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Soil quality — Determination of dehydrogenases activity in soils —

Part 1: Method using triphenyltetrazolium chloride (TTC)

Qualité du sol — Détermination de l'activité des déshydrogénases dans les sols —
Partie 1: Méthode au chlorure de triphényltétrazolium (CTT)

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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).

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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*.

This second edition cancels and replaces the first edition (ISO 23753-1:2005), which has been technically revised. The main changes compared to the previous edition are as follows:

— a new Clause 5 “Limitations” has been added;

— in Clause 6, reagents and their preparation have been updated to new results [e.g. use of less toxic solvent (ethanol), substrate concentration of 60 mmol/l of TTC, concentration of Tris buffer of 100 mmol/l at pH 7,6, incubation time of 6 h];

— Tables 1 and 2 have been added;

— Clause 10 "Validity criteria" has been added;

— a new Annex A “Results of modified parameters” has been added;

— Clause 2 “Normative references” and the Bibliography have been updated.

A list of all the parts in the ISO 23753 series can be found on the ISO website.

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

The soil microflora is responsible for the decomposition and conversion of organic substances, carbon, nitrogen, sulfur and phosphorus cycles, soil aggregates stability and as a food source for microbivores. Dehydrogenases, as intracellular enzymes and respiratory chain components of the microbial cells, play a major role in the production of energy by organisms. They oxidize organic compounds by transferring electrons to an acceptor (e.g. NAD^+). Dehydrogenases are essential components of the enzyme system of microorganisms. Dehydrogenases activity can therefore be used as an indicator of biological redox systems and as a measure of the viable and physiologically active soil microbial community.

Microbial oxidative activity in soil is linked to respiratory activity, which could be approached with the determination of dehydrogenases activity. Basal and induced respiration in soil could be affected by soil management, practices and contamination.

Soil quality — Determination of dehydrogenases activity in soils —

Part 1:
Method using triphenyltetrazolium chloride (TTC)

1 Scope

This document specifies a method for determining the activity of dehydrogenases enzymes in soil using 2,3,5-triphenyltetrazolium chloride (TTC).

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

ISO 18400-206, *Soil quality — Sampling — Part 206: Collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory*

3 Terms and definitions

No terms and definitions are listed in this document.

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

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

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

4 Principle

TTC solution is added to a soil sample and the mixture is incubated at 25 °C ± 1 °C for 6 h. The triphenylformazan (TPF) released is extracted with ethanol and quantified by spectrophotometry at a wavelength of 485 nm.

NOTE 1 The method is based on a modified version of the method reported in Reference [2].

NOTE 2 Other extraction liquids than ethanol can be used (e.g. acetone).

5 Limitations

— The storage can affect the enzyme activity and hence dehydrogenases activity of samples with different storage times should not be compared.

— Abiotic components, such as iron(II) compounds or sulfides can reduce TTC and consequently interfere with the measurement of dehydrogenases activity.

— Presence of coal or coal-like carbonaceous material can lead to significant bias making the dehydrogenase activity (DHA) method unsuitable. To overcome this bias, DHA method should be preliminary tested in these cases with TPF standard solution added in sample. After samples are shaken for 1 h in the dark and centrifugation, supernatant is used to spectrophotometric measurement at 485 nm. The difference between measured and expected absorbance and TPF concentration should not be higher than 20 %.

— For organic litter layers, peat and organic rich lacustrine mud, a sample mass of 0,5 g and a test tube of 2 cm diameter are recommended[3]. However, organic solvents can extract excessive amounts of organic matter from these organic rich substrates, giving blank values (ρ_{bs}) that may not differ from measured values (ρ_{cs}). Hence, the method cannot be unrestrictedly recommended for such substrates.

6 Reagents and materials

6.1 Soil.

Soil samples shall be collected and prepared as specified in ISO 18400-206. If samples which have been sieved in the fresh state cannot be analysed immediately, they may be kept for up to seven days at 4 °C ± 2 °C before measuring the dehydrogenases activity. Determine the dry matter content of the sample in accordance with ISO 11465.

6.2 Hydrochloric acid (HCl), *c* = 1 mol/l.

6.3 Tris buffer solution, *c* = 100 mmol/l, pH 7,6.

— Tris(hydroxymethyl)aminomethane (CAS N°: 77-86-1 - 121,14 g/mol): 12,12 g;

— Deionized water, *ad:* 1 000 ml;

— Hydrochloric acid (CAS N°7647-01-0) (1 mol/l).

Dissolve 12,12 g of Tris base into 800 ml deionized water and adjust pH to 7,6 with hydrochloric acid (1 mol/l). Fill in to 1 000 ml. The storage duration cannot exceed one month at 4 °C ± 2 °C.

6.4 Substrate solution (TTC), *c* = 300 mmol/l (10 %).

— 2,3,5-triphenyltetrazolium chloride -TTC (CAS Number: 298-96-4 – 334,80 g/mol): 1 g;

— Tris buffer 100 mmol/l, pH 7,6.

Dissolve 1 g of 2,3,5-triphenyltetrazolium chloride in 10 ml of Tris buffer solution (6.3). The solution can be stored for a maximum of one week at 4 °C ± 2 °C in the dark.

6.5 Analytical grade ethanol (96 %).

6.6 Triphenylformazan (TPF) solutions.

6.6.1 TPF stock solution (CAS Number: 531-52-2 – 300,36 g/mol), *c* = 33 mmol/l.

Dissolve 100 mg of triphenylformazan (TPF) in ethanol (6.5) to make up 10 ml.

6.6.2 TPF working solution, *c* = 330 nmol/ml.

Dilute 0,5 ml of TPF stock solution (6.6.1) to 50 ml with ethanol (6.5).

7 Apparatus

7.1 Visible light spectrophotometer.

7.2 pH meter.

7.3 Suitable U-bottom tubes (35 to 50) ml, volumetric flasks, pipettes and cuvettes.

For example, 2 cm diameter and at least 35 ml to 50 ml capacity (test portion 5 g). There is no linear correlation between soil mass and TTC reduction if the same test tubes are used. If the test tube diameter is fitted to the soil mass a linear correlation between TTC reduction and mass of test portion is obtained. Optimum test tube diameters are 2 cm for 5 g of soil.

7.4 Incubator, capable of being set to 25 °C ± 1 °C.

7.6 Test tube shaker set to 25 °C ± 1 °C.

7.7 Centrifuge, with temperature regulation at 20 °C ± 2 °C and acceleration of 2 000*g*.

8 Procedure

8.1 Establishment of standard curve

Standard curve require several concentrations of triphenylformazan (TPF), at least in duplicates, preferably in triplicates; all the volumes are given per tubes.

The working solution of TPF with a concentration of 330 nmol/ml is used for establishing the standard curve. Distribute the volumes needed into tubes for the preparation of the following concentrations: 0 nmol/ml; 11 nmol/ml; 22 nmol/ml; 55 nmol/ml; 110 nmol/ml, in triplicate according to Table 1.

Table 1 — Preparation of standard curve of triphenylformazan in tubes

[TPF] nmol/ml	0	11	22	55	110
TPF (ml)	0	0,1	0,2	0,5	1
Ethanol (ml)	3	2,9	2,8	2,5	2

Since TTC and TPF are sensitive to light, the solutions should be protected from exposure to light throughout the analysis.

8.2 Sampling

According to Reference [1], optimum conditions are given at a soil/solution ratio of 1:1.

Weigh 5,00 g of moist sieved soil into each of four tubes (7.3). Add 4 ml of Tris 100 mmol/l pH 7,6, and 1 ml of substrate solution (6.4) to three test tubes . Final substrate concentration is 60 mmol/l (2 %). Add only 4 ml of Tris 100 mmol/l pH 7,6 (6.3) into control tube. Shake each tube manually for a few seconds, seal the tubes with rubber stoppers and incubate at 25 °C ± 1 °C for 6 h. To extract the triphenylformazan formed, add 25 ml of ethanol (6.5) to the samples and allow them to stand for 1 h in the dark under orbital agitation (250 min^{-1}, 25 °C ± 1 °C). At the end of the extraction, add 1 ml of substrate solution (6.4) to control tubes. Tubes are then centrifuged 5 min at 2 000*g* and the supernatants are transferred in cuvettes for absorbance reading on a spectrophotometer at a wavelength of 485 nm. Table 2 summarizes the experimental protocol.