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Soil quality — Contact test for solid samples using the dehydrogenase activity of *Arthrobacter globiformis*

Qualité du sol — Essai contact pour échantillons solides utilisant l'activité déshydrogénase de Arthrobacter globiformis

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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.

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 on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information

The committee responsible for this document is ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

Introduction

This International Standard describes the miniaturized solid contact assay with *Arthrobacter globiformis* that allows the preliminary assessment of solid material (i.e. soil and waste materials) within 6 h. The principle of the assay relies on dehydrogenase activity inhibition of an added test organism, caused by bioavailable toxic substances in soil and waste samples. This is an ecologically relevant assay as far as it uses a ubiquitous soil bacteria species with high affinity to surfaces[16][6] which dehydrogenases are involved in different biological mechanisms withstanding bacteria integrity (e.g. respiratory chains). Moreover, it has been noticed that this parameter (dehydrogenase activity inhibition) is quite sensitive to different toxic substances.[19][10][14][15]

Overall, this assay is non-labour-intensive, rapid, cost-effective and sensitive, providing results that improve the physical and chemical assessment of natural samples while allowing a quick indication of their biological effects.

The miniaturized solid contact assay is based on the solid contact assay established by Reference [7].

This International Standard is also based on Reference [23].

The results of an interlaboratory trial towards the evaluation of test variability to assess different waste and soil samples, as well as chemicals, are presented in Annex A.

Soil quality — Contact test for solid samples using the dehydrogenase activity of *Arthrobacter globiformis*

1 Scope

This International Standard specifies a rapid method for assessing solid samples in an aerobic suspension, by determining the inhibition of dehydrogenase activity of *Arthrobacter globiformis* using the redox dye resazurin.

It is applicable for assessing the effect of water-soluble and solid matter bounded non-volatile contaminants of natural samples, such as soils and waste materials. The test yields a result within 6 h and can therefore be used for screening potentially contaminated material.

2 Normative references

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

ISO 5667-15, *Water quality — Sampling — Part 15: Guidance on the preservation and handling of sludge and sediment samples*

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*

CEN/TR 15310-1, *Characterization of waste — Sampling of waste materials — Part 1: Guidance on selection and application of criteria for sampling under various conditions*

EN 14735, *Characterization of waste — Preparation of waste samples for ecotoxicity tests*

3 Terms and definitions

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

3.1
contact time
exposure period of the bacteria to a suspension of solid matter

3.2
negative control
sample of a *control substrate* (3.6) with a mixture of known solutions [distilled water, medium B or *inoculum* (3.12)].

Note 1 to entry: It is used to standardize the analysis.

3.3
positive control
sample of a *control substrate* (3.6) with a mixture of known solutions [distilled water, medium B or *inoculum* (3.12)] and a reference substance

Note 1 to entry: It is used to check the sensitivity of the test organism.

3.4
blank A
blank, which sets the own fluorescence of the substrate after being deactivated

Note 1 to entry: Blank is not added with bacteria.

3.5
blank B
blank, which sets the natural fluorescence of the substrate without being deactivated

Note 1 to entry: Blank is not added with bacteria.

3.6
control substrate
reference or standard substrate used as a control and as *medium* (3.13) for preparing dilution/concentration series with *test substrates* (3.7) or a reference substance

EXAMPLE Quartz sand or LUFA standard soil type 2.2.

3.7
test substrate
natural or artificial substrate that is naturally contaminated or spiked with a test chemical

Note 1 to entry: The test substrate is the *test material* (3.8) after being prepared for testing (e.g. sieved) and/or diluted with a *control substrate* (3.6).

3.8
test material
original sample of soil or waste material without any changes (e.g. sieving)

3.9
dehydrogenase activity
activity of hydrogen-abstracting enzymes which are involved in many energy and biosynthesis metabolic processes (e.g. the respiratory chain) and which require cell integrity to be produced

Note 1 to entry: These enzymes can reduce resazurin into resorufin in the extracellular environment.[6]

Note 2 to entry: See Reference [21].

3.10
effect concentration for *x* % effect
EC*x*
concentration (mass fraction) of a test substance or sample that causes *x* % of an effect on a given endpoint within a given exposure period when compared with a control

EXAMPLE An EC50 is a concentration estimated to cause an effect on a test end point in 50 % of an exposed population over a defined exposure period.

Note 1 to entry: The EC*x* is expressed as a percentage of soil or waste tested per dry mass of soil mixture. When chemicals are tested, the EC*x* is expressed as mass of the test substance per dry mass of soil, in milligrams per kilogram.

3.11
freeze-dried bacteria
bacterial culture preserved through the water removing of a frozen cell suspension by sublimation under reduced vacuum pressure

Note 1 to entry: The preserved cultures can be stored at (-20 ± 2) °C. The bacteria are active after being reconstituted with sterilized distilled water [20 min to 30 min at (6 ± 2) °C] and ready to be used in the test, see 7.3.4 b).

3.12
inoculum
suspension of bacteria used to inoculate a nutrient solution

3.13
medium
aqueous nutritive solution required for bacterial growth

3.14
optical density of bacterial inoculum
measurement of the attenuation of a light beam passing through a bacterial suspension at 600 nm (used to determine the cell count indirectly)

Note 1 to entry: In a bacterial test, the absorbance is usually measured as FAU (formazine attenuation units) at 600 nm (see Reference [3]).

3.15
test start
moment when the substrates, reagents and the bacterial *inoculum* (3.12) are prepared immediately before the incubation and reaction period

Note 1 to entry: Here is when preparing the test and *control substrates* (3.6) for incubation (i.e. Table 1, day 0).

3.16
reaction time
time it takes for the enzyme to react (from the addition of the resazurin solution until the end of the reaction)

3.17
slope
quotient of the *relative fluorescence* (3.18) variation along the *reaction time* (3.16) between 15 min and 45 min

Note 1 to entry: The slope (expressed as min^{-1}) results from fitting a linear regression model to the fluorescence readings over time.

3.18
relative fluorescence
fluorescence measured for each treatment (control and test) after subtracting the fluorescence of the respective *blank A* (3.4)

3.19
stock culture
bacterial culture obtained from a pure strain culture acquired from a certified laboratory

Note 1 to entry: This stock culture provides an *inoculum* (3.12) for the pre-culture in the test procedure.

3.20
lowest ineffective dilution
LID-value
lowest value of the dilution factor (LID) for which the test does not give an ecotoxicological relevant reduction

Note 1 to entry: The LID is expressed as the reciprocal value of dilution.

EXAMPLE An often used dilution series is 1/2/4/8/16 [= 100 %/50 %/25 %/12,5 %/6,25 % *test substrate* (3.7) to *control substrate* (3.6)]. A LID 8 corresponds to a dilution of soil or waste of 1 : 8.

4 Principle

The bacteria *Arthrobacter globiformis* is added to the solid material and incubated at (30 ± 1) °C for 2 h. After this contact time, the non-toxic redox dye resazurin is added. Due to the dehydrogenase activity, resazurin is transformed into resorufin, in the extracellular environment.[6] Resorufin can be detected fluorometrically (excitation at 535 nm, emission at 590 nm) in the presence of solid matter. The increase of resorufin is determined by measuring the fluorescence every 15 min for a period of 1 h. In order to determine the inhibition of the dehydrogenase activity, the rate of resorufin increase in the sample is compared with the rate of resorufin increase in the control. In the presence of toxic substances, an inhibition of dehydrogenase activity is expected. This is reflected by the reduction of resorufin production and subsequent lowering of fluorescence emission.

5 Reagents and material

5.1 Test organisms

The test organism is *Arthrobacter globiformis* (Conn 1982) Conn and Dimmick 1947 (strain number ATCC 8010), which is common in soils. *Arthrobacter* species belong to the Micrococcaceae family. They are mostly obligate aerobic organisms, although some species may exhibit anaerobic metabolism under limiting oxygen conditions.[9] *Arthrobacter* spp. are chemoheterotrophic, and present pleomorphic characteristics, since they show a rod-to-coccus morphology change as they enter in the stationary phase. Although *Arthrobacter* is gram-positive, it can stain gram-negative during the log-phase. Variations in the cell wall thickness along the bacteria growth can lead to gram variability by differential staining of the granules.[22] However, this characteristic does not induce a differential sensitivity between assays, as far as an inoculum in exponential growth phase is used during the reaction time. *Arthrobacter globiformis* is classified in the risk group 1 – non-pathogenic organism.

The bacteria strain can be achieved from commercially available freeze-dried or liquid-dried reagents, or from culture collections, e.g. Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, or ARS Culture collection NCAUR.[1] The bacterial suspensions used for toxicity measurements shall be freshly prepared from stock cultures or directly used from a ready-to-use freeze-dried batch. The stock culturing and freeze drying process of the bacteria is described in Annex B.

5.2 Control substrates

5.2.1 General

The control substrates selected from the options presented below are to prepare both the negative (addition of distilled water, see 5.2.2, 5.2.3) and positive (addition of the reference substance, see 7.2) controls. The moistening of the control substrates (soil or waste material) shall be made one or two days before the test start (see Table 1). Store the substrate(s) at (4 ± 2) °C until the test start.

5.2.2 Control for soils

There are three possibilities for the choice of the control soil (see also Reference [4]). The reference soil a) is preferred, but if such a soil is not available, either a standard natural soil or a standard artificial soil may be used. In any case, the water content of the control soil should be adjusted to 20 %.

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

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1) Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ) GmbH, Mascheroder Weg 10, D-38124 Braunschweig, Germany; or ARS (Agricultural Research Service) Culture collection (also known as NRRL) belonging to the National Center for Agricultural Utilization Research (NCAUR), 1815 N, University Street, Peoria, Illinois 61604, USA are examples of firms that sell this bacteria. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these firms.

b) Standard natural soil with the following characteristics: C_{org} ≤ (1,7 to 2,6) %; sand (particle size 0,063 mm to 2 mm) content of 50 % to 75 %; <20 % of particles less than 0,02 mm; pH 5 to 7,5.

EXAMPLE LUFA standard soil type 2.2.[2)]

c) Standard artificial soil or quartz sand (with 50 % to 75 % of sand with particle size between 0,063 mm and 2 mm).

The substrate called artificial soil[17] has the following composition:

	Percentage expressed on dry mass basis
— Sphagnum peat finely ground and with no visible plant remains (particle size ≤1 mm)	5 %
— Kaolinite clay containing not less than 30 % kaolinite	20 %
— Industrial quartz sand (dominant fine sand with 50 % to 75 % of particle size 0,063 mm to 2 mm)	74 %
— Calcium carbonate	1 %

Artificial soil prepared with modified peat and quartz sand particle size should be analysed more in detail. The presence of low density particles (e.g. peat) in this artificial substrate that are likely to float can influence the fluorescence readings.

5.2.3 Control for waste material

Quartz sand, see 5.2.2 c). The quartz sand should have a water content of 20 %.

5.3 Test substrates

The samples (soil or waste material) should be tested as soon as possible after sampling. Collect samples as specified

— for soil in ISO 10381-6, or

— for waste materials in ISO 5667-15, EN 14735 and CEN/TR 15310-1.

Store them in the dark at (4 ± 2) °C for not more than two weeks. For long-term storage, the samples may be frozen at (-20 ± 2) °C.

Soil and waste samples shall be passed through a sieve of 2 mm square mesh. Waste raw material (e.g. construction waste material) should be grounded before testing (refer to EN 14735). The screening of metals and organic contaminants in the samples is strongly advised as it provides helpful information for data interpretation.

For interpretation of test results, the following characteristics should be determined for each sample:

a) pH in accordance with ISO 10390 for soil samples, EN 15933 for biowaste samples and ISO 10390 for other solid wastes;

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

c) water content in accordance with ISO 11465 for soil samples and EN 15934 for biowaste samples;

d) water holding capacity according to ISO 11268-2;

e) cation exchange capacity in accordance with ISO 11260;

2) LUFA Standard soil type 2.2 is the trade name of a product supplied by LUFA Speyer. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

f) organic carbon in accordance with ISO 10694 for soil samples and EN 15936 for biowaste samples.

Only samples with a pH between 5 and 9 will be appropriately assessed by this contact test (see 7.4). The water content shall be adjusted to 20 % (soil, waste material; see 5.2.2 and 5.2.3). This adjustment shall be calculated according to the original water content of samples, which should be determined before the preparation of the test. The moistening of samples shall be done one or two days before the test start (see Table 1). Store the samples at (4 ± 2) °C until the test start.

When using natural samples, dilutions may also be prepared (see 7.1).

In case of testing a chemical substance in soil, a different procedure should be followed (see Annex C).

Waste materials consisting of sewage sludge with high organic matter (OM) or total organic carbon (TOC) content should be added with more water (e.g. water content adjusted to 33 %). More tests are being developed as to define the appropriate percentage of water content according to the level of OM or TOC in this type of samples.

5.4 Chemicals

Unless otherwise specified, only analytical-grade reagents shall be used.

5.4.1 Water, sterilized and non-sterilized, deionized, distilled or of equivalent purity (conductivity < 10 $\mu S \cdot cm^{-1}$).

5.4.2 Dimethyl sulfoxide solution (DMSO), C_2H_6OS, volume fraction of 4 % in distilled water.

Sterilize the solution by filtration through a polyamide membrane filter having a pore size of 0,2 µm and using a syringe.

5.4.3 Sodium hydroxide solution, NaOH of, e.g. 1 $mol \cdot l^{-1}$.

NOTE For the adjustment of the media pH, it can be necessary to use bases of lower or higher concentration.

5.4.4 Hydrochloric acid, HCl of, e.g. 1 $mol \cdot l^{-1}$.

NOTE For the adjustment of the media pH, it can be necessary to use acids of lower or higher concentration.

5.4.5 Medium A, for *A. globiformis* stock culture (pH 7,2 to 7,4).

Dissolve

— 10 g casein peptone,

— 5 g yeast extract,

— 5 g D(+)-glucose, and

— 5 g NaCl

in water (5.4.1), make up to 1 000 ml with water and autoclave for 20 min at (121 ± 3) °C. If stored sterilized (never opened) at (4 ± 2) °C in the dark, the solution is stable up to 12 months.

5.4.6 Medium B, for preparing the lyophilizates and the test solution.

Dilute 333,3 ml of medium A (5.4.5) in 666,6 ml of sterilized water. Or, dissolve

— 3,33 g casein peptone,

— 1,67 g yeast extract,

— 1,67 g of D(+)-glucose, and

