



**International  
Standard**

**ISO 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**

**Second edition  
2024-05**

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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)).

ISO draws attention to the possibility that the implementation of this document may involve the use of (a) patent(s). ISO takes no position concerning the evidence, validity or applicability of any claimed patent rights in respect thereof. As of the date of publication of this document, ISO had not received notice of (a) patent(s) which may be required to implement this document. However, implementers are cautioned that this may not represent the latest information, which may be obtained from the patent database available at [www.iso.org/patents](http://www.iso.org/patents). ISO shall not be held responsible for identifying any or all such patent rights.

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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*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 444, *Environmental characterization of solid matrices*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

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  $(-20 \pm 2) ^\circ\text{C}$ . The bacteria are active after being reconstituted with sterilized distilled water [20 min to 30 min at  $(6 \pm 2) ^\circ\text{C}$ ] 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  $(30 \pm 1) ^\circ\text{C}$  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](#) should be used 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  $(4 \pm 2) ^\circ\text{C}$  until the test start.

#### 5.2.2 Control for soils

There are three possibilities for the choice of the control soil (see also Reference [\[15\]](#)). 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.

1) Strain number ATCC 8010<sup>TM</sup> *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.

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- 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  $(4 \pm 2)^\circ\text{C}$  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  $(-20 \pm 2)^\circ\text{C}$ .

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  $(4 \pm 2)^\circ\text{C}$  can be considered upon the judgment of the operator (as per EN 14735).

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). Metals and organic contaminants in the samples should be screened as this provides helpful information for data interpretation.

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

- a) pH in accordance with ISO 10390<sup>[17]</sup> for soil samples, EN 15933<sup>[18]</sup> for biowaste samples and ISO 10390<sup>[17]</sup> for other solid wastes;
- b) texture (sand, loam, silt) in accordance with ISO 11277<sup>[19]</sup>;

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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.

- c) water content in accordance with ISO 11465<sup>[20]</sup> for soil samples and EN 15934<sup>[21]</sup> for biowaste samples;
- d) water holding capacity according to ISO 11268-2<sup>[22]</sup>;
- e) cation exchange capacity in accordance with ISO 11260<sup>[23]</sup>;
- f) organic carbon in accordance with ISO 10694<sup>[24]</sup> for soil samples and EN 15936<sup>[25]</sup> for biowaste samples.

Only samples with a pH between 5 and 9 are 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  $(4 \pm 2)$  °C until performing the test.

When using soil or waste natural samples, dilutions can 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 to define the appropriate percentage of water content according to the level of OM or TOC in this type of samples.

## 5.4 Reagents

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  $< 10 \mu\text{S} \cdot \text{cm}^{-1}$ ).

**5.4.2 Dimethyl sulfoxide solution (DMSO)**,  $\text{C}_2\text{H}_6\text{OS}$ , volume fraction of 4 % in distilled water.

Sterilize the solution by filtration through a polyamide membrane filter having a pore size of 0,2  $\mu\text{m}$  and using a syringe.

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

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**,  $\text{HCl}$  of, e.g.  $1 \text{ mol} \cdot \text{l}^{-1}$ .

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  $(121 \pm 3)$  °C. If stored sterilized (never opened) at  $(4 \pm 2)$  °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 D(+)-glucose, and
- 1,67 g NaCl

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

**5.4.7 Agar (casein-peptone soymeal-peptone) slant**, for a dense cell suspension of *A. globiformis*.

Dissolve 40 g of tryptic soy agar in water (5.4.1) and make up to 1 000 ml with water. After being dissolved, distribute 7 ml of this medium into culture tubes (6.15). The tubes should be filled until allowing the agar to flow just 50 mm below the neck, when the neck is laid over a horizontal 10 ml glass pipette. Seal them with caps and then autoclave for 20 min at  $(121 \pm 3)$  °C. After cooling, the tubes are ready for use.

**5.4.8 Protective medium**, for freeze-drying bacteria.

Dissolve

- 20 g skim milk, and
- 5 g myo-inositol

in water (5.4.1), make up to 100 ml with water and autoclave for 10 min at  $(121 \pm 3)$  °C. After autoclaving and cooling down to  $(80 \pm 2)$  °C, place the medium immediately in an ice bath to avoid caramelization. If stored sterilized (never opened) at  $(4 \pm 2)$  °C in the dark, the solution is stable up to 12 months.

**5.4.9 Phosphate buffer.**

Dissolve

- 8,2 g  $\text{KH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ,
- 13,24 g  $\text{K}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ ,
- 2 g sodium acetate,  $\text{C}_2\text{H}_3\text{NaO}_2$ , and
- 2 g D(+)-glucose

in water (5.4.1) and make up to 1 000 ml with water. Adjust the pH value of the buffer to  $7,0 \pm 0,2$  with diluted hydrochloric acid (5.4.4) or sodium hydroxide solution (5.4.3). Autoclave the medium for 20 min at  $(121 \pm 3)$  °C. If stored at  $(4 \pm 2)$  °C in the dark, the solution is stable for up to 12 months.

**5.4.10 Resazurin dye solution.**

Dissolve  $(45 \pm 1)$  mg of resazurin in 1 000 ml of phosphate buffer (5.4.9). If stored at  $(4 \pm 2)$  °C in the dark, the blue-coloured solution is stable up to one week.

**5.4.11 Benzyltrimethylhexadecylammonium chloride**, reference substance [also called cetalkonium chloride (C16-BAC)],  $\text{CH}_3(\text{CH}_2)_{15}\text{N}(\text{Cl})(\text{CH}_3)_2\text{CH}_2\text{C}_6\text{H}_5$  [CAS Registry Number® (CAS RN)<sup>4)</sup>: 122-18-9].

It is a quaternary ammonium salt often used as an anti-microbial agent. C16-BAC is a homologue of BAC (benzalkonium chloride, a C12 homologue) which is made of a mixture of alkylbenzyltrimethylammonium chlorides, composed mainly of cetalkonium chloride. C16-BAC is moderately soluble in water (between  $2 \text{ g} \cdot \text{l}^{-1}$  and  $85 \text{ g} \cdot \text{l}^{-1}$ ), and whenever needed, the solution can be sonicated to be homogenized. If stored at  $(4 \pm 2) \text{ }^\circ\text{C}$  in the dark, the solution is stable for up to one week. Its mode of action targets mainly gram-negative bacteria by affecting the permeability of their cytoplasmic membrane and leading to cytolysis.<sup>[26]</sup>

**5.4.12 Copper sulfate (II) pentahydrate**, additional reference substance (CAS RN 7758-99-8).

Prepare a stock solution in water (5.4.1) before spiking the quartz sand with its appropriate volume to have a final concentration of  $500 \text{ mg Cu} \cdot \text{kg}^{-1}$  soil dry mass.

**5.4.13 3,5-dichlorophenol (3,5-DCP)**, additional reference substance (CAS RN 591-35-5).

Prepare a stock solution in water (5.4.1) before spiking the quartz sand with its appropriate volume to have a final concentration of  $100 \text{ mg 3,5-DCP} \cdot \text{kg}^{-1}$  soil dry mass.

**5.4.14 Zinc sulfate heptahydrate**, additional reference substance (CAS RN 7446-20-0).

Prepare a stock solution in water (5.4.1) before spiking the quartz sand with its appropriate volume to have a final concentration of  $100 \text{ mg Zn} \cdot \text{kg}^{-1}$  soil dry mass.

## 6 Apparatus

Use laboratory equipment and the following.

**6.1 Autoclave.**

**6.2 Ultrasonic bath.**

**6.3 Sterile bench.**

**6.4 Freezer**,  $-20 \text{ }^\circ\text{C}$  and  $-80 \text{ }^\circ\text{C}$  for storing lyophilizates and the stock cultures.

**6.5 Conical flask**, with cellulose stopper as a culture vessel, nominal capacity of 100 ml (e.g. Reference [27]).

**6.6 Temperature controlled incubator**, capable of being maintained at  $(30 \pm 1) \text{ }^\circ\text{C}$ .

**6.7 Horizontal shaker.**

**6.8 Photometer**, suitable for measuring the optical density of bacterial inoculum at 600 nm.

**6.9 Fluorometer for microplates**, emission at 590 nm, excitation at 535 nm.

NOTE As an example, the fluorometer can have the following features: sensitivity around 5 pM of fluorescein (top and bottom reading), dynamic range of five decades, filter-dependent bandpass, tungsten quartz halogen lamp with a photomultiplier (PMT) detection system.

**6.10 pH-meter.**

4) Chemical Abstracts Service (CAS) Registry Number® is a trademark of the American Chemical Society (ACS). This information is given for the convenience of users of this document 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.

**6.11 Multipette or micropipettes**, 10 000 µl, 1 000 µl, 200 µl, and 20 µl.

**6.12 Tips or combitips**, (suitable for 10 ml) autoclavable.

**6.13 Microplates**, 24 well with lid, transparent and flat bottom.

**6.14 Round-bottom glass bottle and adaptable top filter**, with 0,22 µm pore, if necessary for the lyophilizer.

**6.15 Glass culture tubes with caps**, 16 mm × 160 mm, for agar slant.

**6.16 Screw-cap vials**, resistant to -80 °C, nominal capacity of 1,5 ml or 2 ml.

**6.17 Membrane polyamide filter**, pore size 0,2 µm, diameter 25 mm, and syringe.

**6.18 Water bath.**

**6.19 Lyophilizer.**

**6.20 Sieve**, pore size 2 mm.

## 7 Procedure

### 7.1 Preparation of dilutions

If very toxic samples are being tested, a geometric dilution series can be prepared by mixing different proportions of sample (soil or waste material, see [5.3](#)) with the respective control substrate (see [5.2](#)). Mixture ratios of 100 %, 50 %, 25 %, 12,5 %, 6,25 % and 0 % of sample dry mass are suggested. At the end, adjust the water content as stated in [5.2](#) and [5.3](#).

### 7.2 Reference substances and positive control preparation

A positive control should be performed every time a contact test is run.

C16-BAC (see [5.4.11](#)) should be used as a reference substance in the testing of soil samples. In an interlaboratory test (see Reference [6]), LUFA standard soil type 2.2 [see [5.2.2](#), b)] was spiked with C16-BAC at a concentration of 600 mg kg<sup>-1</sup>. The mean value of the calculated inhibition was 58,2 % (BAC).<sup>[5]</sup>

For preparation of the C16-BAC positive control, spike LUFA standard soil type 2.2 [see [5.2.2](#), b)] with C16-BAC (see [5.4.11](#)) in a concentration of 600 mg · kg<sup>-1</sup> (soil dry mass). Prepare a stock solution by dissolving (600 ± 2) mg of C16-BAC (see [5.4.11](#)) in 100 ml of distilled water. Afterwards, spike 10 g of LUFA standard soil type 2.2 with 1 ml of the stock solution (6 mg · ml<sup>-1</sup>). Mix the soil until the substance is homogeneously distributed. If stored at -20 °C, the prepared positive control substrate is stable for up to six months. If using a freshly made positive control substrate, it should be prepared one or two days before the test start for allowing soil-chemical equilibrium (see [Table 1](#)). Adjust the water content of the substrate (see [5.2](#)).

For the testing of waste materials (or chemicals, see [Annex C](#)), the positive control should also be prepared in the recommended control substrate [i.e. quartz sand; see [5.2.2](#), c)]. Other reference substances should be used, depending on the laboratory resources, or on the type of contaminants in the solid sample (if known) or testing chemicals. For these situations, and in accordance with the interlaboratory test results (see [Annex A](#)), copper (II) sulfate pentahydrate (see [5.4.12](#); at 500 mg Cu · kg<sup>-1</sup>), 3,5-DCP (see [5.4.13](#); at 100 mg 3,5-DCP · kg<sup>-1</sup>) or zinc sulfate heptahydrate (see [5.4.14](#); at 100 mg Zn · kg<sup>-1</sup>) should be used as additional reference substances spiked into quartz sand. Spike this control substrate one or two days before the test start (see [Table 1](#)). As a guidance, the concentrations mentioned cause an average inhibition of *A*.

*globiformis* dehydrogenase activity between 40 % and 100 % for copper sulfate, and between 50 % and 80 % for 3,5-DCP and zinc sulfate.

### 7.3 Contact test procedure

#### 7.3.1 General

One or two days (day -2 or day -1, see [Table 1](#)) before the test start, weigh ( $600 \pm 6$ ) mg of the pre-moistened controls (negative and positive) (see [5.2](#)) and test substrates (see [5.3](#)) into the wells of a 24-well microplate. Use at least four replicates per treatment. Weigh the same amount of each substrate (controls and test) to prepare two (preferably three) replicates for each blank A and B (see [3.4](#), [3.5](#), [Table 1](#)).

- Blank A – This blank sets the remaining dehydrogenase activity measured (as fluorescence) in the different treatments after deactivation, or the own fluorescence of the substrate material (e.g. constrained by the organic matter type). The fluorescence of the blank A (controls and test samples) shall be subtracted from the fluorescence of the respective treatments exposed to the bacteria (see [8.1.1](#)).
- Blank B – This blank indicates the activity level of dehydrogenase in natural samples and the efficiency of the deactivation step by comparing with blank A. Hence, blank B is a useful measurement for data interpretation.

The blank B shall be performed in separate microplates. Store the microplates closed with the lid in the dark at  $(4 \pm 2)$  °C until further testing.

**Table 1 — Overview of the test procedure**

Test day	Step	Blank B	Blank A	Negative control	Positive control	Test sample	
-2 or -1	Preparation of dilutions	—	—	—	—	If needed	
	Moistening of the substrates	x	x	x	x	x	
	Spiking of the substrates	—	—	—	x	x (if chemicals are tested)	
	Uncontaminated material (control) (mg wet mass)	$600 \pm 6$	$600 \pm 6$	$600 \pm 6$	—	—	
	Contaminated or spiked substrate (mg wet mass)	$600 \pm 6$	$600 \pm 6$	—	$600 \pm 6$	$600 \pm 6$	
Store microplates in the dark at 4 °C until the test start (day 0)							
-1	Aeration of samples	—	—	—	—	if anaerobic	
0	Water (ml)	0,6	0,6	0,6	0,6	0,6	
		—	Pasteurization/Deactivation of blank A and test plates <sup>a</sup>				
	Inoculum (ml)	—	—	0,4	0,4	0,4	
	Medium B (ml)	0,4	0,4	—	—	—	
		Two hours of incubation at $(30 \pm 1)$ °C in the dark					
	Resazurin solution (ml)	0,8	0,8	0,8	0,8	0,8	
Measurement of the kinetic (every 15 min) during 1 h							

<sup>a</sup> Test plates are the 24-well microplates containing the negative and positive controls, and the test samples, which are all added with *A. globiformis* inoculum. The blank A and B plates are not added with the inoculum, but only with medium B. The blank B plates are not subjected to the pasteurization/deactivation step.

#### 7.3.2 Aeration

In case of anaerobic soils and waste materials, aerate them one day before the test start (day -1, see [Table 1](#)), by shaking the mixtures ( $600 \text{ mg} \pm 6 \text{ mg}$  of sample wet mass plus 0,6 ml of sterile distilled water) up to 24 h on a horizontal shaker at a suitable frequency, e.g.  $150 \text{ min}^{-1}$ , in order to achieve the first validity criterion

(see [Clause 9](#)). The water shall not be removed after aeration, if no more water is added for the incubation (see [7.3.3](#)).

### 7.3.3 Deactivation

At the test start (day 0, see [Table 1](#)) add 0,6 ml of sterilized distilled water in all replicates and proceed with the deactivation step.

The natural dehydrogenase activity of the substrates should be suppressed as far as possible by pre-treating the samples. This is made by heating (except the blank B microplates) the closed microplates with the test and control (positive and negative) substrates in a water bath at  $(85 \pm 2) ^\circ\text{C}$  for 10 min. Afterwards, cool them down to room temperature for about 15 min in an iced water bath. The heating-cooling process should be repeated once more.

NOTE This step, together with the incubation at  $(30 \pm 1) ^\circ\text{C}$  (see [7.3.5](#)), makes the contact assay inappropriate to test the effect of volatile compounds in natural contaminated or spiked substrates, since they can easily evaporate at room temperature ( $21 ^\circ\text{C} \pm 3 ^\circ\text{C}$ ). Moreover, heating the sample as proposed can cause the degradation of other contaminants. An additional blank (e.g. blank C) that does not undergo deactivation and is exposed to the test organism can be performed to improve data interpretation.

### 7.3.4 Preparation of the inoculum

The inoculum can be prepared a) from a stock culture or b) from the freeze-dried bacteria batch.

- a) The inoculum preparation shall start one day prior to the test start. A 1 ml aliquot of the stock culture of bacteria prepared according to [B.2](#) shall be grown in 50 ml of medium B (see [5.4.6](#)) for 16 h at  $(30 \pm 1) ^\circ\text{C}$  and  $150 \text{ min}^{-1}$ . After that, transfer 1 ml to 50 ml of fresh medium B and incubate 3 h at  $(30 \pm 1) ^\circ\text{C}$  and  $150 \text{ min}^{-1}$  in a horizontal shaker. Adjust the optical density of bacterial inoculum in log-growth phase to  $(0,4 \pm 0,1)$ .
- b) If using freeze-dried bacteria (see [B.3](#)) reconstitute a vial with 0,5 ml of sterilized iced cold water (see [5.4.1](#)) and place the vial for approximately 20 min to 30 min at  $(6 \pm 2) ^\circ\text{C}$ . Transfer the suspension into 20 ml of sterilized medium B (see [5.4.6](#)) previously warmed up to  $(30 \pm 1) ^\circ\text{C}$  to have the inoculum ready to use for the contact test. The preparation of the inoculum by reconstituting freeze-dried bacteria is less time-consuming.

### 7.3.5 Incubation and fluorescence measurement

Add 0,4 ml of inoculum (see [7.3.4](#)) to all replicates, except for the blanks A and B in which 0,4 ml of medium B shall be added instead of the inoculum. Incubate the plates on a horizontal shaker at a speed of  $150 \text{ min}^{-1}$  and a temperature of  $(30 \pm 1) ^\circ\text{C}$  for 2 h in the dark. Afterwards, add 0,8 ml of resazurin solution (see [5.4.10](#)) and measure immediately the fluorescence of the formed product resorufin directly in the microplate [excitation at  $(535 \pm 20) \text{ nm}$ , emission at  $(590 \pm 20) \text{ nm}$ ]. This measurement shall be repeated every 15 min for a period of 1 h. In the interval, the plates shall be incubated again at  $(30 \pm 1) ^\circ\text{C}$  on the horizontal shaker. The increasing of the resorufin product is proportional to the dehydrogenase activity of the test organism.

The pH of the samples shall be measured at least in one replicate of each treatment at the end of the reaction time, in order to confirm pH stability during the contact reaction (see [7.4](#)).

## 7.4 Interferences

The test is not applicable to assess samples or substrates with pH-values (measured in  $\text{CaCl}_2$ ) below 5 (e.g. acid forest soil) and higher than 9.

Anaerobic samples can cause inhibition and shall be aerated up to 24 h before testing. Aerobic samples do not need to be aerated (see [7.3.2](#)).

The natural community of microorganisms in the solid samples can show dehydrogenase activity. To inactivate these microorganisms, the samples are heated at least twice at  $(85 \pm 2) ^\circ\text{C}$  for 10 min (see [7.3.3](#)). In case of waste materials with a negligible microbial community associated, e.g. plastics, the inactivation

step can be skipped. Yet, care should be taken if the waste materials (such as plastics) are not fully aseptic, since the metabolic activity of surface-associated and transient microorganisms can affect the test outcome.

NOTE Blank B can be performed to remove any influence of the samples' background fluorescence from the treatments inoculated with the bacterium. The blank B of substrates presenting no-to-negligible microbial activity usually presents an increase of the average fluorescence by a factor below 1.1 between time 0 min and 60 min.

The type and amount of organic matter in the samples can affect the outcome of the test because of fluorescent light-emitting or light quenching effects. Moreover, the resazurin dye can adsorb to the solid particles or be reduced by the contaminants in the samples, what can also decrease or increase the fluorescence intensities.<sup>[2]</sup> In fact, resazurin is quite dependent on the redox status of the sample. This can be overcome by subtracting the blank A from the respective treatment (see [8.1.1](#)).

Waste materials presenting low density in aqueous phase can float on the liquid surface in the wells and interfere with the fluorescence reading. For such cases, the floating materials can be disregarded by transferring the reaction liquid to new microplate wells before adding the resazurin dye solution ([5.4.10](#)). The same procedure should be performed in the controls and blanks to avoid discrepancies in final results.

## 8 Calculation and expression of results

### 8.1 Calculation

#### 8.1.1 Relative fluorescence

For all measurement periods, the average fluorescence value obtained for each blank A shall be subtracted from the fluorescence value of the respective treatment (controls and samples) exposed to the test organism inoculum. This allows calculating the relative fluorescence of each treatment by rejecting the own fluorescence of the substrates tested. In the control replicates, the absolute value of the relative fluorescence at 0 min should be considered for the calculations.

#### 8.1.2 Determining the percentage of inhibition

Calculate the slope of the relative fluorescence for each replicate and treatment (controls and samples) between 15 min and 45 min, in  $\text{min}^{-1}$ , by means of the linear regression model. Consider only the slope values, which  $R^2 > 0,95$  (computed from the regression adjusted to the three reading points, i.e. at 15 min, 30 min and 45 min), at least in the negative and positive controls.

Calculate the inhibitory effect,  $I$  (as a percentage), for individual replicates of the positive control and samples, using [Formula \(1\)](#):

$$I = 100 - \left[ \frac{S_s}{S_c} \right] \times 100 \quad (1)$$

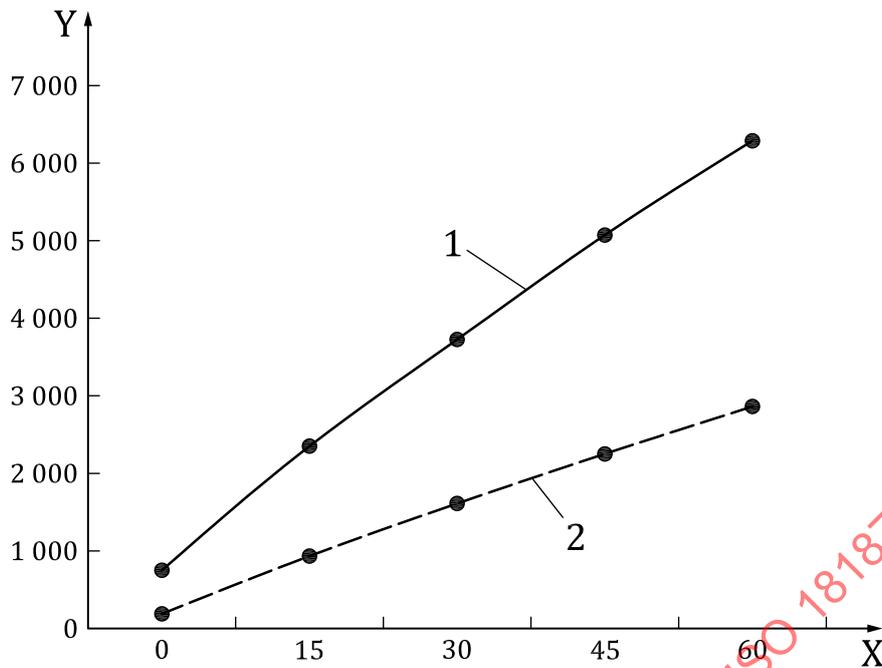
where

$S_s$  is the slope of the relative fluorescence of resorufin in the sample (or positive control) replicate between 15 min and 45 min;

$S_c$  is the average slope of the relative fluorescence of resorufin in the negative control between 15 min and 45 min.

### 8.2 Expression of results

Plot the average ( $\pm$ SD) relative fluorescence of the treatments for each measuring period (0 min, 15 min, 30 min, 45 min, and 60 min) (see [Figure 1](#)).

**Key**

- X reaction time, min  
 Y relative fluorescence  
 1 LUFA standard soil type 2.2  
 2 LUFA standard soil type 2.2 + C16-BAC

**Figure 1 — Example of a measurement of the relative fluorescence versus time in control soil LUFA standard soil type 2.2 and control soil LUFA standard soil type 2.2 spiked with C16-BAC**

Additionally, plot the average ( $\pm$ SD) percentage of dehydrogenase activity inhibition (upon inhibition of resorufin fluorescence) for the positive control and treatments tested, including the dilutions or concentrations (if chemicals are tested according to [Annex C](#)) evaluated. If dose-response curves are obtained indicate the LOEC (i.e. the lowest observed effect concentration) or LID calculated (see [Clause 10](#)).

## 9 Validity of the test

The test is valid if:

- the absolute value of the average relative fluorescence of the negative control increases by a factor of more than 5 from the measuring time 0 min to 60 min;
- the reference substance C16-BAC used in soil testing purposes induces an average inhibition between 30 % and 80 % at 600 mg kg<sup>-1</sup> C16-BAC/LUFA standard soil type 2.2 dry mass; if waste materials (or chemicals, see [Annex C](#)) are tested, other reference substances should be used instead in quartz sand, which should induce an average inhibition between 50 % and 80 % at 100 mg of Zn · kg<sup>-1</sup> quartz sand dry mass, between 50 % and 80 % at 100 mg 3,5-DCP · kg<sup>-1</sup> quartz sand dry mass, or between 40 % and 100 % at 500 mg Cu · kg<sup>-1</sup> quartz sand dry mass;
- the coefficient of variation for the average slope of relative fluorescence in the negative control replicates is less than 15 %.

## 10 Statistical analysis

For performing the statistical analysis either use the % inhibition data or slope values expressed as a percentage of the control slopes.

If the test substrate differs from the control substrate only in contamination, then it can be applied a linear model (e.g. probit analysis, quantal data, or preferably logit, continuous data) for the determination of the EC<sub>x</sub> (where *x* can be 10 %, 20 % or 50 %) point estimates and their respective 95 % confidence intervals (see Reference [28]). These models apply to a dose-response curve.

If the test substrate differs from the control substrate in contamination and also in its intrinsic properties, then a threshold value can be more appropriate to avoid masking the effects of substrate properties over the contamination impacts. Hence, test substrates that induce an inhibition of the *A. globiformis* dehydrogenase activity higher than 30 % relatively to the negative control (e.g. References [4] and [5]) can be considered as toxic for the metabolic functions of the test organism.

In both situations, a one-way analysis of variance (ANOVA) followed by the Tukey or Dunnett's (against the control) multiple comparison tests can be used to ascertain significant differences between the different treatments. This analysis allows the determination of the LOEC value only for the dose-response curves.

NOTE The test can also be designed and evaluated according to the LID (lowest ineffective dilution). However, details of this evaluation approach are still under discussion.

## 11 Test report

The test report shall include the following information:

- a) a reference to this document, i.e. ISO 18187:2024;
- b) the identification of the samples, as well as information on their contamination type (when known), and physical and chemical properties;
- c) the description of control substrate characteristics;
- d) the details on the storage temperature and period of the samples;
- e) the identification of the test substance if a chemical is evaluated;
- f) the date of test performance;
- g) the description of the pre-treatment of samples;
- h) the preparation of chemical solutions and concentrations, and test substrate dilutions;
- i) the detailed description of the followed contact test procedure (e.g. water content, pH value, bacterial inoculum), if amendments to the procedure are done;
- j) a reference to the origin and nature of the test organism, batch number, culture and storage procedures, species purity control;
- k) the date of preparation of the bacterium;
- l) an indication of possible changes in pH values at the end of the contact test;
- m) the inclusion of relevant preliminary results;
- n) the test results expressed as the inhibitory effect of the sample material on the dehydrogenase activity of the test organism relatively to the activity in the negative control;
- o) the evaluation of the inhibitory response in relation to the threshold value (see [Clause 10](#)) and/or through the statistical calculation of the point-estimates (e.g. EC<sub>x</sub> and 95 % confidence limits, LOEC) for the analysed end point; indicate the statistical methods followed;
- p) the concentration-response or dilution-response curves (whenever possible) for inhibition and relative fluorescence data determined to the different treatments.

## Annex A (informative)

### Results on the interlaboratory test

#### A.1 Aim

This annex evaluates the variability of the method described in this document to assess the quality of soil and waste samples, as well as the toxicity of chemicals (metals and organic chemicals). The results of the interlaboratory test were published in Reference [10].

#### A.2 Background

The interlaboratory test on this document started in June 2013 and finished in March 2014. Nine laboratories participated in the interlaboratory test:

- a) INERIS, France;
- b) Welience Agro-Environnement, INRA, France;
- c) LUBW Landesanstalt für Umwelt, Messungen und Naturschutz Baden-Württemberg, Referat Medienübergreifende Umweltbeobachtung, Germany;
- d) ECT-Oekotoxikologie, Germany;
- e) Central Institute for Supervising and Testing in Agriculture (CISTA), Czech Republic;
- f) Research Centre for Toxic Compounds in the Environment (RECETOX), Czech Republic;
- g) University of Aveiro and Centre of Environmental and Marine Studies (CESAM), Portugal;
- h) University of Alcalá, Spain;
- i) CSIRO Land and Water, Australia.

The participants had to select among two testing frameworks (Basics and/or Extended, see [Table A.1](#)) or both of them, depending on their availability. At the end, nine laboratories performed the Basics framework and five finished the Extended one.

#### A.3 Test materials and methodology

The participants were provided with four soil and four waste samples, three control substrates (quartz sand, QS [see [5.2.2](#), c)], LUFA standard natural soil type 2.2 soil [see [5.2.2](#), b)], OECD standard artificial soil [see [5.2.2](#), c)], testing chemicals, resazurin, and vials containing the lyophilized bacterium (*A. globiformis*).

The selection of the samples was made according to previous studies that allowed discriminating between non-contaminated (or low-contaminated) and contaminated samples. Waste and soil samples were processed according to [5.3](#).

Upon arrival, the participants were asked to keep the samples at  $(4 \pm 2)$  °C in the dark and to adjust their water content before starting the assays (see [5.3](#)). A series of five dilutions had to be prepared by the participants, using the respective control substrates (see [5.2](#)). Regarding the chemicals tested (see [Table A.1](#)), also five concentrations were prepared for each one, using quartz sand or artificial OECD soil as substrates.

The *A. globiformis* batches were cultured and lyophilized under the same conditions (see [Annex B](#)) before sending to the participants in individual vials. After reception, the participants were recommended to store

them at -20 °C and proceed with the reconstitution of bacteria [see 7.3.4 b)] whenever a test was started. The performance and sensitivity of lyophilized bacteria was previously tested at the University of Aveiro by running negative and positive controls with different vials. The validity criteria (see Clause 9) were always met, being the  $R^2$  of slope values > 0,95.

**Table A.1 — Substrates and chemicals tested in each framework**

Test substrate/chemical	Control substrate <sup>a</sup>	Framework		Dilution concentration (-/mg kg <sup>-1</sup> )
		Basics	Extended	
Waste no. W1, W2, W3, W4	QS	x	—	G2/G4/G8/G16/G32 G1/G2/G4/G8/G16 <sup>b</sup>
Soil no. S1	LUFA, OECD <sup>d</sup>	x	—	G1
Soils No. S2, S3, S4	LUFA, OECD <sup>d</sup>			G1/G2/G4/G8/G16 <sup>e</sup>
Cu <sup>c</sup>	QS	x	—	500, 100, 50, 10, 5
	OECD	—	x	
3,5-DCP	QS	x	—	300, 200, 100, 50, 25
	OECD	—	x	
Boric acid	QS	—	x	1 000, 750, 500, 250, 125
	OECD	—	x	
Diclofenac sodium salt	QS	—	x	300, 200, 100, 50, 25
	OECD	—	x	
Zinc <sup>c</sup>	QS	—	x	1 000, 500, 100, 50, 10
	OECD	—	x	
C16-BAC (positive control)	LUFA, OECD <sup>d</sup>	x	x	600
<b>Key</b>				
QS quartz sand				
OECD standard artificial soil				
LUFA standard natural soil type 2.2				
<sup>a</sup> Either used as negative controls for preparing dilutions, or as control substrate to be spiked with chemicals.				
<sup>b</sup> Two laboratories performed this range of concentrations, though the first range (i.e. G2/G4/G8/G16/G32) was the suggested one.				
<sup>c</sup> Concentrations in mg Cu or Zn · kg <sup>-1</sup> substrate.				
<sup>d</sup> In this case, the OECD standard artificial soil was only used as a negative control.				
<sup>e</sup> The dilutions of S2, S3 and S4 were prepared with LUFA 2.2 soil.				

#### A.4 Data analysis

The data sets received from the laboratories were thoroughly checked and only accepted if:

- at least one validity criterion defined in this document was met (see Clause 9);
- no significant deviations from the protocol were reported.

The within-laboratory variation (i.e. assay repeatability) of *A. globiformis* response to the negative control substrates (QS, LUFA standard soil type 2.2 and OECD artificial soil) was analysed by the coefficient of variation ( $C_{V,r}$ ) of the slope values obtained for each substrate. Accordingly, the variability of *A. globiformis* response to the reference substance C16-BAC was determined through the  $C_V$  calculated for the average % inhibition of dehydrogenase activity (DHA), either within ( $C_{V,r}$  repeatability) and between ( $C_{V,R}$ , estimation of reproducibility) laboratories.

For the assays performed with test substrates and chemicals, the slope values obtained were expressed as a percentage of the negative control slope, to conduct further statistical analyses. A one-way ANOVA followed

by the Dunnett's test was used to determine the LID or LOEC values for each test substrate/chemical analysed ( $p < 0,05$ ). If the one-way ANOVA assumptions were not met, even after data transformation, a non-parametric ANOVA followed by the Dunn's test was used to determine treatments significantly different from the control ( $p < 0,05$ ). The minimum detected difference (MDD) was computed for the standard deviation of the average response (i.e. % inhibition or slope) corresponding to the LID/LOEC values obtained in each treatment and expressed as a percentage of the respective negative control. The EC20 and EC50 values and respective 95 % confidence limits were derived by fitting the least-squares regression model to the data. The tests in which the ECx or LID/LOEC could be derived were not considered in the statistical analysis.

The variability of the data sets generated for test substrates and chemicals by each laboratory were analysed according to Reference [29]. However, some adaptations had to be made, since this procedure does not take into consideration the testing of concentrations. Besides, it was not possible to calculate the repeatability of the assay by the method in Reference [29] since most laboratories reported only one test result for each substrate/chemical (and concentration ranges); otherwise, it would become too laborious. Thereby, in order to estimate the repeatability of the assay, the logarithmic within-laboratory standard deviation (WLSD) was computed, using the log-transformed 95 % confidence limits of the ECx.[30] The reproducibility of the contact test was analysed by the standard deviation ( $SD_R$ ) of the logarithmic ECx values determined for each test substrate and chemical in the different laboratories.[31] The  $C_{V,R}$  calculated from the arithmetic average and standard deviation of ECx values was used as well to evaluate the reproducibility of the assay. Similarly, the warning limits approach[31] and the min-max factor (obtained from the minimum and maximum values of the ECx) were also determined to complement the statistical evaluation.

### A.5 Evaluation of the results

At least one validity criterion was fulfilled through all the assays performed, but more than 56 % of laboratories met two or three criteria (see Table A.2). The first, second (considering only the C16-BAC reference substance) and third validity criteria (according to their order in Clause 9) were accomplished in 88 %, 72 % and 93 % of the interlaboratory tests performed, respectively. The participants had generally carried out the tests in compliance with the standard procedures, although one laboratory (L9) did not measure the fluorescence at 30 min of reaction time in the soil samples S3 and S4.

**Table A.2 — Summary of the interlaboratory test results on waste and soil samples, and chemical compounds — Test validity, acceptance and sensitivity (MDD)**

Test material/ chemical	Control substrate	≥ 2 validity criteria met (%) <sup>a</sup>	Total no. of tests	No. of tests with outliers <sup>b</sup>	No. of accepted tests	No. of tests for MDD calculation	Mean MDD	SD
W1	QS	89	9	1	8	8	1,2	2,18
W2	QS	89	9	1	8	5	5,4	3,92
W3	QS	78	9	1	8	5	2,9	2,08
W4	QS	78	9	0	9	6	6,8	8,88
<b>Key</b>								
n.d. not determined								
QS quartz sand								
LUF4 standard natural soil type 2.2								
OECD standard artificial soil								
3,5-DCP 3,5-dichlorophenol								
MDD minimum detected difference (%)								
SD standard deviation								
<sup>a</sup> Percentage of valid tests for which at least two validity criteria were met.								
<sup>b</sup> Computed in accordance with ISO 5725-2[29].								

Table A.2 (continued)

Test material/ chemical	Control substrate	≥ 2 validity criteria met (%) <sup>a</sup>	Total no. of tests	No. of tests with outliers <sup>b</sup>	No. of accepted tests	No. of tests for MDD calculation	Mean MDD	SD
S1	LUFA	89	9	0	9	0	n.d.	n.d.
S2	LUFA	89	9	0	9	6	3,8	1,78
S3	LUFA	78	9	1	8	6	6,5	3,87
S4	LUFA	89	9	1	8	4	7,1	1,23
Copper sulfate	QS	100	9	1	8	7	3,5	1,79
	OECD	80	5	0	5	5	13,7	13,85
3,5-DCP	QS	89	9	1	8	8	2,2	0,99
	OECD	80	5	0	5	5	6,3	3,64
Boric acid	QS	80	5	0	5	3	6,8	2,54
	OECD	80	5	0	5	0	n.d.	n.d.
Zinc sulfate	QS	80	5	0	5	4	6,6	4,92
	OECD	80	5	0	5	3	9,5	4,80
Diclofenac sodium salt	QS	80	5	0	5	4	3,2	1,80
	OECD	80	5	0	5	3	3,5	0,18

**Key**  
n.d. not determined  
QS quartz sand  
LUFA standard natural soil type 2.2  
OECD standard artificial soil  
3,5-DCP 3,5-dichlorophenol  
MDD minimum detected difference (%)  
SD standard deviation  
<sup>a</sup> Percentage of valid tests for which at least two validity criteria were met.  
<sup>b</sup> Computed in accordance with ISO 5725-2 [29].

The  $C_{v,r}$ 's for the slopes of negative control substrates were generally < 30 % (see Table A.3), thereby strengthening the low variability of DHA within laboratories.

Table A.3 — Within-laboratory variability ( $C_{v,r}$ ) calculated for slope values obtained to three control substrates in nine laboratories (L1 to L9)

$C_{v,r}$ of slope values	Participant laboratories %								
	L1	L2	L3	L4	L5	L6	L7	L8	L9
<b>QS</b>	10	10	3	25	13	98	12	18	9
No. of tests	9	7	3	8	2	7	4	6	3
<b>LUFA</b>	11	10	12	3	19	92	11	12	23
No. of tests	5	2	2	2	2	2	2	2	2
<b>OECD</b>	12	19	19	13	17	38	12	24	25
No. of tests	6	6	2	5	2	5	2	6	2

**Key**  
QS quartz sand (see 5.2.3)  
LUFA standard natural soil type 2.2 [see 5.2.2 b)]  
OECD standard artificial soil [see 5.2.2 c)]

Except for L6, all laboratories attained inhibitions of *A. globiformis* DHA  $\geq 30$  % and  $< 80$  % for the reference substance (C16-BAC) (see [Table A.4](#)), thereby validating or reinforcing the inhibition range established in this document (see [Clause 9](#)). Although in laboratory L3, the  $C_{V,r}$  was  $> 30$  %, the overall repeatability ( $C_{V,r}$ ) and reproducibility ( $C_{V,R}$ ) of the contact assay regarding C16-BAC was good.

**Table A.4 — Variability calculated for the percentage of dehydrogenase activity inhibition under the reference substance C16-BAC in nine laboratories (L1 to L9)**

	L1	L2	L3	L4	L5	L6	L7	L8	L9	
Mean % inhibition	50,2	30,0	30,5	65,7	53,9	< 0,0	57,0	50,0	43,3	$C_{V,R}$ (%)
No. of tests	5	1	3	2	2	2	2	2	2	26
$C_{V,r}$ (%)	8	22	36	8	13	n.a.	23	22 <sup>a</sup>	29	
<b>Key</b>										
$C_{V,r}$ intralaboratory coefficient of variation (estimation of repeatability)										
$C_{V,R}$ interlaboratory coefficient of variation (estimation of reproducibility)										
n.a. not applicable										

At least eight valid data sets were obtained for waste and soil samples, while for chemicals, there were between five and nine test results (see [Table A.2](#)). Only 5 % of outliers were identified in the whole interlaboratory test results (seven in 130 tests), thereby indicating data consistency. The MDD varied between 1,2 % and 6,8 % for wastes, while for soils, it was between 3,8 % and 7,1 %. The EC20 and EC50 values were always within the respective warning limits calculated for each test, as well as the min-max factors (should be  $< 4,0$ ) and WLSD were generally acceptable [31]. The latter measure provides an estimation of test repeatability, which in this case can be considered acceptable (see [Table A.5](#)).

In what concerns the variability between laboratories estimated from EC20 mean values, seven tests presented a  $C_{V,R} \leq 30$  % (limit recommended by Reference [31]), five had  $31$  %  $< C_{V,R} \leq 65$  % and two were above 65 % variation. For the EC50 mean values, seven tests had  $C_{V,R} \leq 30$  %, and five had  $31$  %  $< C_{V,R} \leq 65$  % (see [Table A.5](#)). An equivalent trend was obtained for the reproducibility standard deviation ( $SD_R$ ), thereby indicating a satisfactory variability between laboratories.

Therefore, the outcome of the interlaboratory test allows concluding that this solid contact assay is valid to assess the quality of soils and wastes with good repeatability and reproducibility. For the tests with chemicals, however, the higher variability between laboratories (particularly the EC20 mean values) observed for tests with Cu, 3,5-DCP and Zn is mainly associated with one of the control substrates used – the standard artificial soil (OECD). This can in part be due to the influence of its constituents (e.g. peat particles) on fluorescence readings. Besides, the use of this substrate had substantially and consistently decreased the average toxicity of the chemical compounds tested. A note was added to this document to inform about the effect of this artificial soil on test outcomes; hence helping with the selection of the control substrate according to the objectives of each study, which can integrate the use of the contact test. The tests performed with spiked quartz sand evidenced higher reproducibility. Moreover, the possibility of including copper sulfate, 3,5-DCP or zinc sulfate as reference substances spiked into quartz sand in this document seems to be applicable.

Table A.5 — Summary of the interlaboratory test results on waste and soil samples, and chemical compounds — Evaluation of test variability based on ECx point estimates

Test material/ chemical	Control substrate	EC20						EC50									
		No. of tests	Mean EC20	LWL	UWL	WLS D (min-max range)	C <sub>V,R</sub> <sup>d</sup> (%)	S <sub>R</sub>	Min-max factor	No. of tests	Mean EC50	LWL	UWL	WLS D (min-max range)	C <sub>V,R</sub> <sup>d</sup> (%)	S <sub>R</sub>	Min-max factor
W1	QS	0	< 1,6/3,1/6,3 <sup>a</sup>	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	< 1,6/3,1/6,3 <sup>a</sup>	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
W2	QS	4	24,8	15,92	37,32	0,06 to 0,25	19	0,09	1,6	0	> 50,0/100,0 <sup>b</sup>	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
W3	QS	6	5,5	2,68	10,15	0,002 to 0,05	33	0,14	2,2	7	6,9	3,37	12,85	0,002 to 0,03	30	0,14	2,5
W4	QS	5	37,9	20,85	64,54	0,02 to 0,06	30	0,12	1,9	3	62,1	27,81	125,70	0,008 to 0,03	40	0,16	2,0
S1 <sup>c</sup>	LUFA	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S2	LUFA	5	69,5	38,08	119,40	0,03 to 0,27	26	0,12	2,1	0	> 100,0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S3	LUFA	6	40,5	10,98	114,16	0,04 to 0,14	52	0,25	4,6	6	67,4	35,41	119,17	0,02 to 0,07	29	0,13	2,2
S4	LUFA	3	32,5	12,37	74,16	0,04 to 0,11	47	0,19	2,4	7	46,9	11,01	144,59	0,02 to 0,50	55	0,28	4,5
Copper sulfate	QS	6	69,5	39,76	114,76	0,02 to 0,03	25	0,12	2,0	6	109,8	53,11	208,06	0,01 to 0,02	29	0,15	2,5
	OECD	5	192,7	25,58	831,85	0,06 to 0,12	77	0,38	8,6	2	237,7	170,80	326,55	0,04 to 0,16	16	0,07	1,3
3,5-DCP	QS	6	39,6	20,51	70,80	0,02 to 0,10	30	0,13	2,1	7	76,2	36,57	143,64	0,01 to 0,06	35	0,15	2,6
	OECD	5	142,2	26,82	521,63	0,04 to 0,09	57	0,32	5,6	4	238,8	135,89	398,07	0,02 to 0,04	26	0,12	1,7

**Key**  
n.d. not determined  
QS quartz sand  
LUFA standard natural soil type 2.2  
OECD standard artificial soil  
3,5-DCP 3,5-dichlorophenol  
No. of tests number of tests included in the statistical analysis for which an ECx could be calculated  
LWL lower warning limit  
UWL upper warning limit  
WLS D logarithmic within-laboratory standard deviation (minimum-maximum range of values)  
S<sub>R</sub> warning limit onefold standard deviation [21]  
Min-max factor factor between minimum and maximum ECx values  
The ECx values, LWL, UWL and SD are expressed as per cent of test substrate and soil sample, or as mg kg<sup>-1</sup> for chemicals.  
<sup>a</sup> The minimum dilution tested by some laboratories was G64 (= 1,6 % of test substrate), G32 (= 3,1 % of test substrate) or G16 (= 6,3 % of test substrate).  
<sup>b</sup> The maximum dilution tested by two laboratories was G1 (= 100,0 % of test substrate), while for the others it was G2 (= 50,0 % of test substrate).  
<sup>c</sup> Only the 100 % of S1 was tested, thereby no point estimates were computed for this sample.  
<sup>d</sup> Coefficient of variation that indicates the variability between laboratories (i.e. estimation of assay reproducibility).

Table A.5 (continued)

Test material/ chemical	Control substrate	EC20						EC50									
		No. of tests	Mean EC20	LWL	UWL	WLSd (min-max range)	$C_{v,R}^d$ (%)	$S_R$	Min-max factor	No. of tests	Mean EC50	LWL	UWL	WLSd (min-max range)	$C_{v,R}^d$ (%)	$S_R$	Min-max factor
Boric acid	QS	0	> 1 000,0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	> 1 000,0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	OECD	0	> 1 000,0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	> 1 000,0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Zinc sulfate	QS	3	30,0	23,82	37,47	0,04 to 0,18	12	0,05	1,3	3	100,6	70,92	139,98	0,02 to 0,09	17	0,07	1,4
	OECD	3	388,3	109,73	1 124,32	0,04 to 0,27	48	0,25	3,0	2	745,4	322,25	1 593,72	0,02 to 0,09	39	0,17	1,8
Diclofenac sodium salt	QS	4	90,0	12,38	375,12	0,04 to 0,10	88	0,37	7,4	3	240,3	109,28	484,95	0,02 to 0,07	33	0,16	2,0
	OECD	3	110,7	65,67	179,18	0,03 to 0,06	23	0,11	1,6	3	219,9	151,65	293,59	0,02 to 0,03	16	0,07	1,3

**Key**

n.d. not determined

QS quartz sand

LUFA standard natural soil type 2.2

OECD standard artificial soil

3,5-DCP 3,5-dichlorophenol

No. of tests number of tests included in the statistical analysis for which an ECx could be calculated

LWL lower warning limit

UWL upper warning limit

WLSd logarithmic within-laboratory standard deviation (minimum-maximum range of values)

$S_R$  warning limit onefold standard deviation [21]

Min-max factor factor between minimum and maximum ECx values

The ECx values, LWL, UWL and SD are expressed as per cent of test substrate and soil sample, or as mg kg<sup>-1</sup> for chemicals.

<sup>a</sup> The minimum dilution tested by some laboratories was G64 (= 1,6 % of test substrate), G32 (= 3,1 % of test substrate) or G16 (= 6,3 % of test substrate).

<sup>b</sup> The maximum dilution tested by two laboratories was G1 (= 100,0 % of test substrate), while for the others it was G2 (= 50,0 % of test substrate).

<sup>c</sup> Only the 100 % of S1 was tested, thereby no point estimates were computed for this sample.

<sup>d</sup> Coefficient of variation that indicates the variability between laboratories (i.e. estimation of assay reproducibility).