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Water quality — Determination of the acute toxicity to the freshwater rotifer *Brachionus calyciflorus*

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National foreword

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Qualité de l'eau — Détermination de la toxicité aigue envers le rotifère d'eau douce Brachionus calyciflorus

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Foreword

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

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Introduction

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

Rotifers, and especially the species *Brachionus calyciflorus*, are of interest from the ecotoxicological standpoint, 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 freshwater rotifer *Brachionus calyciflorus*, after 24 h exposure.

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Water quality — Determination of the acute toxicity to the freshwater rotifer *Brachionus calyciflorus*

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 calyciflorus* after 24 h exposure.

The method is applicable to:

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) freshwaters;

d) aqueous extracts.

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/TS 20281, *Water quality — Guidance on statistical interpretation of ecotoxicity data*

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, definition 3.1]

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

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

[SOURCE: ISO 6341:2012, definition 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, with subsequent calculation of the 24 h LC_{50}.

The test is carried out in one or two stages:

— a “range-finding test” to determine the range of concentrations or dilutions needed for calculation of the 24 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}.

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 calyciflorus*, obtained from a laboratory culture (see References [2],[3],[4]), or hatched from commercially available cysts[1].

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

6.2 Culturing and dilution medium

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

An example of artificial freshwater 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 calyciflorus* 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.

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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 wells (6 x 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 8 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 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 freshwater 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 2 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 half way 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.

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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 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 10 (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 6 replicates for each dilution + a control (i.e. the test medium without sample) also in 6 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

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).

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.

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.

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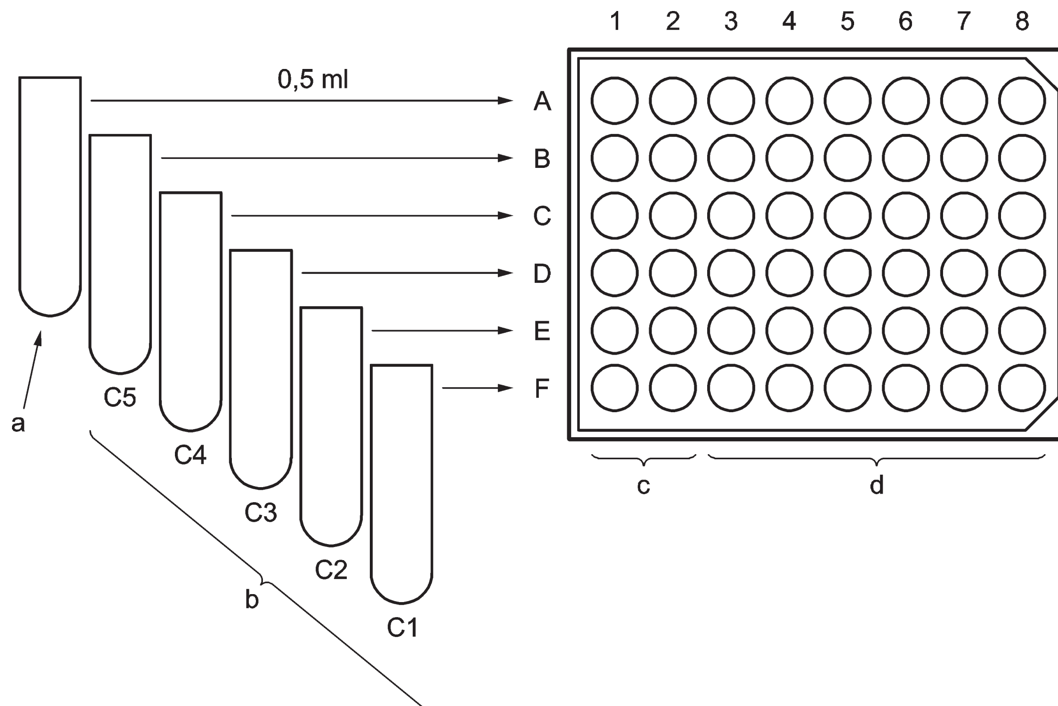

Key

a control medium

b toxicant dilutions

c rinsing wells

d replicates

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

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.

The 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 freshwater.

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

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Put the Petri dish with the rotifers 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 5 rotifers from the rinsing wells in row A (control batch) into the 6 wells of this row.

Repeat this operation for the 5 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 wells to the 6 test wells in each individual row.

On completion of the transfers, cover the microplate with a sheet of, e.g. 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.

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.

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.

Explanation to Table 1:

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)

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Table 1 — Data report template

	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						

10 Estimation of the LC_{50}

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

Determine the 24 h LC_{50} (plus if deemed necessary, other effect percentages, e.g. LC_{10} or LC_{90}) by an appropriate statistical method (see ISO/TS 20281] and Reference [5] 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} of potassium dichromate or copper sulfate (6.3) 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 7,58 mg/l to 21,34 mg/l for a reference test with potassium dichromate ($K_2Cr_2O_7$) and in the range 0,011 mg/l to 0,040 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 (ISO 19827:2016);

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

c) the methods of preparation of the samples:

   1) for effluents and waters, the method and the storage time of the samples;

   2) for chemical substances, the method of preparation of the stock 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. calyciflorus* cysts used;

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

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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 performance of the reference test;

i) data to prove that the validity criteria given in Clause 12 are met;

j) 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;

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

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Annex A
(informative)

Procedure for hatching of *Brachionus calyciflorus* cysts

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

Cyst hatching is performed either in natural or in artificial freshwater.

The composition of an artificial freshwater suitable for hatching *B. calyciflorus* cysts is given in Annex B.

Put 10 ml natural or artificial freshwater in a Petri dish (7.2) and add approximately 15 mg cysts.

Incubate the Petri dish at (25 ± 1) °C for 16 h to 18 h with continuous illumination (light source of at minimum 3 000 lx to 4 000 lx).

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Annex B
(informative)

Preparation of artificial water

A suitable artificial freshwater for *Brachionus calyciflorus* culturing and hatching of cysts is the medium prescribed in Reference[7] and which is prepared as follows:

Dissolve the following mineral substances in 1 l of pure water (3.4):

$NaHCO_3$	96 mg
$CaSO_4 \cdot 2H_2O$	60 mg
$MgSO_4$	60 mg
KCl	4 mg

This test medium corresponds to a synthetic water of moderate hardness, i.e. 80 mg $CaCO_3$ to 100 mg $CaCO_3$ per litre. Thus prepared, the medium has a pH of 7,6 ± 0,3.

When stored in the refrigerator (4 ± 1) °C in the dark, the solution can be used for several months.

Aerate the test medium until the dissolved oxygen concentration has reached the air saturation value and until the pH has stabilized. If necessary, adjust the pH to 7,6 ± 0,3 using a sodium hydroxide or hydrochloric acid solution. The concentration of the acid or base required shall be selected so that the volume to be admixed is as small as possible. Bring the temperature of the test medium up to (25 ± 1) °C prior to use.

Vials with concentrated solutions of the former reagents for preparation of 1 l 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 artificial freshwater. This information is given for the convenience of the users of this document and does not constitute an endorsement by ISO of this supplier.

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Annex C
(informative)

Performance data

C.1 General

In 1989 an extensive international interlaboratory comparison on the 24 h acute toxicity test with *B. calyciflorus* has been organized, with participation of more than 150 laboratories from Europe, the USA and Canada. The reference chemical used for this ringtest was $CuSO_4 \cdot 5H_2O$.

Table C.1 gives the mean 24 h LC_{50} results obtained by the participants in Europe, the USA and Canada respectively. The mean values are relatively close for the 3 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 on the acute *B. calyciflorus* test organized in 1989

	Europe	USA	Canada	Overall
Number of laboratories	102	38	30	170
Mean 24 h LC_{50} (mg/l $CuSO_4 \cdot 5H_2O$)	0,033	0,038	0,026	0,033
Coefficient of variation %	41,5	67,8	27,8	48,5

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

The intralaboratory repeatability of the acute *B. calyciflorus* test has been assessed during 7 training courses on toxicity tests which have been organized between 1990 and 2001 at the Laboratory of Environmental Toxicology and Aquatic Ecology of the Ghent University in Belgium, and at the Biological Station of the Université Blaise Pascal in France. All the assays have been performed on the reference chemical potassium dichromate ($K_2Cr_2O_7$).

Table C.2 gives an overview of the mean 24 h LC_{50} and the variation coefficients obtained by the trainees from a large number of countries.

Table C.2 — Results of the acute *B. calyciflorus* tests from 7 training courses

Year	Place	Number of participants	Mean 24 h LC_{50} mg/l	Coefficient of variation %
1990	Ghent University - Belgium	25	13,7	14,0
1992	Ghent University - Belgium	23	10,8	18,6
1994	Ghent University - Belgium	26	11,3	14,0
1996	Ghent University - Belgium	14	14,1	35,8
1999	Université Blaise Pascal – France	13	10,3	20,8
2000	Université Blaise Pascal – France	13	10,0	12,1
2001	Université Blaise Pascal - France	18	15,7	18,0

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The intralaboratory repeatability of the acute *B. calyciflorus* test has also been determined by two companies in the Netherlands and in Belgium, on reference tests performed with potassium dichromate. Table C.3 shows the 24 h LC_{50} for 9 tests and for 14 tests respectively obtained in the 2 companies.

Table C.3 — Repeatability results of the acute *B.calyciflorus* test obtained by 2 laboratories

Country	Period	Number of tests	Mean 24 h LC_{50} mg/l	Coefficient of variation %
The Netherlands	1993 to 1994	9	13,0	22,4
Belgium	2003 to 2013	14	12,7	22,0

The data in Tables C.2 and C.3 for the very large number of tests carried out over a period of more than 20 years, show that the LC_{50} figures are all in a range of 10 mg/l to 14 mg/l with variation coefficients of (all but one value) between 12 % and 22 %, and hence confirm the high repeatability of the acute *B. calyciflorus* toxicity test.

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