INTERNATIONAL STANDARD

ISO 11267

First edition
1999-04-01

Soil quality — Inhibition of reproduction of Collembola (*Folsomia candida*) by soil pollutants

Qualité du sol — Inhibition de la reproduction de Collembola (Folsomia candida) *par des polluants du sol*

Reference number
ISO 11267:1999(E)

Contents

International Organization for Standardization
Case postale 56 • CH-1211 Genève 20 • Switzerland
Internet iso@iso.ch

Printed in Switzerland

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 3.

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.

International Standard ISO 11267 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods.*

Annexes A to E of this International Standard are for information only.

Introduction

This International Standard describes a method for testing the effects of chemicals on the reproduction of Collembola in artificial soil. It can be adapted for use for testing or comparing soils to assess, for example, the effects of remediation treatments, and for assessing sublethal effects and no-effect levels for pesticides or other added chemicals.

Soil quality — Inhibition of reproduction of Collembola (*Folsomia candida*) by soil pollutants

1 Scope

This International Standard describes a method for determining the effects of substances on the reproduction of *Folsomia candida* by dermal and alimentary uptake in a defined artificial soil substrate.

The method is not applicable to volatile substances, i.e. substances for which H (Henry's constant) or the air/water partition coefficient is greater than 1, or for which the vapour pressure exceeds 0,013 3 Pa at 25 °C.

NOTE 1 The stability of the test substance cannot be assured over the test period. No allowance is made in the test method described for possible degradation of the test substance over the course of the experiment..

NOTE 2 Recommendations for adapting the method for comparing or monitoring soil quality are given in annex E.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 10390:1994, *Soil quality — Determination of pH.*

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

ISO 11274, *Soil quality — Determination of the water retention characteristic — Laboratory methods.*

3 Terms and definitions

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

3.1
LOEC
lowest observed effect concentration
lowest concentration of the test substance which is observed to have a significant effect when compared with the control

NOTE All test concentrations above the LOEC have a harmful effect equal to, or greater than that observed at the LOEC.

3.2
NOEC
no observed effect concentration
highest tested concentration of a test substance at which no lethal or other effect (such as mass alteration) is observed

3.3
EC10
concentration estimated to reduce the reproduction rate at the end of the test by 10 % compared to the control

NOTE All effect concentrations are expressed as mass of test substance per dry mass of the test substrate (5.2.1).

3.4
EC50
concentration estimated to reduce the reproduction rate at the end of the test by 50 % compared to the control

NOTE All effect concentrations are expressed as mass of test substance per dry mass of the test substrate (5.2).

3.5
reproduction
increase in the mean numbers of offspring in each test vessel after 28 days incubation under the specified test conditions

4 Principle

The effects of different concentrations of test substance on the reproduction of 10- to 12-day old springtails (*Folsomia candida*) in a defined artificial substrate are determined. The springtails are incubated until offspring (F_1) emerge from eggs laid by mature adults, and the number of offspring are determined. Normally offspring emerge after 28 days in control experiments.

5 Reagents

5.1 Biological material.

In this test, 10- to 12-day old juvenile springtails of the species *Folsomia candida* (Willem) are used (see A.1. for details on synchronization of breeding).

5.2 Test substrate, consisting of the wet basic soil substrate (defined artificial soil in accordance with ISO 11268-1) the test substance and deionized water.

5.2.1 Soil substrate.

Component	Dry mass fraction
Sphagnum peat, air-dried, finely ground and with no visible plant remains	10 %
Kaolinite clay (air-dry) containing not less than 30 % kaolinite	20 %
Industrial quartz sand (air dried, and predominantly fine sand with more than 50 % of particle size 0,05 mm to 0,2 mm)	70 %

Add sufficient (usually 0,5 % to 1 %) calcium carbonate ($CaCO_3$), pulverized and of recognized analytical grade, to bring the pH (as measured in 1 mol/l KCl solution) to 6,0 ± 0,5 at the start of the test. The pH shall be determined in accordance with ISO 10390.

NOTE 1 The amount of calcium carbonate required will depend on the components of the soil substrate and should be determined by measurements on subsamples (see annex D) immediately before the test.

The dry constituents are blended in the correct proportions and mixed thoroughly in a large-scale laboratory mixer or a household mixer. A portion of the deionized water required is added while mixing is continued. This is the basic soil substrate. The overall water content of the test and control substrates respectively should be adjusted to give the substrate a crumbly structure to enable springtails to penetrate substrate cavities. This is normally at a water content of 40 % to 60 % of the total water-holding capacity determined in accordance with ISO 11274, or with the method given in annex C. Water content and pH are determined before the start of the test and at the end of the test for the control and for each concentration tested.

NOTE 2 Allowance should be made for any water or quartz sand used for introducing the test substance into the soil.

5.2.2 Control substrate, consisting of the basic substrate and deionized water. If the preparation of the test requires the use of an auxiliary agent, then an additional control will be necessary.

5.3 Reference substance.

To ensure the quality of the test system, tests should be performed regularly (once or twice a year) with a reference substance.

The agricultural chemicals Betanal plus (a.i. 160 g/l Phenmedipham) and E 605 forte (a.i. 507,5 g/l Parathion) have been tested in a ring test, and are recommended as reference substances.

WARNING — When handling these chemicals, appropriate precautions should be taken to avoid ingestion or skin contact.

NOTE Betanal plus**:** Effects on reproduction (α = 0,05) were observed at concentrations of between 100 mg and 200 mg of the product per kilogram dry mass of the substrate.

E 605 forte: Effects on mortality and reproduction were observed at concentrations of between 0,18 mg and 0,32 mg product and between 0,1 mg and 0,18 mg product per kilogram dry mass of the substrate respectively.

6 Apparatus

Standard laboratory equipment, and:

6.1 Glass containers (able to be closed tightly) of about 100 ml capacity and with a diameter of about 5 cm.

6.2 Apparatus capable of measuring pH and water content of the substrate.

6.3 Exhaustor for transfer of springtails (see A.2).

6.4 Enclosure, capable of being controlled to a temperature of 20 °C ± 2 °C.

6.5 Light source, capable of delivering a constant light intensity of 400 lx to 800 lx at the substrate surface at a controlled light:dark cycle of between 12 h:12 h and 16 h: 8 h.

7 Procedure

7.1 Preparation of the test

7.1.1 Test concentrations

a) NOEC approach:

At least five concentrations in a geometric series at a factor not exceeding 2 (e.g. $\sqrt[4]{10} \sim 1{,}8$) should be selected to give an estimation of the LOEC/NOEC of the reproduction rate.

b) ECx approach:

For the ECx approach, a higher number of concentrations should be used (e.g. 12), and each tested with two replicates. The spacing of concentrations may be variable (i.e. smaller at low concentrations, and larger at high concentrations).

The concentrations of the test substance shall be expressed as mass of substance per dry mass of soil substrate (mg/kg) (see 5.2).

If no other relevant toxicity data are available, the concentrations selected to provide the LOEC/NOEC or EC10 will be based on the results of a preliminary test (see 7.2).

7.1.2 Introduction of the test substance

7.1.2.1 Water-soluble substances

Immediately before starting the test, dissolve the quantity of test substance in the water required for the replicates of one concentration (or that portion of it necessary to wet the soil substrate in order to meet the requirements of 5.2.1), and mix it thoroughly with the basic soil substrate before introducing it into the test containers.

Continue as described in 7.1.3.

7.1.2.2 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 mix it with a portion of the quartz sand required. After having evaporated the solvent by placing the container in a fume hood, add the remainder of the basic substrate (allowing for the amount of sand used to prepare the test substance) and the water, and mix thoroughly before introducing it into the test containers.

Continue as described in 7.1.3.

NOTE Ultrasonic dispersion, organic solvents, emulsifiers or dispersants may 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.

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

7.1.2.3 Substances insoluble in water or organic solvents

For a substance insoluble in a volatile solvent, prepare a mixture of 10 g of finely ground industrial quartz sand (see 5.2) and the quantity of the test substance required to obtain the desired concentration. Add the remainder of the basic substrate (allowing for the amount of sand used to prepare the test substance) and the water, and mix it thoroughly before introducing it into the test containers.

Continue as described in 7.1.3.

7.1.3 Introduction of the test organisms

Ten juvenile springtails (10 to 12 days old) are placed in each test container.

Springtails are tapped or sucked from the breeding containers to transfer them to the test containers. This can easily be done using an exhaustor as described in clause A.2. Before they are transferred to the test containers, organisms are counted and checked for damage both to reduce control mortality and to avoid systematic trial errors.

7.1.4 Control container

Prepare control containers in the same way as test containers, but without addition of the test substance. If the preparation of the test requires the use of auxiliary substances (see 7.1.2.2), use additional control containers. Treat these containers in the same way as those without the test substance.

7.2 Preliminary test (optional)

If it is necessary to determine the range of concentrations to be used in the final test, perform a preliminary acute test to determine mortality, using four concentrations of the test substance and a control (e.g. 0 mg/kg, 1 mg/kg, 10 mg/kg, 100 mg/kg and 1 000 mg/kg). Use 10 specimens of 10- to 12-day old springtails per concentration and per container. Prepare the test containers as indicated in 7.1.2. Place the test containers in the test enclosure (6.4) with the light source (6.5).

At the beginning of the test, add about 2 mg of granulated dry yeast to each test container, and cover the containers tightly (e.g. using plastic, glass discs or parafilm). Open the test containers briefly twice a week to allow aeration.

After 14 days, count the live springtails in each container, and determine the percentage mortality for each test substance concentration. Also observe surviving springtails and record any symptoms. Due to the rapid degradation of dead springtails, missing springtails are assumed to have died during the test period.

NOTE To obtain additional information for the determination of the concentration range for the final test, the test period can be extended to four weeks to allow qualitative determination of effects at concentrations at which effects on reproduction could be expected.

7.3 Final test

The concentrations selected for use in the test will be based on the results of the preliminary test, or on other toxicity data. Substances do not need to be tested at concentrations higher than 1 000 mg/kg dry mass of test substrate.

After mixing the test substance (see 7.1.2) into the test substrate for one concentration, each test container (replicate) is filled with 30 g wet mass of the test substrate. To ensure easy migration of springtails, the substrate in the test container should not be compressed.

For each concentration and control five replicates are prepared. If the ECx approach is applied prepare at least two replicates for the selected concentrations and five for the control. To facilitate checking of the pH and humidity of the test substrate, use of additional containers for each concentration and for the control is recommended.

At the beginning of the test and after a period of 14 days, add about 2 mg of granulated dry yeast to each test container, and cover the containers tightly (e.g. using plastic, glass discs or parafilm). Open the test containers briefly twice a week to allow aeration.

Determine the water content and the pH (in the presence of 1 mol/l KCl) of the artificial soil at the beginning and end of the test. When acidic or basic substances are tested, do not adjust the pH.

After two weeks, check the water content by reweighing the additional test containers, and compensate for water loss if it exceeds 2 % of the initial water content.

7.4 Determination of surviving springtails

Determine the number of springtails present four weeks after introducing the parental springtails onto the test and control substrates. Pour the test substrate into a 500 ml to 600 ml container and add water. After gentle stirring of the suspension with a spatula, springtails will drift to the water surface. Count adults and juveniles, if present, by a suitable procedure (see annex B) and report the numbers.

NOTE Other extraction methods (e.g. high-gradient extraction) may be used if they have proved to be effective.

8 Calculation and expression of results

8.1 Calculation

8.1.1 General

The data should be presented in tabular form, indicating the mean number of adults and juveniles for each concentration. Further statistical testing will depend on

a) whether the NOEC or the ECx approach has been chosen, and

b) whether the replicate values are normally distributed and are homogeneous regarding their variance.

8.1.2 NOEC approach

For each concentration, a statistical analysis of the homogeneity and normality of replicate results shall be made, e.g. by using Kolmogoroff-Smirnov's and Bartlett's test procedures respectively. With normally distributed and homogeneous data, an appropriate statistical analysis, e.g. multiple *t*-tests such as Dunnett or Williams test (α = 0,05, one-sided) should be performed. If these requirements are not fulfilled, it is recommended to use non-parametric methods, e.g. the Mann & Whitney or the Bonferroni U-test.

8.1.3 ECx approach

To compute any ECx value, the treatment means are used for regression analysis after an appropriate dose-response function has been found. A desired ECx is obtained by inserting a value corresponding to x % of the control mean into the equation found by regression analysis. Confidence limits can be calculated after Fieller (in Finney [11]).

Alternatively, treatment results may be expressed as percentages of the control result or as percent inhibitions relative to the control. The normal (logistic) sigmoid can then be fitted to the results by means of the probit regression procedure.

8.2 Expression of results

Indicate, in milligrams per kilogram dry mass soil:

— the lowest concentration tested with significant difference versus control(s) (LOEC),

— the test concentration immediately below the LOEC (NOEC),

and optionally,

— the concentration at which the reproduction rate is reduced by 10 % compared to the control (EC10),

— the concentration at which the reproduction rate is reduced by 50 % compared to the control (EC50).

All observations should be considered in interpretation of the data obtained from the test.

9 Validity of the test

The mortality of the adults in the control(s) should not exceed 20 % at the end of the test.

The reproduction rate should reach a minimum of 100 instars per control vessel.

The coefficient of variation of reproduction in the control should not exceed 30 %.

10 Test report

The test report shall refer to this International Standard and, in addition to the results expressed as in 8.2, shall provide the following information:

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

b) complete description of the biological material employed (species, age, breeding conditions, supplier);

c) method of preparation of the test substrate together with an indication of the auxiliary substances used for a low-/non-water-soluble substance;

d) results obtained with the reference substance, if performed;

e) detailed conditions of the test environment;

f) a table giving the percent mortality of adults at each concentration and in the control(s);

g) number of dead or missing adults and number of offspring per test container at the end of the test;

h) depending on the statistical approach selected, list the lowest concentration causing significant effects (LOEC), the highest concentration causing no observed effects (NOEC), EC10 and EC50 for the inhibition of reproduction and the method used for calculation (optional);

i) description of any pathological or other symptoms, or distinct changes in behaviour observed in the test organisms per test container;

j) water content and pH of artificial soil at the start and at the end of the test for the control and for each concentration;

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

Annex A
(informative)

Techniques for rearing and breeding of Collembola

A.1 Conditions for rearing and breeding

A.1.1 Breeding substrate

Plaster of Paris (plaster for stucco, pH 6,4) and activated charcoal (pulverized chemically activated charcoal, pH 6 to 7), are mixed in a mass ratio of 8:1, but higher ratios, (9:1 or 10:1) may also be used. Depending on the type of plaster, 60 g to 100 g water are added to 100 g of the mixture. This provides a highly moist substrate, while the charcoal adsorbs waste gases and excretion products. The dark background facilitates observation.

The presence of water on the saturated substrate surface is essential for breeding springtails, and the pH can readily be determined by using pH indicators placed on this wet substrate surface.

A.1.2 Breeding containers

Commercial plastic containers with a volume of about 400 ml should be used. Fill the containers to a depth of about 1 cm with the breeding substrate, and add deionized water to almost saturation. The moisture content can be maintained automatically by using an absorbent wick, implanted in the substrate and running to a water bath below the container, or by supplying distilled water with a pipette until the substrate is saturated but there is no water standing on the substrate surface.

Close the breeding containers tightly using suitable covers, and aerate periodically (e.g. in combination with feeding) by lifting the cover for a short time.

The covers may also be perforated for aeration by a needle.

CAUTION — Care should be taken that predacious mites do not penetrate the containers.

A.1.3 Climatic conditions

For keeping and breeding the springtails, a climatic chamber with a controlled temperature of 20 °C to 22 °C and 70 % to 80 % relative humidity with constant lighting at 400 lx to 800 lx (or light:dark cycle 16 h:8 h) is most suitable.

A.1.4 Food

For breeding and for the test, use granulated dry yeast as food supply. Feeding the breeding containers once or twice a week is recommended, but to avoid spoilage by fungi, food should be applied in small amounts at frequent intervals.

A.1.5 Transfer

After about eight weeks, transfer the springtails to fresh breeding containers by tapping or blowing. The transfer to fresh containers usually induces oviposition.

A.1.6 Test organisms of a standard age

To obtain 10- to 12-day old juvenile springtails for the test, transfer egg clusters from breeding containers to a freshly prepared breeding substrate, using a fine spatula or hair brush. After 48 h remove the egg clusters and feed instars hatched from the eggs.

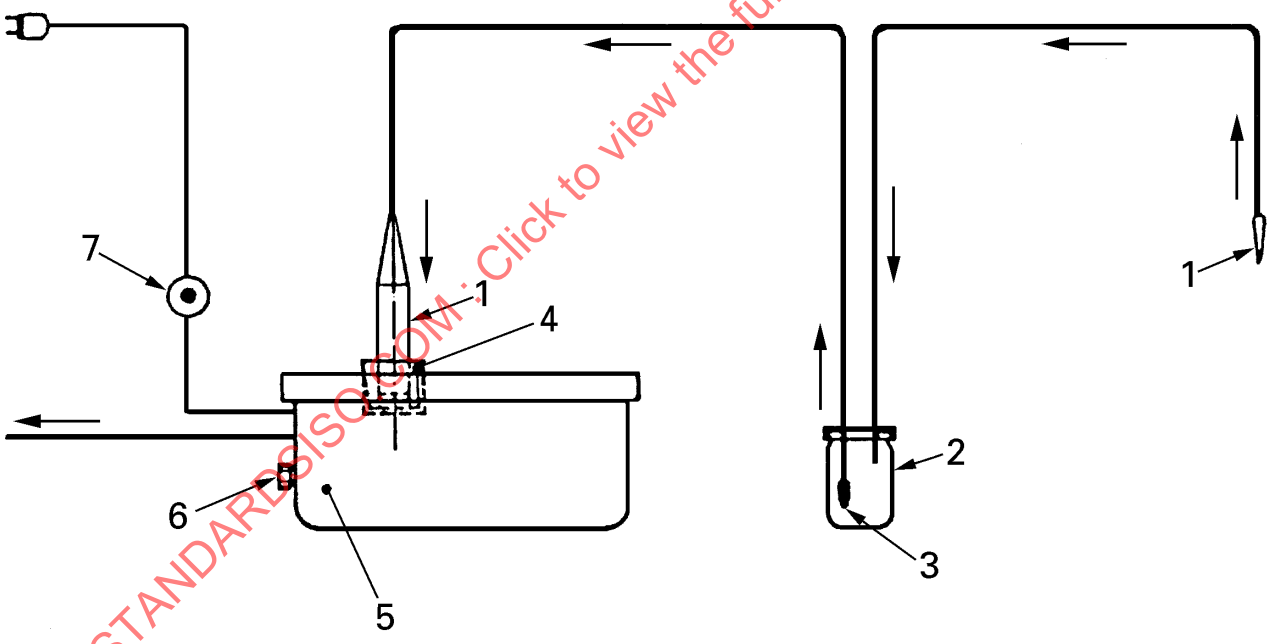
NOTE The egg clusters are easily removed if they are placed on small pieces of breeding substrate or cover glasses laid on the breeding substrate, and juvenile springtails collected after a further 10 days incubation.

Alternatively, 10- to 12-day old juvenile springtails may be obtained by placing a number of adult springtails in small containers with plaster of Paris in the base, and allowing them to lay eggs over a two-day period. After this time remove the adults. Twelve days after the first juveniles have emerged from the eggs, they can be used for the test. To ensure successful synchronization, it is advisable to check the containers for egg production before removing the adults. In some cases, the adults do not start laying eggs immediately, and only few eggs will be produced in two days. If this is observed to be the case, then keep the adults in the containers for an additional day or more.

For both methods, avoid overcrowding in the containers, as this may lead to reduced growth. As a consequence, the 10- to 12-day old animals used for the test may be too small and not yet able to produce a sufficient number of eggs to meet the requirements of the test.

A.2 Transfer of springtails to the test containers

The springtails are easily transferred from the breeding substrate to the test substrate by an exhaustor. An example is shown in Figure A.1.



Key

1 Eppendorf-pipette tip
2 Cylinder (10 ml volume) with rolled flange and plastic cover for control of sucked springtails
3 Gauze
4 Rubber stopper
5 Commercial aquarium pump
6 Regulator
7 Foot switch

Figure A.1 — Low-suction exhaustor apparatus for transfer of springtails

The springtails are sucked individually through a pipette tip to a small covered container to control damage of springtails. Care shall be taken to ensure that the suction of the pump is low to avoid damage to the springtails. After removing the cover, springtails provided for one test container are transferred onto the substrate surface of the test container.

Alternatively, a manual exhaustor can be hand-made from a small glass tube and a piece of flexible plastic tube. For this purpose a Pasteur pipette can be used, from which the small end has been removed and the narrow aperture annealed in a flame to smooth the cut edges. The other end of the tube is covered with a piece of very fine-mesh gauze, and a piece of flexible plastic tube is fixed to it. With such an exhaustor, the juveniles can be sucked up by mouth and transferred to the test containers.

Annex B
(informative)

Techniques for counting juvenile springtails

For evaluating effects on reproduction, the juvenile springtails swimming on the surface of the watered substrate are counted. When reproduction is high, the use of technical devices to facilitate counting is recommended.

If the swimming juveniles are distributed evenly over the water surface, a counting grid may be used and a sample for counting taken at random. Aggregations of instars on the water surface can be a problem and can be prevented by adding a drop of sewing-machine oil.

A culture-counter may also be used to count the animals on a projected picture (slide) of the water surface. Normal photographic equipment is adequate for this purpose (e.g. single-lens reflex camera, macro-lens or other device for close-up photography). Film speed has to be adjusted according to light intensity (e.g. flash or cold light source) and the desired shutter speed. If a cold light source is used, the minimum film speed value is 400 ASA. A useful relation between a sufficient projection format on a screen and the beaker volume is met with volumes of 50 ml to 100 ml (lens used: Zeiss S-Planar 1:2,8 f = 60 mm, angular field 39°, min. focus 0,24 m). To improve the contrast between white springtails and surrounding water surface, water may be coloured dark with ink.

Overall, the average error of counting should not exceed 10 %.

NOTE To avoid errors in determining mortality of the parental springtails, the number of live adults floating on the water surface should be counted by using a binocular microscope.

Annex C
(informative)

Determination of water-holding capacity of artificial soil

C.1 General

The following method has been found to be appropriate for the purposes of determination of water-holding capacity (WHC) of artificial soil and may be used as an alternative to ISO 11274.

C.2 Apparatus

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

C.2.2 Water bath, at room temperature.

C.2.3 Filter paper.

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

C.2.5 Balance, capable of weighing with an accuracy of ± 0,1 g.

C.3 Procedure

Plug the bottom of the tube with a filter paper, and after filling with the artificial soil substrate 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. Leave the substrate sample in the water for about 3 h.

As not all water absorbed by the substrate 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. The same quartz sand as is used for the soil substrate is satisfactory.

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

C.4 Calculation of water-holding capacity (WHC)

$$\mathrm{WHC} = \frac{S - T - D}{D} \times 100$$

where

WHC is the water-holding capacity, expressed as a percent of dry mass;

S is the mass of water-saturated substrate + mass of tube + mass of filter paper;

T is the tare (mass of tube + mass of filter paper);

D is the dry mass of substrate.

Annex D
(informative)

Guidance on adjustment of pH of artificial soil

To estimate how much $CaCO_3$ is needed to obtain the desired pH (6,0 ± 0,5), artificial soil is prepared by mixing peat, sand, kaolin and water as described in 5.2.1. Small portions are taken and mixed with different amounts of $CaCO_3$, e.g. corresponding with concentrations of 0,2 %, 0,4 %, 0,6 %, 0,8 % and 1,0 % dry mass. From these portions, the pH is determined as described in ISO 10390 and the results are plotted as a graph of pH versus the amount of $CaCO_3$. From this graph, the amount of $CaCO_3$ necessary to obtain a pH of 6,0 ± 0,5 can be estimated.