



# FINAL DRAFT International Standard

## ISO/FDIS 18187

### 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 document 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](http://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](http://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 18187:2016), which has been technically revised.

The main changes are as follows:

- the scope was amended to include the possibility of applying the contact assay for testing the effect of chemicals (as detailed in [Annex C](#));
- further details regarding other potential interfering factors (for the testing of plastic and low-density waste materials) on the performance of the contact assay were addressed, and adequate methodological alternatives were suggested to reduce uncertainties in the assay outcome;
- validity criteria were updated to include the range of dehydrogenase activity inhibitions expected under three other reference substances (i.e. copper sulfate (II) pentahydrate, 3,5-dichlorophenol, zinc sulfate heptahydrate) that are spiked into quartz sand and used as alternative positive controls in the quality testing of waste materials;
- in [Clause B.4](#), genome sequencing is proposed to confirm the identity of *Arthrobacter globiformis*, instead of microbiological identification strips which are no longer recommended.

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](http://www.iso.org/members.html).

## Introduction

This document 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 bacterial species with high affinity to surfaces [1][2] whose 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.[3][4][5][6][7]

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 [8].

This document is also based on Reference [9].

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](#) and in Reference [10].

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

## 1 Scope

This document 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 in natural samples, such as soils and waste materials. Although not the main purpose, the contact test can additionally be used for testing the effect of chemicals, as described in the [Annex C](#). The test yields a result within 6 h and can therefore be used for screening potentially contaminated test material.

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

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*

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.

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

#### **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* (3.13) 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* (3.13) 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 A 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 B is not added with bacteria.

### 3.6

#### **control substrate**

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

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, redox reactions, and biosynthetic metabolic processes (e.g. the respiratory chain), which require cell integrity to be produced

Note 1 to entry: These enzymes can reduce resazurin into resorufin in the extracellular environment<sup>[2]</sup>.

Note 2 to entry: See Reference <sup>[11]</sup>.

### 3.10

#### **EC<sub>x</sub>**

effect concentration for *x* % effect

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 EC<sub>50</sub> 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<sub>x</sub> is expressed as a percentage of soil or waste tested per dry mass of soil mixture. When chemicals are tested, the EC<sub>x</sub> is expressed as mass of the test substance per dry mass of substrate, 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  $(-20 \pm 2)$  °C. The bacteria are active after being reconstituted with sterilized distilled water [20 min to 30 min at  $(6 \pm 2)$  °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 [\[12\]](#)).

**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: It is when preparing the *test* ([3.7](#)) 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 kinetics period)

**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  $\text{min}^{-1}$ ) 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**

lowest value of the dilution factor 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)]. An LID of 8 corresponds to a dilution of soil or waste of 1:8.

## 4 Principle

The bacterium *Arthrobacter globiformis* is added to the solid material and incubated at  $(30 \pm 1)$  °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.<sup>[2]</sup> 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 the 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<sup>1)</sup>, which is common in soils. *Arthrobacter* species belong to the *Micrococaceae* family. They are mostly obligate aerobic organisms, although some species can exhibit anaerobic metabolism under limiting oxygen conditions.<sup>[13]</sup> *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.<sup>[14]</sup> 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 I – non-pathogenic organism.

The bacterium strain can be achieved from commercially available freeze-dried or liquid-frozen aliquots from culture collections, e.g. Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH (strain number 20124), or ARS Culture collection NCAUR.<sup>2)</sup> 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 in [5.2.2](#) and [5.2.3](#) include 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  $(4 \pm 2)$  °C until the test start.

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1) Strain number ATCC 8010™ *Arthrobacter globiformis* (Conn) Conn and Dimmick.

2) 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 bacterium. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these firms.

### 5.2.2 Control for soils

There are three possibilities for the choice of the control soil (see also Reference [15]). The reference soil [item a)] is preferred, but if such a soil is not available, either a standard natural soil or a standard artificial soil can 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.
- b) Standard natural soil with the following characteristics:  $C_{\text{org}} \leq (1,7 \text{ to } 2,6) \%$ ; 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.<sup>3)</sup>

- c) Standard artificial soil or quartz sand (with 50 % to 75 % of sand with particle size between 0,063 mm and 2 mm; pH 5,5 to 6,5).

The substrate called standard artificial soil<sup>[16]</sup> has the following composition:

	Percentage expressed on dry mass basis
— Sphagnum peat finely ground and with no visible plant remains (particle size $\leq 1$ 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 %

Standard 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, but other control soils (as suggested above) can be selected.

### 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 18400-206;
- for waste materials in ISO 5667-15, EN 14735 and CEN/TR 15310-1.

Store them in the dark at  $(4 \pm 2) \text{ }^\circ\text{C}$  for not more than two weeks, to avoid (bio)chemical changes that can interfere with the ecotoxicity results. For long-term storage, the samples can be frozen at  $(-20 \pm 2) \text{ }^\circ\text{C}$ .

NOTE For waste samples not microbially-active or not prone to (bio)chemical changes that can lead to a variation in the ecotoxicity testing results, a storage up to two months at  $(4 \pm 2) \text{ }^\circ\text{C}$  can be considered upon the judgment of the operator (as per EN 14735).

3) 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 can be used if they can be shown to lead to the same results.