to (105 ± 5) °C.

7.8 Test environment.

7.8.1 Area to maintain a sterile environment, work bench with Bunsen burner or biological safety cabinet (optional).

7.8.2 Enclosure, capable of constant temperature control.

8 Procedure

8.1 Experimental design

8.1.1 General

A sample of field collected contaminated soil at a single concentration or a chemically-spiked soil at multiple concentrations are compared to an appropriate reference or control soil. Various test designs are described in 8.1.2 and 8.1.3. However, regardless of the test design chosen, each test concentration and associated control soil is replicated five times to allow for the time-spaced sampling during the duration of this testing standard. A filter paper disk (i.e. organic matter) is added to the test soil and the mass loss of filter paper determined over an incubation period.

8.1.2 Chemically-spiked soil test design

8.1.2.1 Range-finding

A preliminary test is recommended to find the range of concentrations that brackets the effect level of a new test substance (e.g. 0 mg/kg, 1 mg/kg, 10 mg/kg, 100 mg/kg and 1 000 mg/kg). A range-finding test shall be performed using the same batch of soil as the planned definitive test. The testing can be conducted with reduced replication (e.g. 2 or 3 replicates), relative to the definitive test. The duration of the range-finding test is the same as for the definitive test. The concentration range in the definitive test should preferably be chosen so that it includes concentrations that span a wide range, including a low concentration that evokes no adverse effects (similar to the negative control treatment) and a high concentration that results in severe effects. Ideally, the concentrations chosen also brackets the mid-range effects to better estimate the EC50 effect concentration.

When no effects are observed in a range-finding test, either with a 100 % contaminated soil sample or a spiked chemical test, the definitive test can be designed as a limit test (e.g. undiluted contaminated field soil) or a specific high chemical concentration (e.g. 1 000 milligrams of test substance per kilogram of test soil).

8.1.2.2 Chemically-spiked soil

In the case where chemical substances are spiked into the soil at different concentrations, the test can be designed for the following two scenarios:

a) For the EC*x* approach, a minimum of 5 concentrations plus the control treatment(s) shall be used, and more (i.e. ≥ 10 concentrations plus controls) are recommended to improve the likelihood of bracketing each end point sought according to ISO 10694. The dilution factor can be variable; smaller at lower concentrations, larger at high concentrations. A minimum of five replicates for each treatment plus the controls are recommended.

b) For the NOEC hypothesis approach, at least five concentrations in a geometric series shall be used according to ISO 10694. Five replicates for each treatment plus eight controls are recommended.

Regardless of the test design chosen, the test concentrations shall be spaced by a dilution factor not exceeding 2.

A limit test can be sufficient if the results from a range-finding test show no toxic effect. This involves a single test concentration (e.g. 1 000 mg/kg) and the control with a minimum of five replicates for each treatment.

8.1.3 Field-contaminated soil test design

In the case where a field-collected contaminated soil is to be tested, the reference soil should match the test soil as closely as possible. A preliminary performance test is recommended to ensure the reference soil meets the validity criteria. There shall be a minimum of five replicates for both the reference and test soil, however more replicates are recommended. For soils collected as distinct horizons (e.g. boreal or taiga soils), each horizon shall be tested separately in independent definitive tests.

8.2 Preparation of filter paper disks

Filter paper disks are cut from commercially available filter paper using a 25 mm diameter hole punch [i.e. sized to fit into the sterile centrifuge tubes (5.2.1)] or they may be purchased already in this size. Make sure the cut is clean with no frayed edges. Individual filter paper disks are then weighed on an analytical balance to the nearest 0,001 g, and the masses recorded. It is also recommended to record the mass directly on the filter papers using a pencil. The filter papers, normally 10 at a time, are then wrapped in aluminium foil and autoclaved (sterilized) on a dry cycle for 20 min (121 °C and 100 kPa) or heated in an oven at 160 °C for 2 h. Filter forceps used in the test procedure are also wrapped in aluminium foil and sterilized in the same manner.

8.3 Preparation of soil

8.3.1 Contaminated and reference soil

The test and reference soils shall be treated the same. The total mass of the test and reference soils shall be the dry mass equivalent to 20 g in each test container. The test soil shall be wetted with sterile deionized water to reach 40 % to 60 % of the total water holding capacity (i.e. desired water holding capacity), or that which results in a crumbly texture (i.e. 2 mm to 3 mm clumps) that is optimal for testing, determined according to Annex A. In some cases, for example when testing forest soils, higher or lower percentages can be required. The optimal percentage water holding capacity should be determined before the test set-up.

Determine the pH for each test and reference soil (one container per concentration) according to ISO 10390 at the beginning and end of the test (when acid or basic substances are tested, do not adjust the pH).

8.3.2 Chemical substances added to control soil

Control soil is used to prepare the test soil. Prepare enough test soil and control soil by summing the mass required for all replicate and sampling times (minimum of 500 g dry mass). Substances are added to the test substrate and mixed thoroughly.

For the introduction of test substances, use either method a), b) or c), as appropriate:

a) Water-soluble substances

   Immediately before hydrating the soil to the desired water holding capacity for the test (refer to 8.3.1), dissolve the quantity of test substance in an amount of sterile water below that which is required to bring the soil to the desired water holding capacity and add to the control soil. Rinse the container with sufficient sterile water for the desired water holding capacity and add to the spiked-control soil. Mix the test soil thoroughly before introducing it into each of the test containers. Repeat this for each test concentration.

b) Substances insoluble in water but soluble in organic solvents

Dissolve the quantity of test substance required to obtain the desired concentration in a volatile solvent (such as acetone or hexane) and add this to a portion of the control soil. Ultrasonic dispersion, organic solvents, emulsifiers or dispersants can be used to disperse substances with low aqueous solubility. When such auxiliary substances are used, all test concentrations and an additional control should contain the same minimum amount of auxiliary substance. After evaporating the solvent by placing the container under a fume hood, add the remainder of the control soil and mix thoroughly. Add sterile water to bring the spiked-control soil to the desired water holding capacity and mix thoroughly again before introducing it into the test containers. In this case, an additional control is required with just the solvent. Repeat this for each test concentration.

WARNING — Take appropriate precautions when dealing with solvent vapour to avoid danger from inhalation or explosion, and to avoid damage to extraction equipment, pumps, etc.

c) Substances insoluble in water or organic solvents

For a substance insoluble in water or a volatile solvent, prepare 10 g dry mass of control soil and add the quantity of control substance required to obtain the desired concentration directly to the soil and mix thoroughly. Add this "dry mix" to the remainder of the test soil and mix thoroughly. Add sterile water to bring this soil to the desired water holding capacity and mix thoroughly again before introducing it into the test containers. Repeat this for each test concentration.

Substances mixed into control soil do not need to be tested at concentrations higher than 1 000 mg/kg dry soil.

Proceed simultaneously with all replicates per concentration and the control(s) required according to the selected approach.

Determine the pH for each test mixture (one container per concentration) according to ISO 10390 at the beginning and end of the test.

8.4 Test set-up

Since the level of biological activity varies between different soils being assessed, test containers (replicates) for each soil sample are set up to allow for destructive sampling at a minimum of 5 time points with 5 replicates for each concentration and controls. The duration of the test and time between sampling points vary depending on the soil being tested and the microbial activity. It is recommended that initial sampling occur following 7 days of incubation, and then every 2 to 4 weeks thereafter until the decomposed filter paper particles are difficult to clean and recover for mass loss measurements. A decision on test completion is based on mass loss of the filter papers in the control or reference soil where 30 % is the earliest point for considering test completion but ideally within the range of 40 % to 70 % loss. A mass loss in the control soil filter paper of higher than 70 % leads to difficulty in recovering and accurately measuring the degraded filter paper. Additional test containers can be added to the experimental design for tests of longer duration (e.g. up to 140 days). However, if a range-finding test has been completed, only 3 or 4 time points are recommended for definitive testing.

Test containers should be prepared in a biological safety cabinet (BSC) or a sterile environment by adding 10 g (dry mass) of hydrated test soil and reference or control soil to 50 ml sterile centrifuge tubes. The tubes are then gently tapped to allow the first layer of soil to settle. Next, a pre-weighed sterile cellulose filter paper-disk (25 mm diameter) is placed on top of the soil using sterile forceps. An additional 10 g (dry mass) of soil (test, reference or control soil) is added on top of the filter paper; tubes are gently tapped again to settle the soil (see Figure 1).

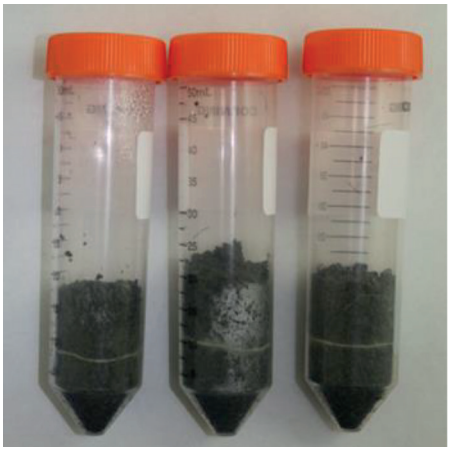


Figure 1 — Test containers showing filter paper placed between 20 g soil (dry mass)

The cap on each tube is screwed on loosely to allow for some air exchange. The total mass of each tube is recorded and the test containers are incubated at a constant temperature (e.g. 20 °C ± 2 °C) in the dark, with weekly hydration on the basis of mass loss of the test container to maintain the desired water holding capacity. If mass loss has occurred, working in the BSC, correct the evaporated water as necessary by adding sterile deionized water as fine droplets on top of the soil using a sterile pipette. Work under the assumption that 1 ml of water weighs 1 g. For example, if a mass loss of 0,48 g has occurred, rehydrate the test container with 480 µl of water.

8.5 Test sampling

Test containers are sampled destructively at a minimum of five different time points over the duration of the test. Incubation period and intervals between sampling days vary depending on the test soil and degree of microbial activity. In the case of a control versus a contaminated soil application, the test duration is dictated by the rate of filter paper mass loss in the control treatment. A decision to end the test shall be based on a mass loss of 30 % at the earliest, but typically is made based on a mass loss within the range from 40 % to 70 %. A mass loss in the control of higher than 70 % leads to difficulty in recovering and accurately measuring the degraded filter paper. For a spiked-chemical study, measurement of a concentration effect influences the test duration. For this method, the test duration should not proceed beyond 140 days.

Before sampling at each time point, label one aluminium weigh-boat (or alternate vessel) per sample and record the mass. One at a time, destructively sample each test container by emptying the entire test container onto a vessel (e.g. a plastic weigh-boat or alternate vessel). Carefully remove the filter paper disk from the soil with fine forceps and place it onto another plastic weigh-boat (or alternate vessel). Then, gently clean the partially decomposed filter paper with a fine-hair paint brush until all easily detachable soil particles are removed. At this time, the soil may be discarded. Always discard contaminated soil through an appropriate waste stream. If the soil is too wet, the filter paper with attached soil may be partially air-dried until the soil more easily detaches. If there is mould attached to the filter paper, it may be gently scraped off so long as it does not disrupt the integrity of the filter paper.

Transfer the partially cleaned filter paper to a glass or disposable petri dish containing deionized water and carefully remove all remaining soil particles with a fine-hair paint brush. Once there are few visible soil particles left on the filter paper, continue to transfer it to clean petri dishes and wash with deionized water as many times as needed until there are no visible soil particles. Take care to gently clean as to not disrupt the integrity of the filter paper. If it is difficult to remove the soil, a dissecting microscope may be used to remove any difficult soil particles. Transfer the soil-free filter paper to the pre-weighed aluminium weigh-boat and oven dry it at 105 °C for 24 h.

After drying, transfer weigh-boats to a desiccator using thermal gloves and allow the filter papers to cool for a minimum of 20 min. Weigh the weigh-boat containing the filter paper on an analytical balance and record the mass. Additionally, comparative photographs of the filter paper decomposition

state between the reference and contaminated soils, or contaminant concentrations, may be taken (see Figure 2). Aluminium weigh boats may be re-used for the different sampling points, so long as the weigh boats are clean and the mass of the weigh boats are recorded each time.

Figure 2 — Example of filter paper decomposition, after oven drying, over incubation period

9 Validity of the test

The results are considered to be valid, if the mean percent effect to the control(s) is ≥ 30 % organic matter mass loss at the end of the test period.

10 Calculation and expression of results

10.1 Calculation

The mass of the dried filter papers is recorded and the percentage mass loss of each filter paper is calculated using the Formula (1):

$$W_L = \frac{W_{TI} - W_{TF}}{W_{TI}} \times 100 \quad (1)$$

where

W_L is the mass loss of each filter paper (%);

W_{TI} is the initial mass of the filter paper (g);

W_{TF} is the final mass of the filter paper (g).

10.2 Expression of results

A graphical presentation of the mean values of the end points including standard deviation of the measured values against the test soil(s) and control soil(s) should be prepared. This comparison or curve gives an impression of the quality of effects and their magnitudes. In the case of a concentration series, express the concentration on a soil dry mass basis.

If a concentration series was performed:

— for the EC*x* approach, indicate in milligrams per kilogram of dried soil; the median nominal or measured concentration of the test substance, which resulted in a reduced effect to 50 % (EC50) compared to the control within the test period[10].

— for the NOEC approach, indicate the nominal or measured concentration of the substance per kilogram of dried soil immediately below the LOEC or highest tested concentration of a test substance which when compared to the control has no statistically significant of effect ($p > 0{,}05$)[10].

11 Precision

Details of an inter-laboratory test on the precision of the method is summarized in Annex B.

12 Statistical analysis

Statistical guidance for evaluation of test results is based on References [7] and [10] and aims to make the investigator aware of problems that can arise in consequence of a test design selected. Computer programs do not necessarily guard against violations of rules that can cause erroneous analyses. It is strongly recommended to look for more information in specific guidance documents (e.g. as provided above) or to contact a statistician.

The mean percentage mass loss of filter paper is compared to control or reference for each test soil over the incubation period.

The mean percentage mass loss of filter paper is compared for each treatment. In the case of comparing reference and contaminated soils, a standard t-test can be used to compare the effect based on the data from the final sampling day or any selected sampling point depending on the experimental protocol. However, if the data from multiple sampling points are considered in the analysis, a two-way ANOVA (analysis of variance) can be performed. For multi-concentration tests using chemically-spiked soils, a concentration-response relationship can be determined using regression analyses to determine the effective concentration of interest (e.g. EC50). In comparing reference and contaminated soil, and the pre-requisites (normality, homogeneity) of parametric test procedures are fulfilled, the Student t-test, otherwise the unequal variance t-test (e.g. Welch t-test) or a nonparametric test, such as the Mann-Whitney U-test, may be used. However, if the data from multiple sampling points are considered in the analysis, a two-way ANOVA can be performed. If a clear concentration-response is obvious, EC*x*-values can be estimated using regression analysis (e.g. logistic model).

13 Test report

The test report shall include the following information:

a) a reference to this document, i.e. ISO 23265:2022;

b) the results, expressed as in 10.2;

c) a detailed description of the test substance and information on physical and chemical properties if helpful for the interpretation of the test result;

d) the origin of the field soil,

e) information on the reference or control soil [soil texture, pH, organic matter (OM) content, and contaminant concentration if applicable], the method of preparation of the test sample together with an indication of the auxiliary substances used for a low-/non-water-soluble substance;

f) detailed conditions of the test environment;

g) a table giving the percent mass loss of filter paper for each treatment and the control(s);

h) depending on the statistical approach selected, report the EC50 for the effect on OM degradation, and statistical methods used for the calculation;

i) in addition to h), it is optional to list the lowest concentration causing significant effects (LOEC) and the highest concentration causing no observed effects (NOEC);

j) water content and pH of the soil to be tested and the control soil at the start of the test for each concentration;

k) any operating details not specified in this document, as well as any factors that may have affected the results.

Annex A
(normative)

Determination of water holding capacity

A.1 General

The following method has been found to be appropriate for laboratory samples of soils to be tested and standard soils.

A.2 Apparatus

A.2.1 Glass tube, approximately 20 mm to 50 mm diameter and at least 100 mm in length.

A.2.2 Water bath, at room temperature.

A.2.3 Filter paper.

A.2.4 Drying oven, set to (105 ± 5) °C.

A.2.5 Balance, capable of weighing with an accuracy of ±0,01 g.

A.3 Procedure

Plug the bottom of the tube with a filter paper, and after filling with the control or test sample to a depth of 5 cm to 7 cm, place the tube on a rack in a water bath. Gradually submerge the tube until the water level is above the top of the soil, but below the upper edge of the tube. Leave the soil sample in the water for about 3 h.

As not all water absorbed by the soil capillary can be retained, the tube containing the sample should be placed for a period of 2 h on very wet finely ground quartz sand for draining.

Weigh the soil sample, dry it to constant mass at 105 °C and reweigh it.

A.4 Calculation of water holding capacity (C_{WH})

$$C_{WH} = \frac{m_S - m_T - m_D}{m_D} \times 100 \quad \text{(A.1)}$$

C_{WH} is the water holding capacity in percentage of dry mass, %;

m_S is the mass of the water-saturated soil plus the mass of the tube plus the mass of the filter paper;

m_T is the tare (mass of tube plus mass of filter paper);

m_D is the dry mass of soil.

Annex B
(informative)

Performance of the method

B.1 General

A summary of results from three (3) phases of an inter-laboratory study is provided in Clauses B.2, B.3, and B.4. Twelve laboratories representing 6 different countries (Canada, France, The Netherlands, Germany, Portugal, and South Korea) participated in phase 1. Fourteen laboratories representing 7 different countries (Canada, France, The Netherlands, Germany, Portugal, Italy, and Czech Republic) participated in phase 2. Twelve laboratories representing 7 different countries (Canada, France, The Netherlands, Germany, Portugal, Czech Republic, and Italy) participated in phase 3. The results of the inter-laboratory ring test are detailed in a report prepared by the ECCC inter-laboratory program coordinator[11].

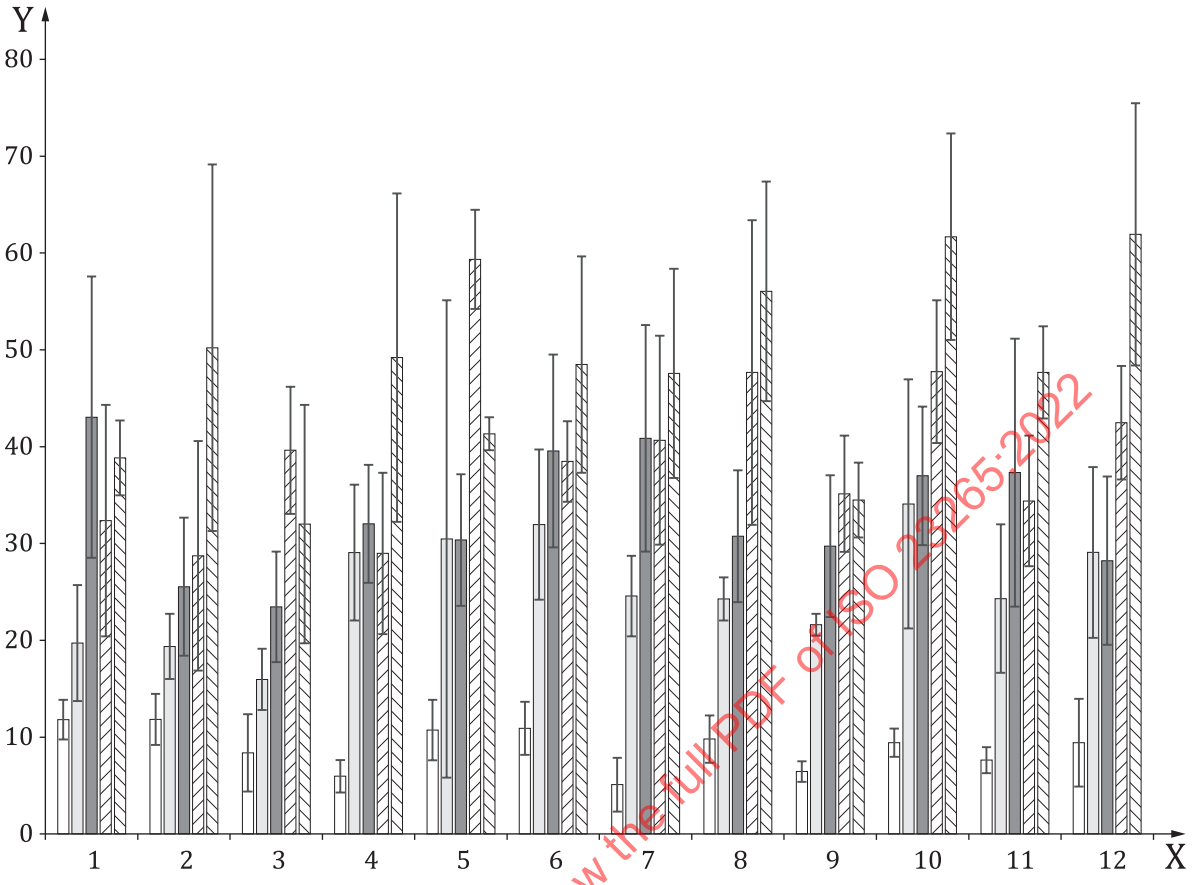
B.2 Phase 1 testing

The purpose of phase 1 was for the laboratories to become comfortable with the method using uncontaminated soil, and address any challenges and concerns associated with the method. Fresh LUFA 2.2 soil was used for this test. One challenge included the recovery of the filter paper pieces from the final sampling time point; therefore, the standard was modified to provide flexibility in the procedure for cleaning soil debris from the filter paper, using drying and a dissecting microscope to remove small soil particles.

All participating laboratories met the validity criteria; the mean percent mass loss of the control soil was ≥30 % by the end of the test. Although the final sampling time point is used to generate the comparison data and reporting, multiple sampling time points were necessary over time to ensure that the filter papers were degrading sufficiently to judge the test progress against the test validity criterion, as well as difficulty in recovering the degraded filter paper.

While calculating the mean mass loss (%) of filter paper for each sampling time point and laboratory, values were excluded from the analysis if they were negative or statistical outliers relative to the treatment mean (e.g. likely if excess soil debris was stuck to the filter papers); values were cross-checked against photographs, when available. The mean mass loss (%) of filter papers for each sampling point within each laboratory is shown in Figure B.1.

The mean mass loss (%) of the filter papers for each sampling time point was compared across laboratories (Table B.1), and the overall coefficient of variation (CV) reported for each sampling point. ISO/TS 5594 acknowledges that different CVs arise depending on the type of biotest standard, but provides a general recommendation of less than 30 %[8]. Based on the recommendations in these documents, the CVs for this phase was acceptable, as they were less than 30 % for each sampling time point.



Key

X	lab number
Y	mean mass loss (%)
	week 2
	week 4
	week 6
	week 8
	week 10

NOTE The test soil used was LUFA 2.2 control soil.

Figure B.1 — Mean (± standard deviation, *n* = 5) mass loss (%) of filter papers recovered from each sampling time point (weeks 2, 4, 6, 8, 10) for the 12 participating laboratories (labs 1 to 12)

Table B.1 — Mean percentage mass loss of filter papers incubated in LUFA 2.2 soil across the 12 participating laboratories

Sampling time (week)	Mean mass loss (%)	Standard deviation	Coefficient of variation (%)
2	9,0	2,3	25
4	25	5,6	22
6	33	6,3	18