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Water and soil quality — Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of *Caenorhabditis elegans* (Nematoda)

Qualité de l'eau et du sol — Détermination de l'effet toxique d'échantillons de sédiment et de sol sur la croissance, la fertilité et la reproduction de Caenorhabditis elegans *(Nematodes)*

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological method.*

This second edition cancels and replaces the first edition (ISO 10872:2010), which has been technically revised. The main changes compared to the previous edition are as follows:

— the title has been changed to achieve a better perception in the field soil toxicity testing;

— for soil testing, the method has been modified in terms of a reduced water content of the test material;

— cited references and standards have been refreshed;

— information on the control soil and restrictions for tested soils has been added;

— the document has been editorially revised.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Nematodes are one of the most abundant and species-rich metazoans in sediments[1] and soils[2] and possess key positions in benthic and soil food webs due to the evolution of various feeding types (bacterial, algal, fungal and plant feeders, omnivores, predators see References [3] and [4]). Moreover, they are well acknowledged as environmental indicators for assessing the toxicity of chemicals and the quality of sediments and soils (see References [5], [6], [7], [8] and [9]).

The test organism *Caenorhabditis elegans* (Maupas, N2 *var*. Bristol) is a bacterivorous nematode that is found primarily in microbe-rich, decaying plant material (see Reference [10]) and belongs to the family of Rhabditidae, frequently found in terrestrial soils and aquatic sediments (see References [11] and [12]). Moreover, individuals of *C. elegans* were already found in sediments of polysaprobial fresh-water systems (see References [13] and [14]). Due to its easy cultivation and short life cycle[15], *C. elegans* has become a well-studied model organism in biomedical and ecotoxicological research (see References [16], [17] and [18]).

The test is designed for measurement of the response to dissolved and particle-bound substances[19]. It applies to the testing of sediments, soils, waste, pore water, elutriates and aqueous extracts (see e.g. References [20], [21], [22] and [23]).

Water and soil quality — Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of *Caenorhabditis elegans* (Nematoda)

WARNING — Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices.

IMPORTANT — It is absolutely essential that tests conducted according to this document be carried out by suitably trained staff.

1 Scope

This document specifies a method for determining the toxicity of environmental samples on growth, fertility and reproduction of *Caenorhabditis elegans*. The method applies to contaminated whole freshwater sediment (maximum salinity 5 g/l), soil and waste, as well as to pore water, elutriates and aqueous extracts that were obtained from contaminated sediment, soil and waste.

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 5667-16, *Water quality — Sampling — Part 16: Guidance on biotesting of samples*

ISO 7027-2, *Water quality — Determination of turbidity — Part 2: Semi-quantitative methods for the assessment of transparency of waters*

3 Terms and definitions

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

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

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

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

3.1
agar plate
Petri dish filled with Nematode Growth Medium (NGM) agar

3.2
aqueous control
water that serves as negative control for tests in aqueous samples

3.3
artificial control sediment
defined artificial sediment

3.4
bacterial stock culture
stock culture of food bacteria

3.5
blank replicate
additional replicate that contains no test organism, but is treated in the same way as the other replicates of a sample

3.6
control
treatment that serves as negative control to which the effect in the respective test material is compared

3.7
control soil
defined standard soil

3.8
dauer larva
developmental stage adopted by *C. elegans* to endure periods of lack of food

Note 1 to entry: Dauer larvae continue normal development if food is supplied.

3.9
exposed test organisms
individuals of *C. elegans* that are introduced at the beginning of the test

3.10
food medium
defined aqueous bacterial suspension

3.11
J_1 stage
first of four juvenile stages (J_1 to J_4) in the development of *C. elegans*

3.12
maximal water holding capacity
WHC_{max}
maximal amount of water a soil sample is able to take up and keep against gravity

3.13
overnight culture
defined culture of *Escherichia coli* in Lysogeny Broth (LB)-medium

3.14
starved plate
agar plate (3.1) with dauer larvae

3.15
test material
discrete portion of a contaminated environmental sample or solution of the reference substance

4 Principle

Juvenile organisms of the species *C. elegans* are exposed to the environmental sample over a period of 96 h. In the controls, the exposed test organisms are able to complete a whole life cycle within this period. Toxicity can be quantified by the intensity of the effect as percentage inhibition. A toxic effect of an environmental sample occurs if the inhibition of growth, fertility or reproduction of *C. elegans* in comparison to a control (aqueous control, control sediment or soil) exceeds a certain threshold value (e.g. as proposed in the following publications: aqueous medium: 10 %, 20 %, 10 % inhibition, respectively (see Reference [24]); freshwater sediments: 25 %, 20 %, 50 % inhibition, respectively (see Reference [21]); soil: 10 %, 20 %, 40 % inhibition, respectively (see Reference [20]), and the performance of toxicity endpoints in the test material is statistically significantly lower compared to the control performance ($p < 0,05$).

5 Reagents and media

Use only reagents of recognized analytical grade.

5.1 Water, distilled or deionized water or water of equivalent purity, conductivity ≤10 µS/cm.

5.2 LB-medium

Dissolve

— 0,5 g of casein peptone;

— 0,25 g of yeast extract;

— 0,5 g of sodium chloride (NaCl);

in 50 ml water in a 250 ml flask and autoclave for 20 min at 121 °C.

5.3 Cholesterol stock solution

Dissolve 500 mg of powdered cholesterol in 100 ml of absolute ethanol (>99 % purity) by stirring and gentle heating (<50 °C). Replace ethanol lost through evaporation with ethanol.

5.4 Calcium chloride stock solution, 1 mol/l $CaCl_2$.

Dissolve 7,35 g of $CaCl_2 \cdot 2H_2O$ in 50 ml water and autoclave for 20 min at 121 °C.

5.5 Magnesium sulfate stock solution, 1 mol/l $MgSO_4$.

Dissolve 12,35 g of $MgSO_4 \cdot 7H_2O$ in 50 ml water and autoclave for 20 min at 121 °C.

5.6 Potassium hydroxide, KOH, pellets.

5.7 Potassium phosphate buffer, 1 mol/l KH_2PO_4.

Dissolve 13,6 g of KH_2PO_4 in 100 ml of water, adjust with KOH (5.6) to pH 6,0 ± 0,2, and autoclave for 20 min at 121 °C.

5.8 Nematode growth-medium agar (NGM agar)

Dissolve

— 2,5 g of casein peptone;

— 17 g of bacteriological agar;

— 3 g of NaCl;

in 900 ml water in a 1 000 ml flask and autoclave for 20 min at 121 °C. After cooling down to 55 °C, add the following sterile solutions:

— 1 ml of cholesterol stock solution (see 5.3);

— 1 ml of calcium chloride stock solution (see 5.4);

— 1 ml of magnesium sulfate stock solution (see 5.5);

— 25 ml of potassium phosphate buffer (see 5.7);

and fill up to 1 000 ml with sterile water.

Transfer portions of NGM agar (about 20 ml to 25 ml) to sterile Petri dishes.

5.9 M9-medium

Dissolve

— 6 g of Na_2HPO_4;

— 3 g of KH_2PO_4;

— 5 g of NaCl;

— 0,25 g of $MgSO_4 \cdot 7H_2O$;

in 1 000 ml of water in a 1 000 ml flask.

5.10 Bengal Rose stock solution

Add approximately 30 mg of Bengal Rose to 100 ml of water and stir thoroughly.

5.11 Ludox suspension

Dilute Ludox TM 50[1)] (colloidal silica; density: 1,4 g/cm^3) with water to a density of (1,13 ± 0,005) g/cm^3 [mix approximately 1 part Ludox TM 50[1)] with 2 parts of water and control the density by weighing 1 ml of the suspension on a balance; 1 ml of the suspension weighs (1,13 ± 0,005) g]. For one 12 or 24-well multidish, approximately 75 ml or 150 ml of Ludox-suspension are required, respectively.

5.12 Artificial control sediment

Mix the following components thoroughly in the given proportions:

— Al_2O_3, 20 % mass fraction;

— $CaCO_3$, 1 % mass fraction;

— dolomite (clay), 0,5 % mass fraction;

— Fe_2O_3, 4,5 % mass fraction;

— silica sand (for example: W4, mean particle size: 0,063 mm), 30 % mass fraction;

— silica sand (0,1 mm to 0,4 mm), 40 % mass fraction;

— peat (decomposed peat from a raised bog, untreated; finely ground and <1 mm sieved), 4 % mass fraction.

The dry sediment is maintainable without restraint.

This sediment serves as negative control for tests with sediments.

WARNING — Artificial sediments with a kaolin content of >5 % mass fraction (e.g. OECD 218) can cause deleterious effects on growth, fertility and reproduction of *C. elegans*. If using a different artificial control sediment than proposed in this standard, the kaolin content shall be ≤5 % mass fraction.

WARNING — If available, the use of a site-specific reference sediment is advised, additionally to the artificial control sediment; the reference sediment shall match following criteria: limit of contamination; important sediment properties shall be similar to the tested sample (e.g. particle size distribution, organic content).

1) Ludox™ 50 is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

5.13 Control soil

Standard soil St. 2.2 from LUFA:

— soil type: loamy sand;

— organic carbon: (2,0 ± 0,5) % mass fraction;

— pH ($CaCl_2$): 5,5 ± 0,5;

— cation exchange capacity: (10,0 ± 0,4) $mmol_c$/100 g;

NOTE $mmol_c$/100 g is synonymous with meq/100 g.

— water holding capacity: (48,2 ± 5) %;

— clay content: (7,5 ± 2,5) % mass fraction particles <0,002 mm;

— silt content: (12,5 ± 2,5) % mass fraction particles 0,002 mm to 0,063 mm;

— sand content: (80,0 ± 5,0) % mass fraction particles 0,063 mm to 2 mm.

This soil serves as negative control for tests with soil.

NOTE LUFA refers to Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer.

WARNING — If tested soils contain >30 % clay even in uncontaminated soils, inhibiting effects on *C. elegans*' growth and reproduction might exceed the toxicity threshold. In these cases, a reference soil with a similar clay content shall be tested additionally to the control soil.

WARNING — Artificial soils with a kaolin content of >5 % mass fraction (e.g. OECD 207) can cause deleterious effects on growth, fertility and reproduction of *C. elegans*. If using a different artificial control soil than proposed in this standard, the kaolin content shall be ≤5 % mass fraction.

5.14 Benzylcetyldimethylammonium chloride monohydrate (BAC-C16) stock solution

Dissolve 30 mg of BAC-C16 ($C_{25}H_{46}ClN{\cdot}H_2O$; CAS No.: 122-18-9) in 1 000 ml of water.

5.15 Glycerol (>98 %; Ph.Eu., water free)

5.16 Formazin: Turbidity 4000 NTU calibration standard

6 Apparatus

6.1 Autoclave.

6.2 Facilities, with constant temperature for 20 °C and 37 °C, for example incubator or temperature-controlled chamber.

6.3 Drigalski spatula, glass spatula for distributing bacteria on an agar plate.

6.4 Erlenmeyer flasks, for example volume 250 ml.

6.5 Plastic vials, autoclavable and sealed, volume 1,5 ml.

6.6 Filter gauze, 5 µm, 10 µm.