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Water quality — Determination of the chronic toxicity to *Brachionus calyciflorus* in 48 h

Qualité de l'eau — Détermination de la toxicité chronique vis-à-vis de Brachionus calyciflorus *en 48 h*

Reference number
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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

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 20666 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Introduction

The evaluation of harmful effects on water quality has for several years involved the performance of biological tests. Rotifera, and especially the species *Brachionus calyciflorus*, are of interest from the ecotoxicological standpoint because they offer the advantage of breeding by parthenogenesis and of possessing a very short generation time: a single mother maintained under favourable conditions over 48 h reproduces several times. *Brachionus calyciflorus* is an organism of the zooplankton, which lives in fresh water. These animals are primary consumers and serve as prey for a large number of fish larvae and invertebrates.

The test specified in this International Standard is carried out over 48 h and therefore involves at least three reproductions from a single parent organism (see Reference [11]).

Water quality — Determination of the chronic toxicity to *Brachionus calyciflorus* in 48 h

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 the determination of the chronic toxicity to rotifer *Brachionus calyciflorus*, based on population growth inhibition in 48 h.

The method is applicable to:

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

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

c) fresh waters;

d) aqueous extracts.

This International Standard is not applicable to the testing of unstable chemicals (hydrolysing, absorbing, etc.) in water unless exposure concentration is measured, nor to the testing of aquatic samples from the estuarine or marine environment.

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 5667-16:1998, *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** (3.2)

[ISO 20665:2008]

NOTE In this International Standard, eight replicates constitute the control batch.

3.2
control solution
mixture of test medium and of food without sample under test

[ISO 20665:2008]

3.3
effective concentration producing x % population growth inhibition
EC_x
estimated concentration of the sample giving rise to x % **population growth inhibition** (3.4) with respect to the **control batch** (3.1)

3.4
population growth inhibition
comparison of the total number of females (offspring and mothers) at the end of the test between the **control batch** (3.1) and the **test batch** (3.5)

3.5
test batch
series of replicates filled with the same **test solution** (3.6)

[ISO 20665:2008]

NOTE In this International Standard, eight replicates constitute a test batch.

3.6
test solution
mixture of test medium, of food and of sample under test

[ISO 20665:2008]

4 Principle

Female *Brachionus calyciflorus*, less than 2 h old at the beginning of the test, are exposed individually to a range of concentrations of the sample under test for a period of 48 h. The test focuses on the population growth of planktonic rotifers by parthenogenetic reproduction. At the end of the test, the number of female rotifers is determined and, by comparison with the control, the population growth inhibition percentages are determined for each concentration.

The data obtained allow, using a regression model, the calculation of the concentration which gives rise to x % population growth inhibition, EC_x, e.g. EC_{10}, EC_{20} or EC_{50}.

5 Test environment

Carry out the test in the dark, in a thermostatically controlled room or chamber so as to obtain a temperature of (25 ± 1) °C in the test containers.

Maintain the atmosphere free from toxic dusts or vapours. This is checked by producing control solutions.

6 Reagents, test organisms and media

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

6.1 Test organisms

Females of the species *Brachionus calyciflorus* (Monogonota, Rotifera) are obtained from a laboratory culture (see References [3], [12], [13]) or born from commercially available cysts[1]. Sensitivity of the test for organisms should be performed with copper sulfate pentahydrate or sodium pentachlorophenolate (NaPCP) (see 6.5).

If cysts are used, employ first generation *Brachionus calyciflorus*, obtained by hatching of cysts under the following conditions.

Transfer the cysts to a container containing the test medium (6.3), e.g. 15 mg of cysts in approximately 10 ml of test medium. Incubate the container at (25 ± 1) °C for 18 h to 24 h, under continuous lighting of intensity 1 000 lx to 4 000 lx (7.7).

A food supply is not necessary for the hatching of the cysts. A better multiplication rate of the rotifers is, however, achieved by adding, just after the emergence of the first neonates, algae in identical quantity to that indicated in 6.4. Alternatively, 100 µg/l of the inert food ROTIRICH[1] can be added as pre-feeding supplement (Reference [18]).

The animals used for the test shall be less than 2 h old, the hatching should therefore be supervised as from 17 h of incubation, then every half hour.

The test is started when the number of young rotifers is considered sufficient to perform a complete test.

EXAMPLE For a test with five concentrations and one control (i.e. 48 rotifers), this condition is generally fulfilled about 1 h after the first hatching has been observed. Hatching time is quite stable within one laboratory, allowing hatching to be planned in advance to provide sufficient offspring during working hours.

6.2 Pure water, having a conductivity below 10 µS/cm[2].

6.3 Test medium, prepared by dissolving the following mineral substances in 1 l of pure water (6.2):

$NaHCO_3$	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 (see Reference [14]). Thus prepared, the medium has a pH of 7,6 ± 0,3.

Store this solution in the dark at ambient temperature and use within 7 d of preparation.

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1) Dehydrated rotifer cysts and ROTIRICH are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

2) 1 mS/m.

Aerate the test medium until the dissolved oxygen concentration has reached the air saturation value and until the pH has stabilised. 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.

6.4 Food, composed of *Chlorella vulgaris* algae.

The algae are grown in any suitable medium (e.g. LC OLIGO, see Annex A). They are used when the culture is in the exponential growth phase. The algal concentration in the test shall be between 2×10^6 and 3×10^6 cells per millilitre. To achieve this, previously adjust the concentration by centrifuging the culture (e.g. for 20 min at 20 000 m s^{-2}) and resuspending the algae by shaking with a sufficient volume of test medium (6.3) in order to obtain a suspension of around 2×10^7 cells per millilitre to 3×10^7 cells per millilitre. This concentration allows the provision of 2×10^6 to 3×10^6 cells by using a volume of 0,1 ml.

The algae culture may be stored at (4 ± 3) °C, in darkness, for a maximum period of 10 d.

The food can also be composed of *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum* or *Raphidocelis subcapitata*) algae[3)]. The algal concentration in the test shall then be between 1×10^6 cells and $1,5 \times 10^6$ cells per millilitre.

The use of algae immobilised in an inert matrix (gelose), in the form of algae beads[4)] is possible. In this case, after dissolving the matrix, centrifuge the algae, discard the supernatant, and resuspend the algae by shaking in the test medium (6.3). Repeat this operation a second time. The algal concentration in the test medium shall be in the range specified above.

6.5 Reference substance

NaPCP (C_6Cl_5ONa) and/or copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) can be used.

CAUTION — If sodium pentachlorophenolate is used as a reference toxicant, the material safety data sheet should be consulted prior to use by laboratory personnel due to the hazardous nature of this substance.

7 Apparatus

Usual laboratory equipment and in particular the following.

7.1 Thermostatically controlled room or **chamber**.

7.2 Test containers; disposable microplates made from chemically inert material, comprising wells with a capacity ⩾1 ml, allowing a water level if possible below the depth of focus of the magnifying glass (7.5) to be obtained. For example, 24 (4 × 6) well microplates with a well diameter of approximately 16 mm, are suitable. Do not use round bottomed microplates. Alternatively, use single closed containers.

7.3 Device for measurement of algal concentration, for example, a microscope equipped with a haemocytometer or particle counter. Indirect methods (e.g. spectrophotometer, turbidimeter, fluorimeter) can be used if an acceptable correlation with the cellular concentration can be established.

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3) The Freshwater Biological Association, Ambleside, UK, is an example of a supplier able to provide these algae commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this supplier.

4) MicroBioTests, Mariakerke (Gent), Belgium, is an example of a supplier able to provide suitable algal beads commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this supplier.

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 Binocular magnifying glass, with a magnification of at least 8 times and, if possible, a continuous magnification.

7.6 Image analysis system, to count and measure *Brachionus calyciflorus*.

7.7 Light source, providing a range of light intensity in the test containers (7.2) of 1 000 lx to 4 000 lx.

7.8 Sample collecting bottles, in accordance with ISO 5667-16:1998, 3.2.

7.9 Sieve, of nominal size of openings of nominal size of openings < 50 µm (a sifting cloth having a nominal size of openings of 10 µm or 20 µm is suitable).

8 Treatment and preparation of samples

8.1 Special precautions for sampling, transportation, storage and treatment of water, effluent, or aqueous extract samples to be tested

Sampling, transportation and storage of the samples should be performed in accordance with the general procedures specified in ISO 5667-16.

Collect the samples in bottles made from chemically inert materials (7.8).

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 4 °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 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, homogenise 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 substances and the surface of the liquid.

If the raw sample or 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. However, this sample manipulation should be avoided unless absolutely necessary since it may change physicochemical characteristics and possibly remove some toxicant from the sample.

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

Measure the pH (as specified in ISO 10523) and the dissolved oxygen concentration (as specified in ISO 5814) and record these values in the test report (Clause 12).

If the aim of the test is to assess the chronic toxicity without considering the pH effects, the test may also be carried out after adjustment of the pH to $7{,}6 \pm 0{,}3$ with hydrochloric acid or sodium hydroxide solutions. Proceed, if appropriate, as indicated above, for the separation of the suspended matter formed following the adjustment of the pH. Mention any pH adjustment in the test report (Clause 12).

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 (6.3) 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 of ISO 5667-16.