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

Qualité de l'eau — 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

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The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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.

ISO 10872 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Introduction

Nematodes are the most abundant and species-rich group of metazoans in sediments and soils[1][2] and play an important role in benthic and soil food webs[3][4]. Nematodes are endobenthic organisms that are found at various trophic levels due to the evolution of different feeding types (bacterivorous, algal feeder, omnivorous, predators).

The test organism *Caenorhabditis elegans* (Maupas, N2 *var*. Bristol) is a bacterivorous nematode that is found primarily in terrestrial soils but it also occurs in aquatic sediments of polysaprobial fresh-water systems[5][6]. *C. elegans* is a well-studied organism and very easy to cultivate[7].

The test is designed for measurement of the response to dissolved and particle-bound substances[8][9][10]. It applies to the testing of sediments, soils, waste, pore water, elutriates and aqueous extracts.

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

WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This International Standard 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 and to ensure compliance with any national regulatory conditions.

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

1 Scope

This International Standard specifies a method for determining the toxicity of environmental samples on growth, fertility and reproduction of *Caenorhabditis elegans*. The method applies to contaminated whole fresh-water sediment (maximum salinity 5 ‰), 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 referenced documents are indispensable for the application 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, *Water quality — Determination of turbidity*

ISO 10390, *Soil quality — Determination of pH*

ISO 10523, *Water quality — Determination of pH*

ISO 11465, *Soil quality — Determination of dry matter and water content on a mass basis — Gravimetric method*

3 Terms and definitions

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

3.1
agar plate
Petri dish filled with NGM agar (5.8)

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

3.3
artificial control sediment
defined artificial sediment (5.12)

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 (3.2, 3.3, 3.7)

3.7
control soil
defined standard soil (5.13)

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

NOTE 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 (10.1)

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

3.12
overnight culture
defined culture of *Escherichia coli* in LB-medium (9.1.2)

3.13
starved plate
agar plate with dauer larvae

3.14
test material
discrete portion of a contaminated environmental sample (10.2) or solution of the reference substance (Clause 7)

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. 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. Toxicity can by quantified by the intensity of the effect as percentage inhibition.

5 Reagents

Use only reagents of recognized analytical grade.

5.1 Water, distilled or deionized water or water of equivalent purity, conductivity $\leqslant$ 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 147 g of $CaCl_2 \cdot 2H_2O$ in 1 000 ml water and autoclave for 20 min at 121 °C.

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

Dissolve 247 g of $MgSO_4 \cdot 7H_2O$ in 1 000 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 136 g of KH_2PO_4 in 1 000 ml of water, adjust with KOH (5.6) to pH 6,0 $\pm$ 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 (5.3);

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

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

— 25 ml of potassium phosphate buffer (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 300 mg of Bengal Rose to 1 000 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 sample, approximately 50 ml of Ludox-suspension are required.

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 (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.

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

The dry sediment is maintainable without restraint.

This sediment serves as negative control for tests in sediments.

WARNING — If a different artificial control sediment is used (e.g. OECD 218), the kaolin content of the > 5 % mass fraction can cause deleterious effects on growth, fertility and reproduction of *C. elegans*.

5.13 Control soil.

Standard soil St. 2.2 from LUFA[2)]:

— soil type: loamy sand;

— organic carbon: (2,16 ± 0,4) % mass fraction;

— pH: 5,4 ± 0,1;

— cation exchange capacity: (10 ± 1) $mmol_c$/100 g;

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

— water holding capacity: (48,2 ± 5) g/100 g;

— clay content: (6,4 ± 0,9) % mass fraction particles < 0,002 mm;

— silt content: (12,7 ± 2,6) % mass fraction particles 0,002 mm to 0,063 mm;

— sand content: (81,2 ± 5,1) % mass fraction particles 0,063 mm to 2 mm.

This soil serves as negative control for tests in soil.

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 (CAS No.: 56-81-5).

6 Apparatus

6.1 Autoclave.

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

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

6.4 Erlenmeyer flasks, e.g. volume 250 ml.

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

6.6 Filter gauze, 5 µm, 10 µm.

6.7 Freezer, capable of being maintained at –20 °C.

2) Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer.

6.8 Micropipette.

Draw a Pasteur pipette over a Bunsen burner to a thin capillary. Plug the Pasteur pipette in a section cup at the thicker end.

6.9 Microscope, 100-fold magnification, with measurement scale.

6.10 Thermometer, minimum-maximum.

6.11 Shaker, for 250 ml Erlenmeyer flasks.

6.12 Stereo microscope, 4-fold to 20-fold magnification, with transmitted light.

6.13 Clean bench.

6.14 Sterile Petri dishes, of diameters 3 cm, 6 cm or 10 cm.

6.15 Spectrophotometer, capable of operating at wavelength 600 nm.

6.16 Test tube mixer.

6.17 Balance, 0,001 g resolution.

6.18 Drying oven, approximately 80 °C.

6.19 Multidishes, with 12 wells, 3,5 cm^2/well.

6.20 Centrifuge with swing-out rotor.

6.21 Piston pipettes, 10 ml to 100 ml, 100 ml to 1 000 ml.

6.22 Sieves, 1 mm and 2 mm.

6.23 Magnetic stirrer and **magnetic stirring bar**.

6.24 pH-meter.

6.25 Inoculating loop.

7 Reference substance

To ensure that the laboratory test conditions (including the condition and sensitivity of the exposed test organisms) are adequate and have not changed significantly, it is necessary to test a reference substance as a positive control in parallel with each test, using one concentration near the EC_{50} for growth. The test parameters “fertility” and “reproduction” are not analysed when testing the reference substance. Use benzylcetyldimethylammonium chloride monohydrate (BAC-C16; 5.14), which has been shown to affect growth of *C. elegans,* as reference substance. The positive control is tested in water according to the instructions for testing aqueous substrates (10.1, 10.2.2, 10.3). The inhibition of growth at a concentration of 15 mg/l (EC_{50} for BAC-C16, 5.14) compared to the control should be in the range of 20 % to 80 %.

Additionally, the EC_{50} of the reference substance shall be determined at least every 12 months. The EC_{50} (growth) in water shall be in the range of 8 mg to 22 mg BAC/l using an EC_x design as specified in ISO 5667-16. Stock solutions of BAC in the concentrations of 7,1 mg/l, 10,6 mg/l, 16 mg/l, 24 mg/l, 36 mg/l, 54 mg/l and 81 mg/l are prepared in water and tested according to the instructions for testing aqueous substrates (10.1, 10.2.2, 10.3).

8 Organisms

8.1 Test organism

Caenorhabditis elegans (Maupas, 1899) is a widespread, free-living soil nematode that feeds primarily on bacteria. Adult worms are about 1,0 mm to 1,5 mm in length and can be distinguished into hermaphrodites and rarely occurring males (see Annex A). Hermaphrodites usually reproduce by self-fertilization although they can also be fertilized by males. After hatching, *C. elegans* develops to the adult stage through four juvenile stages separated by moults. Under starvation conditions, a developmentally arrested stage, the dauer larva, can be formed as an alternative third larval stage. The life cycle for worms grown on *E. coli* is about 3 days at 20 °C. The biology of *C. elegans* has been extensively studied and, in many respects, it is the most thoroughly characterized animal. The “wild type” strain N2 is used as test organism.

8.2 Food organism

As food organism for *C. elegans*, the bacterium *E. coli* (OP50; uracil-deficient strain) is used.

9 Stock- and pre-cultures

9.1 Stock cultures

9.1.1 *Caenorhabditis elegans*

C. elegans[3)] is maintained on agar plates (3.1) with a bacterial lawn (*E. coli* OP 50; 8.2) at (20 ± 2) °C. When bacteria are used up, *C. elegans* forms dauer larvae (3.8) due to lack of food. These starved plates (3.13) serve as stock cultures for *C. elegans* that should be replenished every two months. If too many males occur (⩾ 10 % in tests), new stock cultures should be ordered.

9.1.2 *Escherichia coli*

Inoculate under sterile conditions 50 ml of LB-medium (5.2) in a 250 ml Erlenmeyer flask (6.4) with *E. coli* from a bacterial lawn on agar [as sent by the Caenorhabditis Genetic Center (CGC)] using an inoculating loop (6.25) and incubate for 17 h at 37 °C on a shaker (6.11) (overnight culture). Transfer 200 µl glycerol (5.15) into each 1,5 ml plastic vial (6.5) and sterilize in an autoclave (6.1) at 121 °C for 20 min. Add under sterile conditions 800 µl of the *E. coli* overnight culture (3.12), vortex and freeze immediately at −20 °C. Bacterial stock cultures are thawed only once and discarded after use. Bacterial stock cultures should be replenished after six months.

9.2 Pre-culture

Inoculate under sterile conditions an agar plate (3.1) with approximately 200 µl of an overnight culture (3.12) of *E. coli*, distribute equally using a Drigalski spatula (6.3) and incubate for at least 8 h at (37 ± 2) °C. Cut two or three small pieces (approximately 1 cm^2) out of a starved plate (3.13) and transfer them under sterile

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3) Supplier for *Caenorhabditis elegans* and *Escherichia coli*.

Caenorhabditis Genetic Center
Theresa Stiernagle
University of Minnesota
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321 Church Street S.E.
Minneapolis, MN 55455
E-mail: cgc@umn.edu
http://www.cbs.umn.edu/CGC/

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conditions on to an agar plate with a fresh lawn of *E. coli.* After about 3 days at 20 °C, a lot of gravid hermaphrodites as well as juveniles of stages 1 and 2 are found on the plate. The test starts with worms in the first juvenile stage. In order to obtain worms synchronized to this life stage, rinse the plate with M9-medium (5.9). Then filter the suspension containing the nematodes through a cascade of filter gauze (6.6) of 5 µm and 10 µm mesh size to retain larger juveniles and adults. The filtered suspension contains only first-stage juveniles (J_1). Measure the length of 30 J_1 (killed with heat) to obtain the initial length of the introduced exposed test organisms (mean value).

10 Procedure

10.1 Preparation of food medium

After thawing, vortex (6.20) a vial with the *E. coli* stock vigorously. Inoculate under sterile conditions $x \times 50$ ml of LB-medium (5.2) (depending on the demand) in 250 ml Erlenmeyer flasks (6.4) with approximately 20 µl of the *E. coli* stock each and incubate for 17 h at 37 °C on a shaker (6.11). To control the bacterial density after incubation, dilute an aliquot of the bacterial suspension 1→10 with LB-medium and measure the optical density at 600 nm against the LB-medium. The turbidity of the bacterial suspension is given as specified in ISO 7027 in FAU (formazine absorption units). OD600 is usually > 200 FAU. After centrifugation of the bacterial suspension (20 min, 2 000g), remove the supernatant and resuspend the pellet in M9-medium (5.9). After repeated centrifugation and removing of the supernatant, resuspend the pellet in approximately

— $x \times 8$ ml of M9-medium and adjust the bacterial density to (12 000 ± 600) FAU for testing sediment, soil and waste;

— $x \times 100$ ml of M9-medium and adjust the bacterial density to (1 000 ± 50) FAU for testing pore water, elutriates, extracts or solutions of reference substance.

Finally, the accurate volume of cholesterol stock solution is added (0,2 % of volume of bacterial suspension; e.g. 100 µl of cholesterol stock solution in 50 ml bacterial suspension).

NOTE Densities of bacteria differ between tests with solid and liquid test material due to different exposure conditions.

10.2 Preparation of test material and controls

10.2.1 Sediment, soil and waste

Pass the test material (3.14) through a 2 mm sieve (6.22). Determine the dry mass of the test material and control sediment or control soil in accordance with ISO 11465 by drying a small portion of the test sample. Determine the pH of the test material and control sediment or control soil in accordance with ISO 10523 (aqueous test material, sediments) and ISO 10390 (soils).

Prepare at least four replicates for each test material and the control [artificial control sediment (5.12) or control soil (5.13)]. Prepare one additional blank replicate (without test organisms) to estimate the number of indigenous nematodes in the samples. For artificial substrates, such as the artificial control sediment, it is not necessary to set up a blank replicate. For test material with $\geqslant 40$ % water content (based on total mass), transfer (0,500 ± 0,010) g (wet mass) of test material into each test well (6.19). For test material with < 40 % water content (based on total mass), artificial control sediment and control soil, transfer $m \pm 0{,}010$ g into each test well, add (0,500 − m) ml of M9-medium (5.9), and stir with a spatula to achieve a homogenous suspension. Calculate m, expressed in grams, as given in Equation (1):

$$m = \frac{0{,}5 \times 0{,}60}{m_{\mathrm{o,t}}} \tag{1}$$

where $m_{\mathrm{o,t}}$ is the measured dry mass of the test material.

Store in a refrigerator at (8 ± 2) °C to avoid loss of moisture.

Stir the food medium (3.10, 10.1; 12 000 FAU) to ensure homogeneity and add, immediately before the start of the test, 0,5 ml of homogenized food medium (3.10) to each test well. Mix artificial control sediment or control soil and test material with the added food medium thoroughly with a spatula.

10.2.2 Pore water, elutriate, extract

Prepare at least four replicates for each test material (3.14) and the control (aqueous control; 3.2). Transfer 0,5 ml of test material and aqueous control into each test well (6.19). Stir the food medium (3.10, 10.1; 1 000 FAU) to ensure homogeneity and add, immediately before the start of the test, 0,5 ml of homogenized food medium to each test well.

10.2.3 Solution of reference substance

Prepare at least four replicates for each solution of the reference substance (5.14, Clause 7) and the control (aqueous control; 3.2). Transfer 0,5 ml of the solution of reference substance and aqueous control into each test well (6.19). Stir the food medium (3.10, 10.1; 1 000 FAU) to ensure homogeneity and add, immediately before the start of the test, 0,5 ml of homogenized food medium to each test well.

10.3 Test

At the start of the test, transfer ten first-stage juveniles (J_1; exposed test organisms) from the filtrate by micropipette (6.8) to each of the test wells containing test material (3.14) and food medium (3.10) (after allowing temperature to equilibrate to room temperature). Non-moving organisms should be excluded from the test. After the addition of the test organisms, seal the multidishes (6.19) with an adhesive tape [Parafilm[4)]] and incubate at (20 ± 0,5) °C in the dark. After 96 h, add approximately 0,5 ml of Rose Bengal stock solution (5.10) to each test well to stain the nematode cuticle for better recovery. Heat the multidishes with the lid on in a drying oven for 10 min at 80 °C to terminate the test. This treatment results in straightened, easily measurable worms. Until further processing, samples are stored at (8 ± 2) °C. Samples should be analysed within eight weeks.

10.4 Nematode separation

Transfer the sediment, soil or waste of each test well (10.3) into a separate centrifuge tube using tap water and a Pasteur pipette. Centrifuge the samples using a swing-out rotor (6.20) for 5 min at 800g and decant aqueous supernatant with caution into a separate test tube for further analysis. Add 2 ml of Ludox suspension (5.11), mix thoroughly using a test tube mixer (6.16) and centrifuge the samples using a swing-out rotor for 5 min at 800g. The exposed test organisms and their offspring can be found in the Ludox supernatant that is decanted in a separate test tube. The sediment or soil pellet is again thoroughly mixed with 2 ml Ludox suspension and centrifuged. Repeat the Ludox separation three times. The gathered Ludox suspension with separated nematodes is decanted into a Petri dish (6.14) for further analysis. Both organisms in the aqueous and Ludox supernatant (exposed test organisms and offspring) are considered for measurements and calculations (10.5).

In case of testing aqueous samples, no separation steps are necessary. Rinse the content of one test well in a Petri dish for further analysis using tap water and a Pasteur pipette.

10.5 Measurements and calculations

10.5.1 Recovery

Count the separated exposed test organisms (3.9; 10.4) and calculate the percentage of recovered exposed test organisms by dividing the total number of recovered exposed test organisms (including male exposed test organisms) by the number of introduced test organisms (10) and multiplying by 100. For an example, see Tables 1 and 2.

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4) Parafilm™ is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

10.5.2 Males

Males can occur at low frequencies. Count the males in the replicates and exclude them from further measurement of growth (10.5.3) and calculations of fertility (10.5.3) and reproduction (10.5.4) as adult males are generally smaller than adult hermaphrodites and males are not able to produce eggs and offspring. Determine the percentage of male exposed test organisms by dividing the number of males by the total number of recovered exposed test organisms and multiplying by 100. For an example, see Tables 1 and 2.

10.5.3 Growth and fertility

Transfer the separated exposed test organisms from the Petri dish to a microscopic slide using a stereo microscope (4-fold to 20-fold magnification; 6.12). Measure the body length of the exposed test organisms under a microscope (100-fold magnification) using a measurement scale (males are not measured) and determine if there are eggs inside the body of each exposed test organism. A worm is considered gravid if the number of eggs inside the body is $\geqslant 1$.

Calculate the mean body length for each replicate for further calculations.

Calculate the replicate growth as the difference of the mean measured body length and mean body length of 30 J_1 at the beginning of the test (as determined in 9.2). For an example, see Tables 1 and 2.

Calculate per replicate the percentage of gravid exposed test organisms in relation to the total number of recovered hermaphroditic exposed test organisms (fertility) by dividing the number of gravid exposed test organisms by the total number of recovered exposed test organisms (less the number of males) and multiplying by 100. For an example, see Tables 1 and 2.

Table 1 — Example for the calculation of mean growth and fertility in six replicates of the control

Exposed test organism	Replicates											
	1		2		3		4		5		6	
	l_1	Gravid	l_1	Gravid	l_1	Gravid	l_1	Gravid	l_1	Gravid	l_1	Gravid
1	1 430	+	1 450	+	1 400	+	1 380	+	1 480	+	1 470	+
2	1 480	+	1 500	+	1 450	+	1 430	+	1 530	+	1 520	+
3	1 350	+	1 370	+	1 320	+	1 300	+	1 400	+	1 390	+
4	1 520	+	1 540	+	1 490	+	1 470	+	1 570	+	1 560	+
5	1 410	+	1 430	+	1 380	+	1 360	+	1 460	+	1 450	+
6	1 430	+	1 450	+	1 400	+	1 380	+	1 480	+	1 470	+
7	1 450	+	1 470	+	1 420	+	1 400	+	900	-	1 490	+
8	1 390	+	1 410	+	1 360	+	1 340	+	1 440	+	1 430	+
9	male		n.r.		n.r.		1 410	+	male		1 500	+
10	n.r.		n.r.		n.r.		1 290	+	male		n.r.	
$\overline{l}_1$, µm	1 433		1 453		1 403		1 376		1 408		1 476	
$\overline{l}_{o,J_1}$, µm	290		290		290		290		290		290	
Mean growth, µm	1 143		1 163		1 113		1 086		1 118		1 186	
Fertility, %	100		100		100		100		87,5		100	

Abbreviations:

$\overline{l}_1$ mean body length of the measured exposed test organisms

$\overline{l}_{o,J_1}$ mean initial body length of the introduced J_1 (as calculated for 30 J_1; in micrometres)

\+ gravid

– not gravid

n.r. not recovered

10.5.4 Reproduction

Count the number of offspring (second-generation juveniles) by using a stereo microscope (4-fold to 20-fold magnification). Divide this number by the number of introduced test organisms (10) less the number of males. Express the results as offspring per exposed test organisms (reproduction). For an example, see Table 2.

Table 2 — Example for calculation of test parameters and validation criteria in the control

Parameter	Replicates						Mean
	1	2	3	4	5	6	
Number of introduced test organisms	10	10	10	10	10	10	
Number of recovered exposed test organisms	9	8	8	10	10	9	
Number of males	1	0	0	0	2	0	
Number of measured exposed test organisms	8	8	8	10	8	9	
Number of gravid exposed test organisms	8	8	8	10	7	9	
Number of offspring	675	550	650	450	480	550	
Recovery, %	90	80	80	100	100	90	90
Males, %	11,1	0	0	0	20	0	5
Mean growth, µm	1 143	1 163	1 113	1 086	1 118	1 186	1 135
Fertility, %	100	100	100	100	87,5	100	98
Reproduction	75	55	65	45	60	55	59

10.6 Timetable of the test

Day D−4		Preparation of agar plates with fresh bacterial lawn (9.2)
Day D−3		Transferring of dauer larvae to agar plate with fresh bacterial lawn (9.2)
Day D−2		Determination of dry mass of test material and control (10.2.1)
Day D−1	1.	Weighing of test material and control in test wells (10.2.1)
	2.	Adjustment of water content of test material and control (10.2.1)
	3.	Inoculation of LB-medium with bacterial stock (10.1)
Day D	1.	Preparation of food medium (10.1)
	2.	Mixing of test medium and control with food medium (10.2)
	3.	Synchronization of test organisms (9.2)
	4.	Addition of test organisms to test wells and incubation at 20 °C in the dark (10.3)
	5.	Determination of initial body length (9.2)
Day D+4	1.	Addition of Rose Bengal (10.3)
	2.	Termination of test (heat fixing; 10.3)
	3.	Storage at (8 ± 2) °C till analysis (10.3)

11 Validity criteria

The test is considered valid if

a) the mean recovery of exposed test organisms (10.5.1) from the control is $\geqslant$ 80 % and $\leqslant$ 120 %;

b) the mean percentage of males (10.5.2) in the control is $\leqslant$ 10 %; the percentage of males in a single control replicate is $\leqslant$ 20 % (for an example, see Tables 1 and 2);

c) the mean fertility (10.5.3) in the control is $\geqslant$ 80 %;

d) the mean reproduction (10.5.4) in the control is $\geqslant$ 30 offspring per exposed test organism.

Recovery of exposed test organisms from the control sediment or control soil is an indicator of accuracy when adding test organisms (10.3) and accuracy of nematode separation (10.4). The determination of $\geqslant$ 80 % and $\leqslant$ 120 % recovery as validity criteria ensures a certain accuracy for the calculations of reproduction.

Males do not influence test results substantially if they occur in low frequencies (< 10 %). Above a certain threshold (20 %; 2 of 10 exposed test organisms), calculation of test parameters can be biased. Moreover, mean percentages of males of $\geqslant$ 10 % indicate cultures that are not appropriate for the requirements of this test. In this case, it is recommended that new cultures be ordered.

12 Expression of results

For each treatment, calculate the mean and standard deviation from the replicate data. Moreover, express the results as inhibition of a test parameter, x_t, expressed as a percentage relative to the corresponding value in the control, as given in Equation (2):

$$x_t = (100 - \frac{\bar{x}_A}{\bar{x}_C}) \times 100 \quad (2)$$

where

$\bar{x}_A$ is the mean of the parameter in sample A;

$\bar{x}_C$ is the mean of the parameter in the control.

Table 3 — Example for calculation of the percentage inhibition of test parameters

Parameter	Control	Sample A	Inhibition %
Growth, µm	1 135	700	38
Fertility, %	98	80	18
Reproduction (offspring per exposed test organism)	60	15	75

See Annex B for precision data.

13 Test report

The test report shall contain at least the following information:

a) reference to this International Standard (ISO 10872:2010);

b) name of the laboratory performing the test;

c) date and period of test;

d) test organism (e.g. systematic name, strain, source);

e) food organism (e.g. systematic name, strain, source);

f) designation of test material (batch number, origin, date and period of sampling);

g) sample pre-treatment and characteristics (storage period and conditions, water content, organic matter content as a percentage mass fraction, pH and particle size distribution of sediment or soil);

h) test conditions: bacterial density in FAU; minimal and maximal temperature during the test; initial body length of exposed test organisms (mean and standard deviation); number of indigenous nematodes in the blank replicate;

i) results (mean and standard deviation of test parameters in treatments and control, percentage inhibition of test parameters in all treatments);

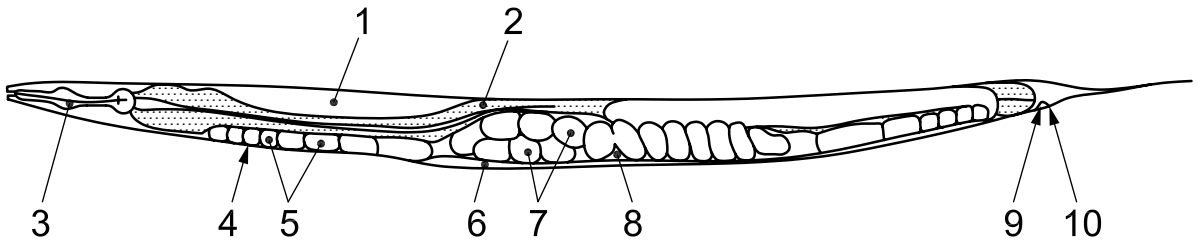
j) details of test results (e.g. replicate data);

k) results from the test with reference substance;

l) any deviations from the test protocol.

Annex A
(informative)

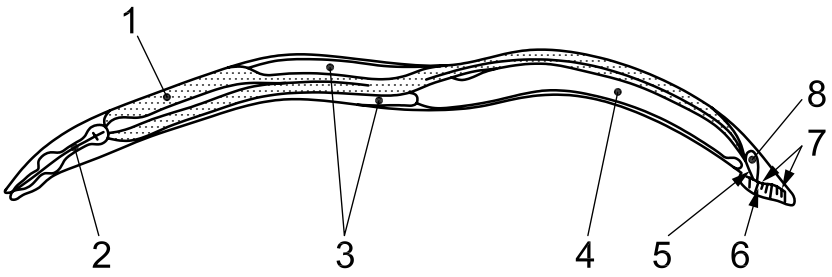
Figures of adult worms *C. elegans*



Key

1 ovary
2 intestine
3 pharynx
4 oviduct
5 oocytes
6 uterus
7 eggs
8 vulva
9 rectum
10 anus

Figure A.1 — Adult hermaphrodite



Key

1 intestine
2 pharynx
3 testes
4 vas deferens
5 cloaca
6 fan
7 rays
8 spicules

Figure A.2 — Male

Annex B
(informative)

Precision data

International interlaboratory tests based on the test described in this International Standard were carried out in 2009. The results with the reference substance BAC-16 for EC_{50} for the parameter growth, as well as results with contaminated sediment and soil samples for the parameters growth and reproduction are shown in Table B.1. Additionally, for the sediment and soil tests, all parameters required for determining the validity criteria (Clause 11) are summarized in Table B.2.

Table B.1 — Interlaboratory test results — Toxicity parameters

Substance/sample	Parameter	p	n	n_{OP} %	$\bar{\bar{\rho}}$ mg/l (EC_{50})	s_R mg/l (EC_{50})	$C_{V,R}$ %	s_r mg/l (EC_{50})	$C_{V,r}$ %
BAC-C16	EC_{50} growth	8	18	0,0	15,1	1,68	11,1	1,14	7,6
Polluted soil	% inhibition growth	8	16	0,0	23,8	7,97	33,5	3,87	16,3
	% inhibition reproduction	7	14	12,5	83,3	11,80	14,2	7,80	9,4
Polluted sediment	% inhibition growth	8	16	0,0	18,1	4,95	27,4	2,41	13,3
	% inhibition reproduction	8	16	0,0	63,7	18,34	28,8	8,62	13,5

p number of laboratories after elimination of outliers
n number of valid measured values after elimination of outliers
n_{OP} percentage of outliers
$\bar{\bar{\rho}}$ overall mean
s_R standard deviation of reproducibility
$C_{V,R}$ coefficient of variation of reproducibility
s_r standard deviation of repeatability
$C_{V,r}$ coefficient of variation of repeatability

Table B.2 — Interlaboratory test results — Validity criteria for sediment and soil tests

	Recovery %		Males %		Fertility %		Reproduction (number of offspring per test organism)	
	Control sediment	Control soil	Control sediment	Control soil	Control sediment	Control soil	Control sediment	Control soil
Labor. 1/1	84	92	0	0	100	100	85	117
Labor. 1/2	92	86	0	0	100	100	153	165
Labor. 2/1	100	92	2	6	100	100	87	55
Labor. 2/2	100	98	0	0	100	100	110	66
Labor. 3/1	100	90	0	0	100	100	90	79
Labor. 3/2	98	100	0	0	100	100	80	93
Labor. 4/1	82	94	0	0	100	100	235	90
Labor. 4/2	100	92	0	0	100	100	109	149
Labor. 5/1	100	98	0	0	100	100	127	127
Labor. 5/2	100	96	0	0	100	100	121	135
Labor. 6/1	92	96	6	0	100	100	114	121
Labor. 6/2	84	96	4	8	100	100	102	130
Labor. 7/1	94	80	0	0	100	100	82	54
Labor. 7/2	80	96	0	0	100	100	71	75
Labor. 8/1	100	86	0	0	100	100	103	104
Labor. 8/2	100	96	0	0	100	100	80	106

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