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Water quality — Determination of the acute toxicity to the marine rotifer *Brachionus plicatilis*

Qualité de l'eau — Détermination de la toxicité aigue envers le rotifère marin Brachionus plicatilis

Reference number
ISO 19820:2016(E)

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Contents

Page

Foreword

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The committee responsible for this document is ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Introduction

The evaluation of harmful effects on chemicals and pollutants on the biota in marine and estuarine environments has, for several years, involved the performance of biological tests.

Rotifers, and especially the species *Brachionus plicatilis*, are of interest from the ecotoxicological view because they are often an important component of the zooplankton and serve as prey for small fish and larger invertebrates.

The test specified in this International Standard involves determination of the lethal effects of toxicants to the marine rotifer, *Brachionus plicatilis*, after 24 h or 48 h exposure (depending on the intended use of the results). Prolongation of the exposure to 48 h is advised since it substantially increases the sensitivity of the assay.

Water quality — Determination of the acute toxicity to the marine rotifer *Brachionus plicatilis*

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 and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests conducted in accordance with this document be carried out by suitably qualified staff.

1 Scope

This International Standard specifies a method for the determination of the lethal effects of toxicants to *Brachionus plicatilis* after 24 h or 48 h exposure.

The method is applicable to the following:

a) chemical substances which are soluble or which can be maintained as a stable suspension of dispersions under the conditions of the test;

b) industrial or sewage effluents, treated or untreated, if appropriate after decantation, filtration, or centrifugation;

c) marine or estuarine waters;

d) sediment elutriates/eluates.

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

ISO 5814, *Water quality — Determination of dissolved oxygen — Electrochemical probe method*

ISO 10523, *Water quality — Determination of pH*

3 Terms and definitions

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

3.1
control batch
series of replicates containing control solution

[SOURCE: ISO 6341:2012, 3.1]

3.2
LC_{50}
concentration of dilution of the test sample which gives rise to 50 % mortality of the test organisms

3.3
test batch
series of replicates filled with the same test solution

[SOURCE: ISO 6341:2012, 3.6]

3.4
pure water
deionized or distilled water with a conductivity below 10 µS/cm

4 Principle

The test organisms are exposed to a range of concentrations of the sample under analysis and the percentage mortality of the test organisms is determined after 24 h exposure and/or 48 h exposure with subsequent calculation of the 24 h LC_{50} and/or the 48 h LC_{50}.

Prolongation of the test to 48 h is recommended since it substantially increases the sensitivity of the assay.

The test is carried out in one or two of the following stages:

— a "range-finding test" to determine the range of concentrations or dilutions needed for calculation of the 24 h LC_{50} or the 48 h LC_{50};

— a "definitive test" conducted when the data of the range-finding test are not sufficient or adequate for calculation of the 24 h LC_{50} or the 48 h LC_{50}.

5 Test environment

The test shall be carried out in the dark, in a temperature-controlled room, or incubator at (25 ± 1) °C in the test containers.

Maintain the atmosphere free from toxic dusts or vapours. The use of control solutions is a double check that the test is performed in an atmosphere free from toxic dusts and vapours.

6 Reagents, test organisms and media

Use only reagents of recognized analytical grade, unless otherwise specified.

6.1 Test organisms

The tests organisms are females of the species *Brachionus plicatilis* obtained from a laboratory culture (see References [5],[7], and [8]) or hatched from commercially available cysts.[1)]

The procedure for hatching of *Brachionus plicatilis* from cysts is described in Annex A.

6.2 Culturing and dilution medium

A natural or an artificial seawater may be used as the water for stock culturing the rotifers or as dilution water for the testing. Natural seawater shall be collected from an unpolluted location and should have salinity between 29 ‰ and 36 ‰. Natural seawater must be filtered (30 µm) and conditioned to test temperature and oxygen saturation prior to use. Natural seawater can be stored cold (4 ± 1) °C for several weeks.

An example of artificial seawater suitable for culturing and testing is given in Annex B.

1) MicroBioTests Inc. Mariakerke, Belgium, is an example of a supplier able to provide suitable *Brachionus plicatilis* cysts commercially. This information is given for the convenience of the users of this document and does not constitute an endorsement by ISO of this supplier.

6.3 Reference substance

Potassium dichromate ($K_2Cr_2O_7$) or copper sulfate ($CuSO_4 \cdot 5H_2O$) are recommended as reference chemicals.

NOTE Since $K_2Cr_2O_7$ is a carcinogenic substance toxic via inhalation, the use of a ready-made solution with a defined concentration of $K_2Cr_2O_7$[2)] for the preparation of the stock solution of the reference substance can reduce the risk of inhalation of the toxic dust in the laboratory.

7 Apparatus

Usual laboratory equipment and, in particular, the following.

7.1 Temperature-controlled room or chamber.

7.2 Petri dishes.

Small Petri dishes (diameter 5 cm) in glass or in inert plastic material.

7.3 Test containers.

Disposable 48 (6 × 8) microplates made from chemically inert material.

7.4 Pipette for sampling rotifers, with a sufficient diameter for capturing the animals while allowing sampling of only a small volume of medium.

For example, single use 1 ml capillary mini-pipettes are suitable.

7.5 Stereomicroscope with incident (bottom) illumination, with a magnification of at least eight times and, if possible, a continuous magnification.

7.6 Light source, providing a range of light intensity in the hatching Petri dish of 3 000 lx to 4 000 lx corresponding to 40 $\mu mol.m^{-2}.s^{-1}$ to 55 $\mu mol.m^{-2}.s^{-1}$.

7.7 Sample collecting bottles, as specified in ISO 5667-16.

8 Treatment and preparation of samples

8.1 Special precautions

Special precautions are required for sampling, transportation, storage, and treatment of seawater or effluent.

Sampling, transportation, and storage of the samples should be performed as specified in ISO 5667-16.

Carry out the toxicity test as soon as possible, ideally within 12 h of collection. If this time interval cannot be met, cool the sample to 0 °C to 5 °C and test the sample within 24 h. If it is not possible to perform the test within 72 h, the sample may be frozen and maintained deep-frozen (below –18 °C) for testing within two months of collection, provided that characteristics are known to be unaffected by freezing. At the time of testing, homogenize the sample to be analysed by shaking manually, and, if necessary, allow to settle for 2 h in a container and sample by drawing off (using a pipette) the required quantity of supernatant maintaining the end of the pipette in the centre of the section of the test tube and halfway between the surface of the deposited matters and the surface of the liquid.

2) Titrisol potassium dichromate solution is an example of a suitable product available commercially. This information is given for the convenience of the users of this document and does not constitute an endorsement by ISO of this product.

If the raw sample of the decanted supernatant is likely to interfere with the test (due to the presence of residual suspended matter, protozoa, microorganisms, etc.), filter or centrifuge the raw or decanted sample.

The sample obtained by either of these methods is the sample submitted to testing.

Measure the dissolved oxygen concentration (as specified in ISO 5814) and record the value (mg/l) in the test report.

8.2 Preparation of the stock solutions of substances to be tested

Prepare the stock solution of the substance to be tested by dissolving a known quantity of substance in a specified volume of test medium (6.2) at the time of use. However, if the stock solution of the substance is stable under certain conditions, it may be prepared in advance and stored under these conditions.

For substances sparingly soluble in the test medium, refer to the specifications given in ISO 5667-16.

9 Procedure

9.1 Selection of test concentrations

The test should comprise at least five concentrations of the sample to be tested. The dilutions shall be selected within a geometric series with a separation factor which depends on the nature of the sample to be analysed (chemical substances, effluents, waters) and of the type of assay (range finding or definitive).

For the range finding test with chemical substances, the separation factor for the serial dilutions is usually ten (one order of magnitude difference between two successive dilutions).

For effluents or waters, a 1:1 dilution factor is normally applied (i.e. dilution of the previous concentration by half).

Dilutions series for the definitive test on chemical substances are prepared with a separation factor not exceeding 3,2, whereas for effluents and waters, a 1:1 dilution factor is normally applied.

The test is carried out with six replicates for each dilution plus a control (i.e. the test medium without sample) also in six replicates.

When using a solvent in order to dissolve or disperse chemical substances, a preliminary test has to be performed to determine whether the highest concentration of the solvent used in the dilution series does not have a negative impact on the test organisms.

9.2 Preparation of the test and control solutions

For testing of samples of lower salinity than seawater (e.g. estuarine water), the salinity of the dilution medium must be adjusted to the salinity of the sample by dilution with pure water.

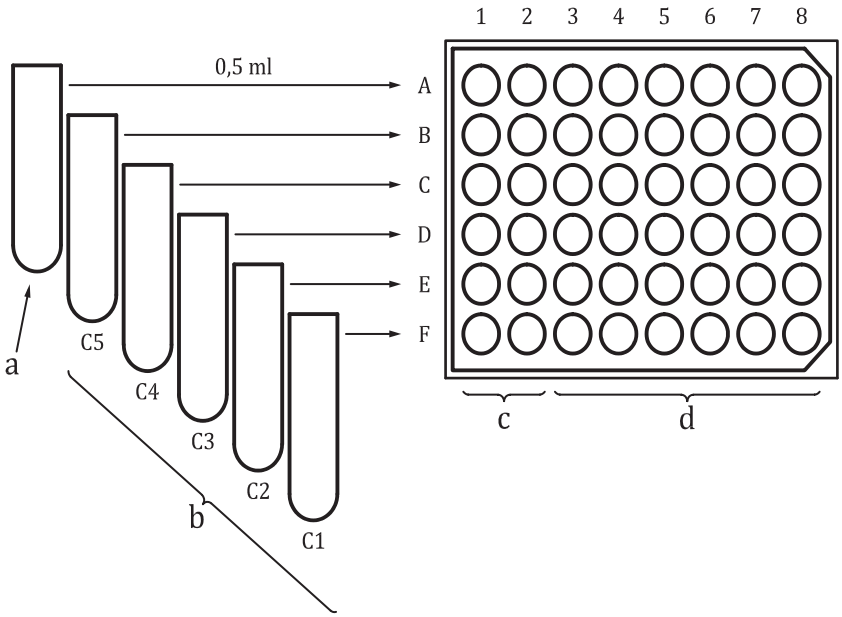
NOTE *Brachionus plicatilis* is a euryhaline species and toxicity tests can even be performed at salinities as low as 5 ‰.

Prepare the test solutions by mixing the appropriate volumes of the sample to be tested (see Clause 8 and 9.1) or of its initial dilution with dilution medium (6.2).

Depending on the purpose of the test, the exposure concentrations used in the test solutions at the start and end of the study should be verified by specific chemical analysis.

Control and test solutions can be prepared in 10 ml containers (e.g. tubes in glass or in inert plastic material).

The containers shall be labelled as control, C1, C2, C3, C4, and C5 in sequence of the highest to the lowest test concentration.



Key

a control medium
b toxicant dilutions
c rinsing wells
d replicates

Figure 1 — Filling of the microplate with control and test solutions

Distribute the test and control solutions in the microplate at the rate of 0,5 ml per well and according to the spatial distribution of the solutions in the wells as shown in Figure 1.

The 48 wells microplate has 6 rows (A to F) and 8 columns (1 to 8).

The 8 wells in the top row (row A) are filled with the control medium [= the dilution medium (6.2)].

The wells of the other rows are filled with the toxicants [test batches (3.3)] as follows: the 8 wells in row B are filled with the lowest toxicant dilution (C5), those of row C with the second lowest toxicant dilution (C4), etc.

Wells 3 to 8 in each row are for the 6 replicates of the control batch columns and the test batch columns, respectively.

The wells in columns 1 and 2 are “rinsing wells” intended to avoid dilution of the toxicant in the test wells during the transfer of the organisms from the Petri dish to the microplate.

9.3 Introduction of the organisms

As indicated in 6.1, rotifers from either laboratory cultures or hatched from cysts can be used for the toxicity test.

If rotifers from live cultures are used, transfer about 300 rotifers in a 5 cm Petri dish (7.2) containing 10 ml natural or artificial fresh water.

In case rotifers hatched from cysts are used, sufficient numbers of neonates for the toxicity test will be present in the hatching Petri dish.

Put the Petri dish on the glass stage of the stereomicroscope (7.5) and collect a number of (actively swimming) rotifers with the pipette (7.4) taking care to suck up as little hatching medium as possible during this operation.

Transfer about 25 test organisms into well 1 of row A of the microplate (first rinsing well of the control row) and repeat this operation for well 2 of row A (second rinsing well of the control row).

Proceed similarly to put about 25 rotifers in the 2 rinsing wells of row B (which is the lowest test concentration) and subsequently, in the 2 rinsing wells of the rows with increasing test concentrations.

Put the microplate on the glass stage of the stereomicroscope (7.5) and transfer five rotifers from the rinsing wells in row A (control batch) into the 6 wells of this row.

Repeat this operation for the five other rows going "from top to bottom", i.e. starting with row B (lowest test concentration) to row F (highest test concentration).

The pipette should be rinsed with dilution medium after the organisms have been transferred from the rinsing cups to the 6 test cups in each individual row.

On completion of the transfers, cover the microplate with a sheet of, for example, polyethylene and the microplate cover.

9.4 Incubation of the test system

Incubate the microplate at (25 ± 1) °C in the dark for 24 h and also for 48 h in case of evaluation of the 48 h effects.

9.5 Measurements

Take the cover and the sheet from the microplate and put the microplate on the glass stage of the stereomicroscope.

Check all the wells of rows A to F and record the number of dead rotifers in each well after 24 h exposure and/or 48 h exposure.

NOTE The organisms are considered dead if they do not show any movement during 10 s of observation.

Score the number of dead rotifers in each well on the data report template for the 24 h exposure and/or for the 48 h exposure (see Table 1).

Explanation to Table 1:

Exposure time:	hours	
Replicates:	The 6 wells in each row containing the same test medium and 5 rotifers	
Test dilution series	C5:	(lowest test concentration)
	C4:	
	C3:	
	C2:	
	C1:	(highest test concentration)

Table 1 — Data report template

Exposure time : hours						
	Control	C5	C4	C3	C2	C1
Replicate 1						
Replicate 2						
Replicate 3						
Replicate 4						
Replicate 5						
Replicate 6						
Total	/30	/30	/30	/30	/30	/30
% mortality						

On completion of the count, collect the contents of the 6 wells of the control row in a suitable container and measure the pH as specified in ISO 10523 and the oxygen concentration as specified in ISO 5814.

Mix the transferred (very small) volumes with great care in order to avoid adding oxygen from the air which could bias the oxygen measurement.

Perform the same operation and measurement for the test wells of the most concentrated test concentration (row F).

10 Estimation of the LC_{50}

Calculate the mean % mortality in the control and in each test concentration.

Determine the 24 h LC_{50} and/or the 48 h LC_{50} (plus, if deemed necessary, other effect percentages, for example, LC_{10} or LC_{90}) by an appropriate statistical method (see ISO/TS 20281 [2] and Reference [6], e.g. moving average or probit depending on the mortality values in the dilution series). Other models may be used depending on the shape of the dose-response curve as the objective is to obtain the best fit to the data (see ISO/TS 20281).

11 Reference test

Periodically, determine the 24 h LC_{50} and/or the 48 h LC_{50} of potassium dichromate (6.3) or copper sulfate in order to verify the sensitivity of the test organisms and the conformity to the test procedure.

Based on the performance data given in Annex C, the 24 h LC_{50} should be in the range 287 mg/l to 402 mg/l for a reference test with potassium dichromate ($K_2Cr_2O_7$) and in the range 0,14 mg/l to 0,42 mg/l for a reference test with copper sulfate ($CuSO_4 \cdot 5H_2O$).

12 Validity criteria

The test is considered valid if the percentage mortality in the negative controls is not higher than 10 %.

13 Test report

This test report shall contain at least the following information:

a) the test method used, together with a reference to this International Standard, i.e. ISO 19820:2016;

b) all information required for the complete identification of the sample or of the substrate under test;

c) the methods of preparation of the following samples:

   1) for effluents and waters, the method and the storage time of the samples, the dissolved oxygen concentration of the initial sample;

   2) for chemical substances, the method of preparation of the stock and test solutions;

   3) details of all chemical analysis performed on control and test solutions;

d) all biological, chemical, and physical information relative to the test specified in this International Standard;

e) all information relative to the test organism and, if need be, the origin and number of the batch of *B. plicatilis* cysts used;

f) all information relative to the test (sample concentrations, etc.);

g) the test results in accordance with Clause 10 and the method with which they were calculated;

h) the results obtained with the reference test (Clause 11) as well as the date of the reference test;

i) all operating details not specified in this International Standard, or regarded as optional, together with details of any incident that may have influenced the results;

j) name and address of the testing laboratory, the persons carrying out the test, and the person approving the report.

Annex A
(informative)

Procedure for hatching of *Brachionus plicatilis* cysts

The transfer of the cysts into the hatching medium shall be started 24 h to 26 h prior to the start of the toxicity test.

Cyst hatching is performed either in natural seawater or in artificial seawater, but at lower salinity (20 ‰) since this gives a higher and better hatching than at regular (35 ‰) seawater salinity.

A volume of 10 ml hatching medium is prepared by mixing 5,7 ml seawater (of 35 ‰ salinity) with 4,3 ml pure water in a test tube.

Pour the 10 ml hatching medium in a small Petri dish (5 cm diameter) and add approximately 15 mg cysts.

Incubate the Petri dish at (25 ± 1) °C for 24 h to 28 h with continuous illumination (light source of at minimum 3 000 lx to 4 000 lx).

NOTE If after 28 h the number of neonates is too small to start the test, incubate the Petri dish further for another 2 h.

Annex B
(informative)

Preparation of artificial seawater

A suitable artificial seawater for *Brachionus plicatilis* culturing and hatching of cysts is the ASPM medium (see Reference [8]).

To prepare 1 l of artificial seawater of 35 ‰ salinity, the following amounts of analytical grade reagents are dissolved one by one and in sequence, in 800 ml pure water in a 1 l calibrated flask:

NaCl:	26,40 g;
KCl:	0,84 g;
$CaCl_2 \cdot 2H_2O$:	1,67 g;
$MgCl_2 \cdot 6H_2O$:	4,60 g;
$MgSO_4 \cdot 7H_2O$:	5,58 g;
$NaHCO_3$:	0,17 g;
H_3BO_3:	0,03 g.

The calibrated flask is then filled to the 1 l mark with pure water and the flask is shaken to homogenize the medium.

Vials with concentrated solutions of the former reagents for preparation of 1 l ASPM artificial seawater are available commercially.[3)]

3) MicroBioTests Inc. Mariakerke, Belgium, is an example of a supplier able to provide commercially concentrated solutions for preparation of 1 l ASPM artificial seawater. This information is given for the convenience of the users of this document and does not constitute an endorsement by ISO of this supplier.

Annex C
(informative)

Performance data

C.1 General

In 1989, an extensive international interlaboratory comparison on the 24 h acute toxicity test with *B. plicatilis* has been organized in which more than 100 laboratories from Europe, USA, and Canada participated. The reference chemical used for this exercise was $CuSO_4 \cdot 5H_2O$.

Table C.1 gives the mean 24 h LC_{50} results obtained by the participants in Europe, USA, and Canada, respectively. The mean values are relatively close for the three geographical areas, but due to problems with the stability of the reference chemical, the variation coefficients were quite high for the European and USA laboratories.

Table C.1 — Results of the international interlaboratory comparison organized in 1989

	Europe	USA	Canada	Overall
Number of laboratories	71	27	22	120
Mean 24 h LC_{50} (mg/l $CuSO_4 \cdot 5H_2O$)	0,33	0,40	0,28	0,32
Coefficient of variation %	50,8	50,2	25,4	49,1

The findings of this international ringtest have been published (see Reference [6]).

The intralaboratory repeatability of the acute *B. plicatilis* test has been assessed over a period of 10 years in a laboratory in Belgium on reference tests with potassium dichromate. Table C.2 shows the 24 h LC_{50} and the 48 h LC_{50} values for 12 tests and for 10 tests, respectively which have been performed during the period 2003 to 2013 in this laboratory.

Table C.2 — Repeatability test results with potassium dichromate

Date of test performance	24 h LC_{50} mg/l	48 h LC_{50} mg/l
24/04/2003	335,63	
24/04/2003	356,14	
25/02/2004	327,97	216,24
01/09/2004	317,91	173,19
15/12/2004	386,10	260,24
22/06/2005	397,94	192,91
10/10/2006	356,87	185,02
10/07/2008	349,61	227,72
12/12/2008	300,41	196,51
27/04/2010	362,42	261,82
11/05/2013	320,00	223,44
11/05/2013	328,05	181,59
Number of tests	12	10
Mean	344,92	211,87
Standard deviation	28,75	31,57
Coefficient of variation %	8,34	14,90

The low variation coefficients (<10 % for the 24 h LC_{50} and 15 % for the 48 h LC_{50}) for the multiple reference tests carried out over a period of 10 years confirm the high repeatability of the acute *Brachionus plicatilis* toxicity test.

Bibliography

[1] ISO 5667-1, *Water quality — Sampling — Part 1: Guidance on the design of sampling programmes and sampling techniques*

[2] ISO/TS 20281, *Water quality — Guidance on statistical interpretation of ecotoxicity data*

[3] Environment Directorate. Current approaches in the statistical analysis of ecotoxicity data. A guidance to application. Paris: OECD, 2006. 147 p. (OECD Series of Testing and Assessments. No **54**. Available at http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2006)18&docLanguage=En

[4] Guillard R. Culture of phytoplankton for feeding marine invertebrates. In: Culture of Marine Invertebrates, (Berg D.J. ed.). Hutchinson-Ross, Stroudsberg, PA, 1983, pp. 108–32.

[5] Hoff F.H., & Snell T.W. Plankton Culture Manual. Florida Aqua Farms, Florida, USA, Fourth Edition, 1989, pp. 141.

[6] Persoone G., Blaise C., Snell T., Janssen C., Van Streertegem M. Cyst-based toxicity tests II. Report on an international intercalibration exercise with three cost-effective Toxkits. *Z. Angew. Zool.* 1992/1993, **79** (1) pp. 17–36

[7] Snell T.W. 1986, Effect of temperature, salinity and food level on sexual and asexual reproduction in *Brachionus plicatilis* (Rotifera). *Mar. Biol.* 1986, **92** pp. 157–162

[8] Snell T.W., & Boyer E.M. 1988, Thresholds for mictic female production in the rotifer *Brachionus plicatilis* (Muller). *Journal of experimental marine biology*, 1988, **124**, pp. 73-85

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