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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

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation 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 I — 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.

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

— 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 ± 3) °C. If stored sterilized (never opened) at (4 ± 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 ± 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 ± 3) °C. After autoclaving and cooling down to (80 ± 2) °C, place the medium immediately in an ice bath to avoid caramelization. If stored sterilized (never opened) at (4 ± 2) °C in the dark, the solution is stable up to 12 months.

5.4.9 Phosphate buffer.

Dissolve

— 8,2 g $KH_2PO_4 \cdot H_2O$,

— 13,24 g $K_2HPO_4 \cdot H_2O$,

— 2 g sodium acetate, $C_2H_3NaO_2$, 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 ± 0,2 with diluted hydrochloric acid (5.4.4) or sodium hydroxide solution (5.4.3). Autoclave the medium for 20 min at (121 ± 3) °C. If stored at (4 ± 2) °C in the dark, the solution is stable for up to 12 months.

5.4.10 Resazurin dye solution.

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

5.4.11 Benzyldimethylhexadecylammonium chloride, reference substance [also called cetalkonium chloride (C16-BAC)], $CH_3(CH_2)15N(Cl)(CH_3)2CH_2C_6H_5$ (CAS no.: 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 alkylbenzyldimethyl-ammonium chlorides, composed mainly of cetalkonium chloride. C16-BAC is moderately soluble in water (between 2 g l^{-1} and 85 g l^{-1}), and whenever needed, the solution can be sonicated to be homogenized. If stored at (4 ± 2) °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.[19]

5.4.12 Copper sulfate (II) pentahydrate, additional reference substance (CAS no. 7758-99-8).

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

5.4.13 3,5-dichlorophenol (3,5-DCP), additional reference substance (CAS no. 591-35-5).

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

5.4.14 Zinc sulfate heptahydrate, additional reference substance (CAS no. 7446-20-0).

Prepare a stock solution in water (5.4.1) before spiking the quartz sand with its appropriate volume as to have a final concentration of 100 mg Zn 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 °C and -80 °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 [1]).

6.6 Temperature controlled incubator, capable of being maintained at (30 ± 1) °C.

6.7 Horizontal shaker.

6.8 Photometer, suitable for measuring the optical density at 600 nm.

6.9 Fluorimeter for microplates, emission at 590 nm, excitation at 535 nm.

NOTE As an example, the fluorimeter 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.

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 may 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 substance and positive control preparation

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

C16-BAC (see 5.4.11) is recommended as a reference substance. In a round robin test (see Reference [7]), LUFA standard soil type 2.2 [see 5.2.2, b)] was spiked with C16-BAC at a concentration of 600 mg kg^{-1}. The mean value of the calculated inhibition was 58,2 % (BAC).[14]

For preparation of the positive control, spike LUFA standard soil type 2.2 (see 5.2.2) with C16-BAC (see 5.4.11) in a concentration of 600 mg kg^{-1} (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^{-1}). 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).

Besides using LUFA standard soil type 2.2, the positive control should also be prepared for the control substrate selected according to the type of sample being tested (see 5.2).

NOTE If another reference substance is needed depending on laboratory resources or on the type of contaminants in the solid sample (if known) or testing chemicals, and in accordance with the ring test results (see Annex A), it is suggested to use copper (II) sulfate pentahydrate (see 5.4.12; at 500 mg Cu kg^{-1}), 3,5-DCP (see 5.4.13; at 100 mg 3,5-DCP kg^{-1}) or zinc sulfate heptahydrate (see 5.4.14; at 100 mg Zn kg^{-1}) as additional reference substances spiked into quartz sand. Spike this control soil 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 ± 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. 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 ± 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 ± 6	600 ± 6	600 ± 6	—	—
	Contaminated or spiked substrate (mg wet mass)	600 ± 6	600 ± 6	—	600 ± 6	600 ± 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[a]			
	Inoculum (ml)	—	—	0,4	0,4	0,4
	Medium B (ml)	0,4	0,4	—	—	—
		Two hours of incubation at (30 ± 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				

[a] 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 mg ± 6 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 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 the closed microplates (except the ones for blank B) with the test and control (positive and negative) substrates in a water bath at (85 ± 2) °C for 10 min. Afterwards, cool them down to room temperature for about 15 min in iced water bath. The heating-cooling process should be repeated once more.

NOTE This step, together with the incubation at (30 ± 1) °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 °C ± 3 °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. However, further testing is needed.

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 A.2 shall be grown in 50 ml of medium B (see 5.4.6) for 16 h at (30 ± 1) °C and 150 min^{-1}. After that, transfer 1 ml to 50 ml of fresh medium B and incubate 3 h at (30 ± 1) °C and 150 min^{-1} in a horizontal shaker. Adjust the optical density of the prepared inoculum in log-growth phase to 0,4 ± 0,1.

b) If using freeze-dried bacteria (see A.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 ± 2) °C. Transfer the suspension into 20 ml of sterilized medium B (see 5.4.6) previously warmed up to (30 ± 1) °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 shall be added 0,4 ml of medium B instead of the inoculum. Incubate the plates on a horizontal shaker at a speed of 150 min^{-1} and a temperature of (30 ± 1) °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 ± 20) nm, emission at (590 ± 20) 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 ± 1) °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/substrates with pH-values (measured in $CaCl_2$) 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 to (85 ± 2) °C for 10 min (see 7.3.3).

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 may also decrease or increase the fluorescence intensities.[6] 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).

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.

8.1.2 Determining the percentage of inhibition

Calculate the slope of the relative fluorescence for each replicate and treatment (controls and samples) using Formula (1):

$$S = \left[\frac{f_2 - f_1}{t_2 - t_1}\right] \quad (1)$$

where

S is the slope of the relative fluorescence (f_1 and f_2) of resorufin in the treatment between 15 min (t_1) and 45 min (t_2), in min^{-1}. Consider only the slope values, which $R^2 > 0{,}95$, 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 (2):

$$I = 100 - \left[\frac{S_s}{S_c} \times 100\right] \quad (2)$$

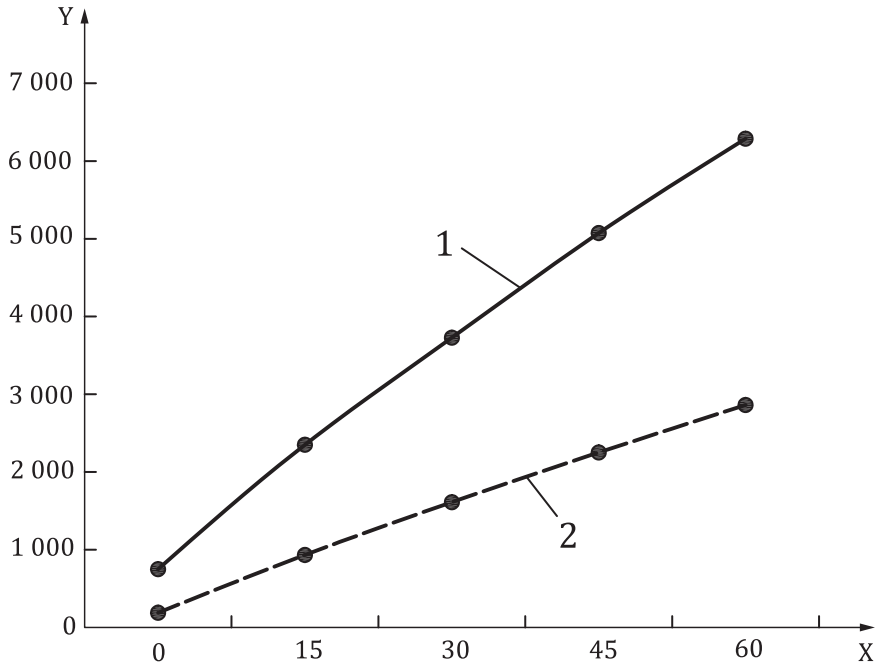
where

S_s is the slope of the relative fluorescence of resorufin in the sample 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 (±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

1 LUFA standard soil type 2.2

2 LUFA standard soil type 2.2 + C16-BAC

X reaction time, min

Y relative fluorescence

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 (±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 B) evaluated. If dose-response curves are obtained indicate the LOEC 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 induces an average inhibition between 30 % and 80 % at 600 mg kg^{-1} C16-BAC/Lufa standard soil type 2.2 soil dry mass, and

— 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 % 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 (e.g. probit analysis, quantal data, or preferably logit, continuous data) for the determination

of the EC*x* (where *x* can be 10 %, 20 % or 50 %) point estimates and their respective 95 %-confidence intervals (see Reference [5]). 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 effects of substrate properties over contamination impacts. Hence, test substrates that induce an inhibition of the dehydrogenase activity higher than 30 % relatively to the negative control (e.g. Reference [14]) 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 may be used to ascertain significant differences between the different treatments. This analysis allows the determination of the LOEC value (i.e. the lowest observed effect concentration) only for the dose-response curves.

NOTE 1 More work is being performed in order to derive a more feasible threshold value to different types of contaminated substrates (see e.g. Reference [12]).

NOTE 2 The test could also be designed and evaluated according to a 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 International Standard, i.e. ISO 18187;

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 bacteria;

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 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 10) and/or through the statistical calculation of the point-estimates (e.g. EC*x* and 95 % confidence limits, LOEC) for the analysed endpoint; 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 ring test

A.1 Aim

Evaluate the variability of the method described in this International Standard to assess the quality of soil and waste samples, as well as the toxicity of chemicals (metals and organic chemicals).

A.2 Background

The ring test on this International Standard started in June 2013 and finished in March 2014. Nine laboratories participated in the interlaboratorial test: a) INERIS, France; b) Welience Agro-Environement, 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 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 soil type 2.2 soil [see 5.2.2, b)], OECD artificial soil [see 5.2.2, c)], testing chemicals, resazurin, and vials containing lyophilized bacteria (*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 °C in the dark, and 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 lyophilised 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 lyophilised 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[a]	Framework		Dilution concentration (-/mg kg^{-1})
		Basics	Extended	
Waste no. W1, W2, W3, W4	QS	x	—	G2/G4/G8/G16/G32 G1/G2/G4/G8/G16[b]
Soil no. S1	Lufa, OECD[d]	x	—	G1
Soils No. S2, S3, S4	Lufa, OECD[d]			G1/G2/G4/G8/G16[e]
Cu (as $CuSO_4 \cdot 5H_2O$)[c]	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 (as $ZnSO_4 \cdot 7H_2O$)[c]	QS	—	x	1 000, 500, 100, 50, 10
	OECD	—	x	
C16-BAC (positive control)	Lufa, OECD[d]	x	x	600

QS — quartz sand; OECD — artificial soil; Lufa — standard soil type 2.2.

[a] Either used as negative controls for preparing dilutions, or as control substrate to be spiked with chemicals.

[b] Two laboratories performed this range of concentrations, though the first range was the suggested one.

[c] Concentrations in mg Cu or Zn kg^{-1} substrate; not in mg $CuSO_4 \cdot 5H_2O$ or $ZnSO_4 \cdot 7H_2O$ kg^{-1} substrate.

[d] In this case, the OECD artificial soil will be only used as a negative control.

[e] The dilutions of S2, S3 and S4 were prepared with Lufa 2.2 soil.

A.4 Data analysis

The datasets received from the laboratories were thoroughly checked and only accepted if: a) at least one validity criterion defined in this International Standard was met (see Clause 9); b) 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 soils) was analysed by the coefficient of variation (CV_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 CV calculated for the average % inhibition of dehydrogenase activity (DHA), either within (CV_r, repeatability) and between (CV_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, as to conduct further statistical analysis. A one-way ANOVA followed by the Dunnett's test was used to determine the LID (see 3.20) 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 EC*x* or LID/LOEC could not be derived were not considered in the statistical analysis.

The variability of the datasets generated for test substrates and chemicals by each laboratory were analysed according to Reference [2]. 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 [2] 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 EC*x* (see 3.10).[13] The reproducibility of the contact test was analysed by the standard deviation (SD_R) of the logarithmic EC*x* values determined for each test substrate and chemical in the different laboratories.[8] The CV_R calculated from the arithmetic average and standard deviation of EC*x* values was used as well to evaluate the reproducibility of the assay. Similarly, the warning limits approach (8) and the min-max factor (obtained from the minimum and maximum values of the EC*x*) 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 could meet two or three criteria (see Table A.4). The first, second and third validity criteria (according to their order in Clause 9) were accomplished in 88 %, 72 % and 93 % of the interlaboratorial 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.

The CV_r's for the slopes of negative control substrates were generally <30 % (see Table A.2), thereby strengthening the low variability of DHA within laboratories.

Table A.2 — Within-laboratory variability (CV_r) calculated for slope values obtained to three control substrates in nine laboratories (L1 to L9)

CV_r of slope values	Participant laboratories %								
	L1	**L2**	**L3**	**L4**	**L5**	**L6**	**L7**	**L8**	**L9**
QS	10	10	3	25	13	98	12	18	9
No. of tests	9	7	3	8	2	7	4	6	3
LUFA	11	10	12	3	19	92	11	12	23
No. of tests	5	2	2	2	2	2	2	2	2
OECD	12	19	19	13	17	38	12	24	25
No. of tests	6	6	2	5	2	5	2	6	2
QS — quartz sand (see 5.2.3); OECD — artificial soil [see 5.2.2 c)]; LUFA standard soil type 2.2 [see 5.2.2 b)].									

Except for L6, all laboratories attained inhibitions of *A. globiformis* DHA ≥30 % and <80 % for the reference substance (C16-BAC) (see Table A.3), thereby validating or reinforcing the inhibition range established in this International Standard (see Clause 9). Although in laboratory L3, the CV_r was >30 %, the overall repeatability (CV_r) and reproducibility (CV_R) of the contact assay regarding C16-BAC was good.

Table A.3 — 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	CV_R (%)
No. of tests	5	1	3	2	2	2	2	2	2	26
CV_r (%)	8	22	36	8	13	n.a.	23	22[a]	29	

CV_r — within-laboratory coefficient of variation (estimation of repeatability); CV_R — between-laboratory coefficient of variation (estimation of reproducibility); n.a. — not applicable.

At least eight valid datasets were obtained for waste and soil samples, whilst for chemicals, there were between five and nine test results (see Table A.4). Only 5 % of outliers were identified in the whole ring 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.[19] 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 $CV_R \leq 30$ % (limit recommended by Reference [21]), five had 31 % < $CV_R \leq 65$ % and two were above 65 % variation. For the EC50 mean values, seven tests had $CV_R \leq 30$ %, and five had 31 % < $CV_R \leq 65$ % (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 ring 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 artificial soil (OECD). This could 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 International Standard as to inform about the effect of this artificial soil on test outcomes; hence helping with the selection of control substrate according to the objectives of each study, which might 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 International Standard seems to be applicable.

Table A.4 — Summary of the ring 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 (%)[a]	Total no. of tests	No. of tests with outliers[b]	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
S1	LUFA	89	9	0	9	0	n.d.	n.d.
S2	LUFA	89	9	0	9	6	3,8	1,78

n.d. Not determined.

QS — quartz sand; LUFA standard soil type 2.2; OECD — artificial soil;

3,5-DCP — 3,5-dichlorophenol; MDD — minimum detected difference (%); SD — standard deviation.

[a] Percentage of valid tests for which at least two validity criteria were met.

[b] Computed in accordance with ISO 5725-2.

Table A.4 *(continued)*

Test material/ chemical	Control substrate	≥2 validity criteria met (%)[a]	Total no. of tests	No. of tests with outliers[b]	No. of accepted tests	No. of tests for MDD calculation	Mean MDD	SD
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

n.d. Not determined.

QS — quartz sand; LUFA standard soil type 2.2; OECD — artificial soil;

3,5-DCP — 3,5-dichlorophenol; MDD — minimum detected difference (%); SD — standard deviation.

[a] Percentage of valid tests for which at least two validity criteria were met.

[b] Computed in accordance with ISO 5725-2.

Table A.5 — Summary of the ring test results on waste and soil samples, and chemical compounds — Evaluation of test variability based on EC*x* point estimates

Test material/ chemical	Control substrate	EC20								EC50							
		No. of tests	Mean EC20	LWL	UWL	WLSD (min-max range)	CV_R[d] (%)	S_R	Min-max factor	No. of tests	Mean EC50	LWL	UWL	WLSD (min-max range)	CV_R[d] (%)	S_R	Min-max factor
W1	QS	0	<1,6/3,1/6,3[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	<1,6/3,1/6,3[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
W2	QS	4	24,8	15,92	37,32	0,06-0,25	19	0,09	1,6	0	>50,0/100,0[b]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
W3	QS	6	5,5	2,68	10,15	0,002-0,05	33	0,14	2,2	7	6,9	3,37	12,85	0,002-0,03	30	0,14	2,5
W4	QS	5	37,9	20,85	64,54	0,02-0,06	30	0,12	1,9	3	62,1	27,81	125,70	0,008-0,03	40	0,16	2,0
S1[c]	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	0,03-0,27	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	0,04-0,14	52	0,25	4,6	6	67,4	35,41	119,17	0,02-0,07	29	0,13	2,2
S4	LUFA	3	32,5	12,37	74,16	0,04-0,11	47	0,19	2,4	7	46,9	11,01	144,59	0,02-0,50	55	0,28	4,5
Copper sulfate	QS	6	69,5	39,76	114,76	0,02-0,03	25	0,12	2,0	6	109,8	53,11	208,06	0,01-0,02	29	0,15	2,5
	OECD	5	192,7	25,58	831,85	0,06-0,12	77	0,38	8,6	2	237,7	170,80	326,55	0,04-0,16	16	0,07	1,3
3,5-DCP	QS	6	39,6	20,51	70,80	0,02-0,10	30	0,13	2,1	7	76,2	36,57	143,64	0,01-0,06	35	0,15	2,6
	OECD	5	142,2	26,82	521,63	0,04-0,09	57	0,32	5,6	4	238,8	135,89	398,07	0,02-0,04	26	0,12	1,7
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	0,04-0,18	12	0,05	1,3	3	100,6	70,92	139,98	0,02-0,09	17	0,07	1,4
	OECD	3	388,3	109,73	1 124,32	0,04-0,27	48	0,25	3,0	2	745,4	322,25	1 593,72	0,02-0,09	39	0,17	1,8
Diclofenac sodium salt	QS	4	90,0	12,38	375,12	0,04-0,10	88	0,37	7,4	3	240,3	109,28	484,95	0,02-0,07	33	0,16	2,0
	OECD	3	110,7	65,67	179,18	0,03-0,06	23	0,11	1,6	3	219,9	151,65	293,59	0,02-0,03	16	0,07	1,3

n.d. Not determined.

QS — quartz sand; LUFA standard soil type 2.2; OECD — artificial soil; 3,5-DCP — 3,5-dichlorophenol; No. of tests — number of tests included in the statistical analysis for which an EC*x* 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;[8] Min-max factor — factor between minimum and maximum EC*x* values.

The EC*x* values, LWL, UWL and SD are expressed as per cent of test substrate and soil sample, or as mg kg^{-1} for chemicals.

[a] 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).

[b] 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).

[c] Only the 100 % of S1 was tested, thereby no point estimates were computed for this sample.

[d] Coefficient of variation that indicates the variability between laboratories (i.e. estimation of assay reproducibility).

Annex B
(informative)

Preparation of test organisms

B.1 General

The following steps shall be done under aseptic conditions in a sterile bench using a burning flame and sterilized material and reagents.

B.2 Stock culturing

The soil bacteria *A. globiformis* (strain number ATCC 8010) can be obtained from the German Collection of Microrganisms and Cell Cultures [Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ; No. 20124 at http://www.dsmz.de/catalogues/)] or from the American ARS Culture Collection, NCAUR.[3)] It shall be reconstituted according to the instructions provided by DSMZ or according to other culture collections supplier. Afterwards, the bacteria is grown in 50 ml of medium A (see 5.4.5) and incubated for 8 h at (30 ± 1) °C on a horizontal shaker (frequency: 150 min^{-1}). Transfer 1 ml of this culture into 50 ml of fresh medium A (see 5.4.5) and incubate for 16 h at (30 ± 1) °C and 100 min^{-1}. Determine the turbidity of the culture at 600 nm and adjust to an optical density of (0,4 ± 0,1) (either by increasing the incubation period or diluting the suspension with medium A). Store the sterile stock culture in 1 ml aliquots with DMSO (4 % volume fraction) and freeze them at -80 °C.

B.3 Freeze-drying of bacteria

Freeze-drying is a convenient method for preservation and long-term storage of microorganisms.[18] Reconstituted freeze-dried bacteria are immediately active and ready for use, allowing results within 6 h in this test procedure. In order to protect the cells against freezing and drying injuries, some protective agents like skim milk and myo-inositol are used.[7][11]

In the first step, freeze-dry the sterile protective medium (see 5.4.8): add 0,5 ml of protective medium in several sterile 1 ml vials and deep-freeze them at -80 °C for at least 15 min. After, introduce the open vials in a sterilized apparatus [e.g. glass bottle (see 6.14) with a screwed neck adapted to a sterile top filter of 0,22 µm pore diameter] that allows water sublimation under sterile conditions during the lyophilisation. Take the whole set to -80 °C during 15 min to ensure that the protective medium is completely frozen and then place the apparatus with the vials in the lyophiliser for 24 h (depending on the type of equipment and functioning options) at a temperature of (-60 to -80) °C. At the end, close the vials and store the aliquots of freeze-dried protective medium at -80 °C until further use.

For the second step of lyophilisation, prepare an overnight culture by taking 1 ml of the unfrozen stock culture and adding 250 µl to 50 ml of medium B (see 5.4.6). Incubate the suspension for 14 h to 16 h at (30 ± 1) °C on a horizontal shaker (frequency: 150 min^{-1}). Determine the turbidity of the culture at 600 nm and adjust to an optical density of 0,2 (either by increasing the incubation period or diluting the suspension with medium B). Incubate for two more hours until the density is 0,4.

Inoculate the agar slants (see 5.4.7) (e.g. 10 tubes) with the fresh culture prepared and incubate the agar slant for 24 h at (30 ± 1) °C. Afterwards, prepare a thick cell suspension (at least 10^8 cells per ml or

3) 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.

absorbance >1,2 at 600 nm) by elutriating the culture of one agar slant with 10 ml medium B and then transfer to the following one. Elutriate the second agar slant and transfer again to the third one. Repeat this procedure for all inoculated agar slants.

If the freeze-dried protective medium is not already stored at -80 °C, it should be placed for 1 h to 2 h at -80 °C. After that, add 0,5 ml of cell suspension to each vial containing the protective medium with care as to prevent touching the sides of the vial. The vials shall be quickly placed in a deep-freezer (-80 °C) for 2 h ± 30 min. Open the vials in a sterile bench and introduce them in the apparatus mentioned before. Dry the protective medium with the suspension in the lyophiliser for about 24 h at (-60 to -80) °C. The freeze-dried cultures can be stored for at least 2 y at -20 °C. If the optical density of the lyophilisates is measured at 600 nm after reconstitution, it should be between 0,2 to 0,4, against a blank constituted by the same amount of protective medium.

B.4 Quality control

To prove the purity of the cultured bacteria, it is recommended to use microbiological identification strips that can specifically identify *A. globiformis*. This step may be applied to the suspensions obtained from the stock cultures or the lyophilisates. It assures that the correct bacteria species is being used and that no contamination occurred. If a more precise confirmation is periodically needed, it can be performed through 16S rRNA gene sequence analysis in a certified laboratory.

Also, each laboratory may develop their own "warning charts"[8] based on the toxic response of *A. globiformis* to the reference substance (C16-BAC, see 5.4.11). These plots present the EC50 values, which are obtained from different tests repeated over months in one laboratory. The "warning charts" allow verifying the sensitivity of the organism over time and can function as an internal quality control as well.

Annex C
(informative)

Testing chemical substances

C.1 General

The contact assay is also suitable for the testing of chemical substances that are not volatile and stable up to (85 ± 2) °C. However, it requires some adaptations, as described below.

C.2 Control and test substrate

For the testing of chemicals, use an artificial soil or quartz sand to prepare both control and test (with chemical substance) treatments (see 5.2 and 5.3). No chemical substance is added to the control substrate. Adjust the water content of both substrates as indicated in 5.2 and 5.3.

NOTE The use of the artificial soil as the spiked substrate for testing chemicals can provide lower toxicities (see Annex A).

C.3 Testing of chemicals

Use one of the following methods:

a) Water soluble test substances. One or two days before starting the test (see Table 1), prepare an emulsion or dispersion of the test substance in distilled water for all replicates of one concentration (have into consideration the final moisture content of the substrates needed). Mix the emulsion or dispersion thoroughly with the whole batch of artificial soil or quartz sand (see 5.2) for all replicates, before weighing it into the microplates.

b) Test substances insoluble in water but soluble in organic solvents. The quantity of test substance required to obtain the desired concentration is dissolved in a volatile solvent (such as acetone or DMSO 5 %) and it is mixed with quartz sand. After evaporation of the solvent by placing the container in a fume hood for at least 1 h, the portion of quartz sand required is mixed thoroughly with the substrate. For soil samples, if the artificial soil is selected, then the amount of quartz sand used for the application of the test substance shall be considered when preparing the substrate. Moist the spiked substrate (see 5.3) before weighing it into the microplates.

Ultrasonic dispersion, organic solvents, emulsifiers, or dispersants can be used to disperse substances with low aqueous solubility. When such auxiliary substances are used, all test concentrations and the control substrate should contain the same minimum amount of the carrier substance. If needed, prepare an additional control without the solvent used to ascertain possible effects on dehydrogenase activity.

c) Test substances insoluble in water or organic solvents. A mixture of finely ground quartz sand and the quantity of the test substance required to obtain the desired concentration is prepared. Afterwards, this mixture is mixed thoroughly with the pre-moistened substrate (see 5.3) and with the amount of distilled water in order to get the final moisture required before weighing it into the microplates.

   NOTE The quantity of quartz sand used for mixing the substance depends on its final proportion in the substrate.

d) Test substances which shall be tested in high concentrations to simulate the conditions of contaminated sites (e.g. mineral oil) may be added directly to the selected substrate. A homogenous

distribution of the test substance in the substrate shall be demonstrated. After that, weigh the substrate to the microplates.

Preliminary tests should be done to a wide concentration range of the test substance, which can then be shortened to improve the accuracy of point estimates calculation. The remaining procedure steps and the contact time are the same as mentioned before.

For the calculation and expression of the results, proceed as described in Clause 8 for the situation in which the control and test substrates differ only in contamination (testing of dose-response relationships).

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