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**Soil quality — Biological methods —  
Determination of nitrogen mineralization  
and nitrification in soils and the influence  
of chemicals on these processes**

*Qualité du sol — Méthodes biologiques — Détermination de la  
minéralisation de l'azote et de la nitrification dans les sols, et de  
l'influence des produits chimiques sur ces processus*





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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 14238 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

This second edition cancels and replaces the first edition (ISO 14238:1997), which has been technically revised.

## Introduction

Soil consists of both living and non-living components which exist in a complex and heterogeneous environment. Microorganisms in the soil are mainly responsible for cycling of some nutrients and thus play an essential role in the maintenance of soil fertility. One of the most important microbial processes in soil is the mineralization of nitrogen contained in organic forms to ammonium (ammonification) and thereafter to nitrite and nitrate (nitrification). Clearly, any long-term interference with this process could influence soil fertility.



# Soil quality — Biological methods — Determination of nitrogen mineralization and nitrification in soils and the influence of chemicals on these processes

## 1 Scope

This International Standard specifies laboratory procedures for measuring the mineralization and nitrification of nitrogen by the soil microbiota.

For investigations on the evaluation of soil quality or on effects of contamination, a procedure is given to measure the rates and extent of N-mineralization in soil or soils of known or unknown quality.

For investigation of the potential toxicity of chemicals to N-mineralization in soils, a simple procedure is given which allows the impact of single chemicals to be assessed and provides a basis for comparison of the toxicities of different chemicals.

## 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 11261, *Soil quality — Determination of total nitrogen — Modified Kjeldahl method*

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

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 mass basis — Gravimetric method*

## 3 Terms and definitions

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

### 3.1

#### **nitrogen mineralization**

#### **N-mineralization**

microbial degradation of an organic substance containing nitrogen, via the processes of ammonification and nitrification, to the respective inorganic endproducts, specifically ammonium and nitrate

### 3.2

#### **ammonification**

microbial degradation of organic nitrogen to ammonium

### 3.3

#### **nitrification**

microbial oxidation of ammonium to nitrite and thereafter to nitrate

### 3.4

#### **inhibitory dose**

##### **ID<sub>x</sub>**

amount of a chemical added to soil that effectively inhibits N-mineralization by a stated percentage, after a given time, in comparison to an untreated control

EXAMPLE ID<sub>25</sub> and ID<sub>50</sub> indicate 25 % and 50 % inhibition of N-mineralization, respectively.

## 4 Principle

The rates or extent of N-mineralization in aerobic soils are determined by measuring the concentrations of ammonium, nitrite, and nitrate released during mineralization of nitrogen contained in the soil organic matter or during mineralization of an added nitrogenous organic compound.

The influence of chemicals on N-mineralization is determined by amending soil with a readily degradable source of organic nitrogen, and measuring the percentage inhibition of product formation in test portions treated with different quantities of a chemical as compared to an untreated control.

## 5 Materials

### 5.1 Soils

#### 5.1.1 Selection of soils

##### 5.1.1.1 Basic mineralization test

For basic tests used for comparing the N-mineralization capacities of different soils or for comparing N-mineralization in one soil collected at different times of the year, ensure that the choice of soil(s) is consistent with the purpose of the determination.

##### 5.1.1.2 Toxicity testing

To determine the influence of chemicals on N-mineralization, use a soil with low content (as a mass fraction) of organic carbon (0,5 % to 1,5 %) and low content of clay.

NOTE Such soil represents a worst-case situation, since adsorption is minimum and availability of the chemical to the microbiota is maximum. For routine testing, soils with a pH(KCl) less than 5 are not satisfactory, as the rate of nitrification is likely to be too low to permit a valid assessment of the effects of the chemical on the process. Sandy loam soils and loamy sand soils are preferred.

#### 5.1.2 Collection, handling and storage of soils

For all tests, the recommendations in ISO 10381-6 for collection, handling and storage of soil shall be followed.

The following information shall be documented:

- date of collection;
- date(s) used in experiments;
- storage conditions, including temperature, moisture content;
- length of storage.



### 5.1.3 Characterization of soils

To facilitate interpretation of data and for comparative purposes, the following characteristics shall be determined:

- a) physical properties:
  - particle size distribution measured in accordance with ISO 11277,
  - water content in accordance with ISO 11465,
  - water retention characteristic in accordance with ISO 11274 and/or water-holding capacity in accordance with Annex A;
- b) chemical properties:
  - pH of soil in accordance with ISO 10390,
  - cation exchange capacity in accordance with ISO 11260,
  - organic matter content in accordance with ISO 10694,
  - total nitrogen content in accordance with ISO 11261.

## 5.2 Reagents and materials

**5.2.1 Quartz sand**, fine and clean, of particle size 0,05 mm to 0,2 mm.

**5.2.2 Potassium chloride**, solution  $c(\text{KCl}) = 1 \text{ mol/l}$

**5.2.3 Nitrogenous substrate**, at a concentration of nitrogen in soil of  $\sim 100 \text{ mg/kg}$ .

For example:

- lucerne meal, with a C-to-N mass ratio of approximately 16 to 1;
- horn meal, with a C-to-N mass ratio of approximately 16 to 1;
- any other appropriate finely ground organic nitrogen source.

Mineralization of nitrogen may also be measured from the organic matter of the soil. In this case, soil is not amended with an organic nitrogen source.

For tests in which nitrification alone is of interest, ammonium [as  $(\text{NH}_4)_2\text{SO}_4$ ] is an appropriate nitrogen source.

## 5.3 Test substance

A test substance is only needed when the purpose of the investigation is to determine whether the substance can influence N-mineralization. Test substances shall be the purest that are commercially available. In many circumstances, it may be appropriate to test technical-grade or commercial-grade chemicals or mixtures.

**NOTE** If carriers or formulation ingredients are mixed with the test substance, their influence on N-mineralization (if any) should be taken into account.

In standard experiments with known test substances, the following data (if applicable) shall be given:

- name (IUPAC);
- structure;
- Chemical Abstracts Service number;
- relative molecular mass;

- purity;
- stability in water;
- solubility in organic solvents;
- vapour pressure;
- octanol/water partition coefficient ( $P_{OW}$ );
- common logarithm of the acid dissociation constant ( $pK_a$ );
- absorption coefficient ( $K_{oc}$ ).

## 6 Apparatus

Usual laboratory apparatus and in particular the following.

### 6.1 Mechanical shaker.

### 6.2 Centrifuge or filter paper (nitrate- and ammonium-free).

### 6.3 Instruments for measurement of concentrations of ammonium, nitrate, and nitrite in soil extracts.

## 7 Procedures

### 7.1 Experimental options

#### 7.1.1 Basic mineralization test

To compare N-mineralization capacities in different soils or N-mineralization in one soil collected at different times of the year, ensure that the design of the experiment and the analyses performed are consistent with the goals of the experiment.

#### 7.1.2 Toxicity testing

To determine the influence of chemicals on N-mineralization, treat a single microbiologically active soil with at least five concentrations of the test substance. For convenience, limit the analyses to measurement of the quantities of nitrate formed in treated and control samples after 0 d and 28 d incubation. Using this simple test design, dose–response relationships can be established. In some cases, e.g. where soil concentrations are known or can be predicted by rough screening (as for pesticides), it is possible that dose–response information is not needed, and an untreated sample and one appropriate concentration of test chemical is sufficient. If the influence of the chemical is not known, it is recommended that a range-finding test be performed before the final test.

### 7.2 Treatment of soils

#### 7.2.1 Basic mineralization test

Choose a substrate from the list given in 5.2.3, although the final choice of specific organic substrate used depends on the purpose of the test. Mix the organic material chosen thoroughly and homogeneously into the soil. If mineralization of nitrogen from the soil organic matter is being investigated, a nitrogenous substrate need not be added.

### 7.2.2 Toxicity testing

To determine the influence of chemicals on N-mineralization, use any of the nitrogenous substrates given in 5.2.3.

NOTE Compounds with low C-to-N ratios (i.e. not much higher than 16 to 1) are probably the best choice as little of the nitrogen released during mineralization is immobilized by the soil microbiota.

Mix the chosen nitrogenous substrate (5.2.3) thoroughly and homogeneously into the soil. Then, divide the soil into six test portions of equal mass. Mix five of these test portions with different concentrations of the substance to be tested. Sufficient soil should be prepared so that the soil can later be split into at least three replicates for each dose. Mix the remaining test portion, but do not add any test chemical (if a carrier is used, mix only into the soil). The chemical-free test portion serves as the untreated control. If possible, select a concentration series which allows ID<sub>25</sub> or ID<sub>50</sub> values to be estimated.

Apply the test substance using an appropriate carrier, for example:

- a) in water, depending upon water solubility of the compound;
- b) on a solid, e.g. mixed with quartz sand (5.2.1) or with a portion of the soil under investigation.

With many organic chemicals, the soil or sand used as a carrier can be coated with the test chemical by dissolving it in a solvent. In such cases, the solvent should be removed by evaporation before mixing with the soil.

When water is used to apply the test substance, care should be taken that the water content (as a mass fraction) does not exceed 60 % of the water-holding capacity or a water pressure of approximately 0,02 MPa.

### 7.3 Incubation of soils

For N-mineralization investigations, incubate the soils in either of these two ways:

- a) as bulk samples of each variant (e.g. soils of different quality or different levels of contamination) or treatment;
- b) as a series of individual test portions of each variant or treatment.

When variants are incubated as bulk samples, prepare large quantities of soil and take test portions (e.g. 10 g to 100 g) during the experiment, as needed. Here, the amounts of soil prepared are determined by the sizes of the samples taken, the number of replicates used (at least three), and the duration of the experiment. Mix soils incubated in bulk thoroughly before taking test portions. With large samples, spread the soil out to a depth of not more than 3 cm to facilitate oxygen transfer. Also mix the soil sample on a weekly basis.

When variants are held as a series of individual test portions, divide each variant into a series of equal test portions and sacrifice these test portions as needed. In studies with more than one sampling interval, prepare sufficient test portions to account for all replicates and sampling times.

NOTE 1 The choice of temperatures, water content of the soil and light conditions during incubation depends on the purpose of the experiment.

For tests to determine the influence of chemicals on N-mineralization, maintain soils at  $(20 \pm 2)$  °C and a pore water pressure of approximately 0,02 MPa to the nearest 5 % [ $(40 \pm 5)$  % to  $(60 \pm 5)$  % of the maximum water-holding capacity] in the dark.

NOTE 2 A temperature of  $(20 \pm 2)$  °C has been chosen as a standard for comparative purposes and because it gives relatively rapid results. Temperatures outside this range can be used if they are more appropriate (e.g. because of local conditions or lack of cooling equipment).

In all experiments, vessels holding soils shall allow free exchange of gases. This helps prevent the development of anaerobic sites which could cause nitrogen loss through denitrification. Minimize water losses from the soil by incubating soils in covered vessels. Determine the moisture content of the soil at regular intervals, and replace losses with deionized water.

NOTE 3 Deionized water can be applied to the surface of the samples as a fine spray.

When the mineralization potentials of different soils are to be compared, the water pressure of the soils shall be kept as similar as possible, e.g. by adjusting it to the same level of water-holding capacity (40 % to 60 %).

## 7.4 Sampling of soils for testing

### 7.4.1 Basic mineralization test

The number of samples and frequency of sampling depend on the purpose of the experiments, but shall be sufficient to allow for accurate measurement of inorganic nitrogen concentrations. For incubation with an organic substrate, an incubation time of 28 d is recommended. For incubation without substrate, an incubation time of 48 d may be necessary. It is possible to carry out intermediate measurements, e.g. after 7 d and 14 d.

### 7.4.2 Toxicity test

To determine the influence of chemicals on N-mineralization, sample the soils directly after treatment (see 7.2) (0 d) and after 28 d incubation (see 7.3). In most microbiologically intact, organic nitrogen-amended, untreated soils, the maximum rate of mineralization is reached within 28 d. Thus, sampling at 28 d usually allows valid estimations of  $ID_{25}$  or  $ID_{50}$  values. It is possible to carry out intermediate measurements, e.g. after 7 d and 14 d.

## 7.5 Extraction of soils

Extract ammonium, nitrite, and nitrate from soil samples directly after treatment (see 7.4) by shaking samples with potassium chloride solution (5.2.2) (5 ml potassium chloride per 1 g dry mass equivalent of soil) at 150 r/min for 60 min. To optimize extraction, do not fill containers holding soil and potassium chloride solution more than half full. Remove fine particles of soil from the extracts by filtration through a filter paper or centrifugation using a centrifuge (6.2). If the measurements are not performed extemporaneously, store particle-free extracts at  $(-20 \pm 5) ^\circ\text{C}$  for up to six months. Where novel analytical methods are used, ensure that interference from extractants is allowed for.

Test substances that contain high quantities of mineralizable nitrogen can contribute to the quantities of ammonium, nitrite or nitrate formed. Where high concentrations of such substances are required for toxicity testing, this should be taken into consideration.

## 7.6 Analyses

Carry out quantitative analyses of ammonium-N, nitrate-N and nitrite-N.

## 8 Expression of results

### 8.1 Basic mineralization test

For basic tests, consider preparing mineralization rate curves using values for the individual nitrogen ions. For this purpose, to allow nitrogen balances to be established, convert the quantities expressed in milligrams per kilogram of ammonium ( $\text{NH}_4^+$ ), nitrite ( $\text{NO}_2^-$ ), and nitrate ( $\text{NO}_3^-$ ), determined analytically (7.6), to ammonium-N ( $\text{NH}_4^+ - \text{N}$ ), nitrite-N ( $\text{NO}_2^- - \text{N}$ ), and nitrate-N ( $\text{NO}_3^- - \text{N}$ ).

In other cases, especially for comparing mineralization rates in different soils, express N-mineralization values at each sampling interval as a single value called " $N_{\text{min}}$ ".

To determine  $N_{\text{min}}$ , use the following equation:

$$N_{\text{min}} = \left[ \left( \text{NH}_4^+ - N_T \right) + \left( \text{NO}_2^- - N_T \right) + \left( \text{NO}_3^- - N_T \right) \right] - \left[ \left( \text{NH}_4^+ - N_S \right) + \left( \text{NO}_2^- - N_S \right) + \left( \text{NO}_3^- - N_S \right) \right]$$

where

- $(\text{NH}_4^+ - \text{N}_T)$  is the concentration of ammonium-N, in milligrams of nitrogen per kilogram of dry soil, at the time of sampling;
- $(\text{NO}_2^- - \text{N}_T)$  is the concentration of nitrite-N, in milligrams of nitrogen per kilogram of dry soil, at the time of sampling;
- $(\text{NO}_3^- - \text{N}_T)$  is the concentration of nitrate-N, in milligrams of nitrogen per kilogram of dry soil, at the time of sampling;
- $(\text{NH}_4^+ - \text{N}_S)$  is the concentration of ammonium-N, in milligrams of nitrogen per kilogram of dry soil, at the start of incubation;
- $(\text{NO}_2^- - \text{N}_S)$  is the concentration of nitrite-N, in milligrams of nitrogen per kilogram of dry soil, at the start of incubation;
- $(\text{NO}_3^- - \text{N}_S)$  is the concentration of nitrate-N, in milligrams of nitrogen per kilogram of dry soil, at the start of incubation.

## 8.2 Toxicity testing

To determine the effects of different concentrations of test substance on N-mineralization to nitrate, compare the rate of formation of nitrate (i.e. milligrams of  $\text{NO}_3^-$  per kilogram of dry mass of soil per day) found in the treated samples after 28 d incubation to that found in the untreated control. For the calculation of the rate of nitrate formation, subtract the mean concentration of nitrate (i.e. mg  $\text{NO}_3^-$  per kg of dry mass of soil) at the start of the test (day 0) from the mean concentration at the end of the test, and divide the value obtained by 28. If intermediate measurements have been carried out (e.g. after 7 d and 14 d), the rates during the periods between these measurements (e.g. 0 d to 7 d, 7 d to 14 d and 14 d to 28 d) should also be calculated. Since nitrogen balances are not necessary for evaluation of toxicity, do not convert milligrams of  $\text{NO}_3^-$  per kilogram of dry mass of soil values to  $(\text{NO}_3^- - \text{N})$  per kilogram of dry mass of soil values.

Calculate inhibition values as a percentage of the control,  $\text{ID}_x$ , for each treatment level as follows:

$$\text{ID}_x = 100 - \frac{\dot{w}_{\text{NO}_3^-,1}}{\dot{w}_{\text{NO}_3^-,2}} \times 100$$

where

$\dot{w}_{\text{NO}_3^-,1}$  is the rate of formation in milligrams of  $\text{NO}_3^-$  per kilogram per day in treated soil;

$\dot{w}_{\text{NO}_3^-,2}$  is the rate of formation in milligrams of  $\text{NO}_3^-$  per kilogram per day in untreated soil.

After making this simple calculation for each concentration of chemical tested, either

- prepare a dose–response curve similar to that shown in Figure 1, in which the  $\text{ID}_{25}$  and  $\text{ID}_{50}$  values for a hypothetical test substance are 1 mg of chemical per kilogram of dry mass of soil and 10 mg of chemical per kilogram of dry mass of soil, respectively;
- use regression analysis of the dependence between the test substance dosed and the content of  $\text{NO}_3^- - \text{N}$ .

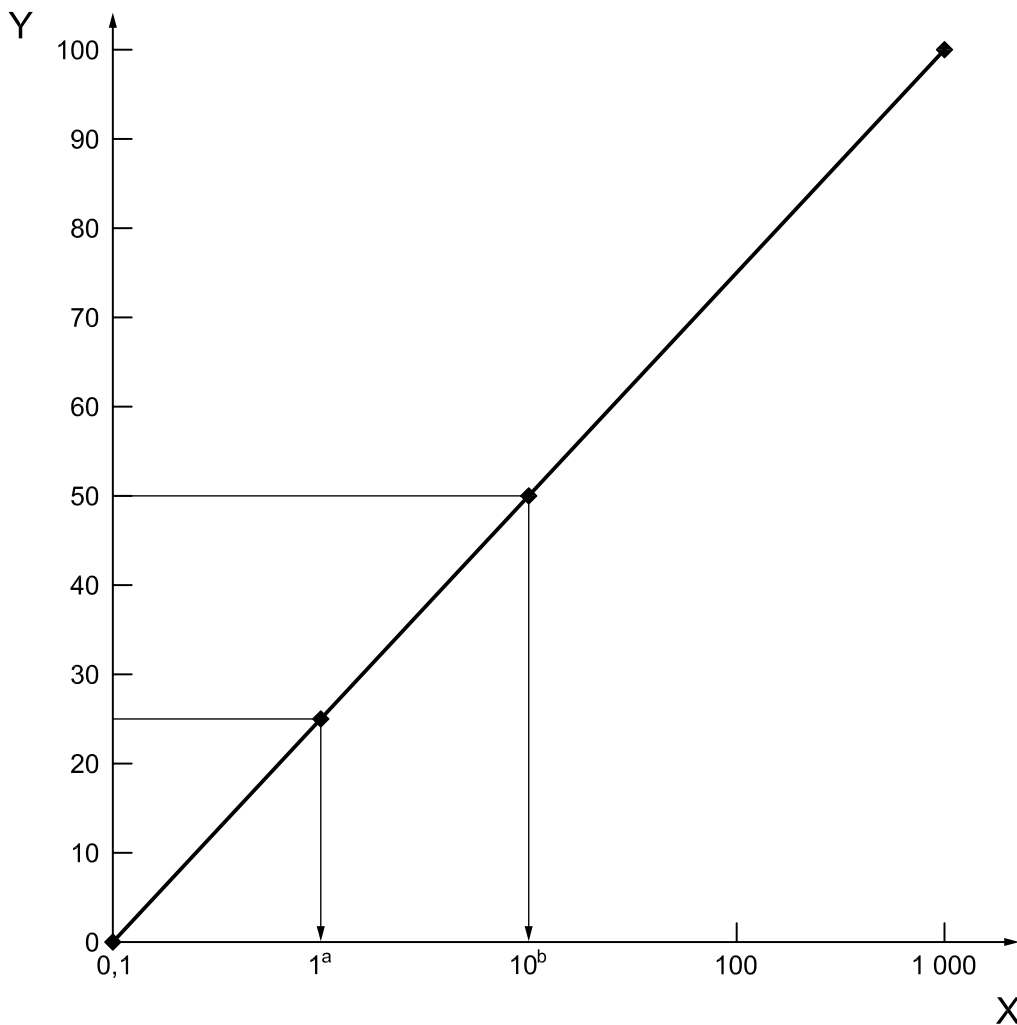
**NOTE** On completion of tests carried out with certain chemicals, the treated soil can contain more nitrate than untreated control samples. This is usually due to mineralization of the nitrogen in microbial cells killed by the chemical. Other causes can be mineralization of nitrogen from the chemical being tested or stimulation of the mineralization of soil organic carbon.

## 9 Test report

The test report shall contain the following information:

- a) a reference to this International Standard (ISO 14238:2012);
- b) soil characteristics (see 5.1.2 and 5.1.3);
- c) description of the test substance (if applicable) (see 5.3);
- d) soil collection, treatment, incubation, including date collected, length of storage, date(s) used in test(s), method of treatment (if applicable), amounts of chemical applied (if applicable), incubation conditions (see 7.1 to 7.6);
- e) sampling dates, extraction procedures and dates;
- f) analytical equipment and methods, detection limits, recovery efficiencies;
- g) figures and/or tables of results;
- h) evaluation of the results and conclusions drawn (if applicable).

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**Key**

- Y  $w_{NO_3^-}$  inhibition of nitrate formation
- X  $w_t$  concentration of test substance
- $^{a}ID_{25}$  dose resulting in 25 % inhibition of N-mineralization
- $^{b}ID_{50}$  dose resulting in 50 % inhibition of N-mineralization

**Figure 1 — Example of a dose–response curve showing concentrations of test substance inhibiting N-mineralization to  $NO_3^-$**

## Annex A (informative)

### Determination of water-holding capacity of soil

#### A.1 General

The method described in this annex has been found to be appropriate for laboratory samples of soils.

#### A.2 Apparatus and materials

Usual laboratory equipment and in particular the following.

**A.2.1 Glass tube**, approximately 20 mm to 50 mm in 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  $(105 \pm 5)$  °C.

**A.2.5 Balance**, capable of weighing with an accuracy of  $\pm 0,1$  g.

#### A.3 Method

Plug the bottom of the tube (A.2.1) with filter paper (A.2.3), weigh (A.2.5) the assembly, and after filling with the soil to a depth of 5 cm to 7 cm, place the tube on a rack in a water bath (A.2.2). Gradually submerge the tube until the water level is above the top of the soil, but below the upper lip of the tube. Leave the soil sample in the water bath 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 sample, dry (A.2.4) it to constant mass at 105 °C and reweigh it.

#### A.4 Calculation of water-holding capacity

Calculate the water-holding capacity,  $w_{\text{H}_2\text{O,c}}$ , as a percentage, using Equation (A.1):

$$w_{\text{H}_2\text{O,c}} = \frac{m_{\text{S}} - m_{\text{T}} - m_{\text{D}}}{m_{\text{D}}} \times 100 \quad (\text{A.1})$$

where

$w_{\text{H}_2\text{O,c}}$  is the water-holding capacity, expressed as a percentage of dry mass, %;

$m_{\text{S}}$  is the mass of water-saturated soil plus the mass of the tube plus the mass of the filter paper, in grams;



- $m_T$  is the tare (mass of tube plus mass of filter paper), in grams;
- $m_D$  is the dry mass of substrate, in grams.

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