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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
ISO 20666:2008(E)

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Foreword

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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.

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.

9 Procedure

9.1 Selection of concentrations

The test should comprise at least five concentrations of the sample to be tested, selected within a geometric series with a separation factor not exceeding 3,2.

Take the following criteria into account for selecting the range of concentrations to be examined: to obtain an EC_x value, it is desirable that at least one concentration higher by x % than this is used and at least one x % lower.

Produce at least 8 replicates for each concentration. These replicates constitute a test batch (3.5).

Include in each test a control batch (3.1) without any sample to be tested. A control batch is also made up of at least eight replicates (better 16).

Where a chemical (solvent, emulsifier, extractant, etc.) is used in order to solubilise or disperse the substances, the concentration of the chemical shall be identical in all containers and a second control batch containing the chemical at the concentration being used shall be included. In this case, when a minimal but still acceptable effect is seen in the solvent control, this one has to be used in statistical endpoint calculations. This is the situation where the effect in the solvent control is minor and the test is still valid.

In the case of samples of waters, effluents, and aqueous extracts, the highest tested concentration cannot be equal to a volume fraction of 100 % of the initial sample on account of the food supply, which shall be added in the lowest possible volume ($\leqslant$10 % of the final volume of the sample and its dilutions).

9.2 Preparation of the test and control solutions

Prepare the test solutions by mixing the appropriate volumes of the sample to be tested (8.1 or 8.2) or of its initial dilution with test medium (6.3) and food (6.4).

Calculate the quantity of food contained in each well of the microplate as given in 6.4. The total volume shall be 1 ml per well.

Prepare the control solutions by mixing the test medium (6.3) and the food (6.4). If a chemical is used according to 9.1, prepare a solvent control for the chemical.

Distribute the test and control solutions at the rate of 1 ml per well, approximately 1 h to 2 h before the end of the hatching period.

NOTE 1 By using two 4 $\times$ 6 well microplates, they can be arranged in order to present 6 columns of 8 wells constituting a batch of 8 replicates. One column is used for the control and the other 5 columns are used for testing the 5 different concentrations.

NOTE 2 It is also possible to perform the dilutions of the sample under test directly in the wells, either in dilution series or from a stock solution or from its dilution. The food is then introduced into the wells according to 6.4. The total volume is 1 ml per well. In this case, in order to avoid well intercontamination, it is advisable to start depositing the food (6.4) in the column of control wells, then to continue adding food from the lowest to the highest concentration. However, if volatile compounds are suspected to be present, it is necessary to replace open microplates by single closed containers for each replicate or an adhesive foil covering all wells with appropriate piercing.

9.3 Introduction of the organisms

Place the microplates or single closed containers containing the test and control solutions in a thermostatically controlled room or chamber (7.1) so as to obtain a test and control solutions temperature of (25 $\pm$ 1) °C.

Under a binocular magnifying glass (7.5), sample a *Brachionus calyciflorus* using a sampling pipette (7.4), then deposit it below the surface of the test solution, minimising the volume of hatching medium introduced together with the rotifer.

Maintain the *Brachionus calyciflorus* at (25 ± 1) °C during the different phases of the test, thus protecting the rotifers against all temperature variations which could subsequently induce the emergence of males.

NOTE 1 In order to facilitate the sampling of the rotifers, particularly if the latter are numerous in the hatching container, they can be diluted in the test medium (6.3), the latter having been previously brought up to the same temperature as the hatching medium.

NOTE 2 Using a 1 ml capillary mini-pipette, the volume used for the transferral of the organism is generally less than 20 µl.

Place one *Brachionus calyciflorus* per well or single closed container by proceeding by increasing concentrations and by changing the pipette between each series.

Begin with series 1 in the order: control; concentration, C_1, concentration C_2, concentration C_3, concentration C_4, concentration C_5. Change the pipette and continue in the same manner for series 2. Change the pipette and continue in this manner up to series 8. See Table 1.

Table 1 — Data report template

Series No	Control	Concentration				
		C_1	C_2	C_3	C_4	C_5
1						
2						
3						
4						
5						
6						
7						
8						

Verify the presence of a single rotifer in each well or single closed container. Possibly withdraw or add the surplus or missing rotifer(a).

Cover the microplate or single closed container with a film, e.g. polyethene and/or with the microplate cover.

9.4 Incubation of the test system

Incubate the plate at (25 ± 1) °C in the dark for 48 h. It is recommended to carry out the incubation in a water saturated atmosphere in order to avoid losses of test solution by evaporation. For example, arrange the microplate in a closed dish at the bottom of which a film of water has been previously deposited.

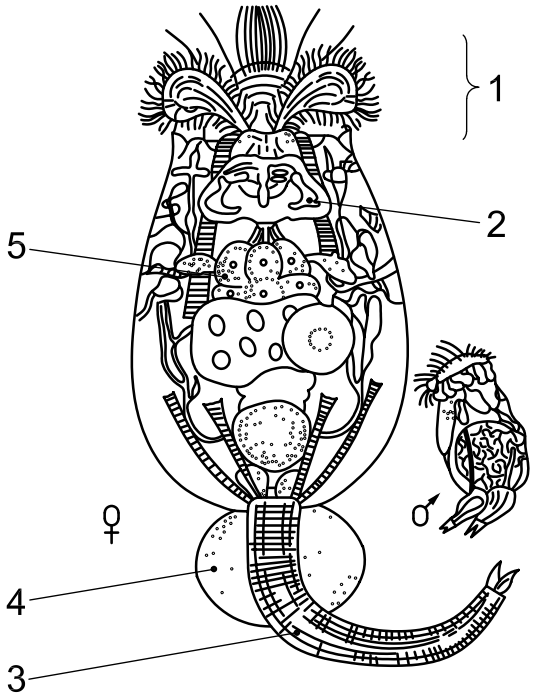
9.5 Measurements

At the end of the 48 h incubation period, count under a binocular magnifying glass (7.5) or image analysis system (7.6), in each well or single closed container, the living female *Brachionus calyciflorus*. The reading diagram (counting) shall follow the diagram for introduction of the rotifers given in 9.3.

In order to determine whether a rotifer is dead, observe it under a binocular magnifying glass (7.5) for about 5 s and check the absence of movement of the mastax and of the ciliary corona (see Figure 1).

NOTE In cases of very good reproduction of the rotifers (more than 10 organisms per well), it may happen that in some wells a few male rotifers are present. This is probably the result of "overcrowding". Since males are only observed under circumstances of "very good reproduction" (i.e. mainly in the controls) their presence is not related to the toxic effect of the test compound(s) under investigation.

Male adult *Brachionus calyciflorus* are 3 to 5 times smaller than females and they move about far more quickly. These two criteria in particular allow a clear differentiation to be made between males and females.



Key

1 ciliary corona
2 mastax
3 foot
4 egg
5 stomach

Figure 1 — Diagram of female and male *Brachionus* rotifers
(according to Reference [8], magnification 300 times)

In the case where, at the end of the test, the high number of rotifers per well would make counting difficult (e.g. over 10), a preliminary stage of concentration of the organisms may be necessary. Filter the contents of the well through a sieve (7.9) of nominal size of openings < 50 µm (a sifting cloth having a nominal size of openings of 10 µm or 20 µm is suitable). Immediately after having sampled the solution contained in the well, add some test medium (6.3) to the empty well in order to maintain the remaining rotifer(a), if any, in an aqueous environment. Count the live female *Brachionus calyciflorus* directly in the sieve. Observe the well again in order to count any remaining rotifers.

On completion of the count, gather the contents of the control batch wells in a suitable container and measure the pH according to ISO 10523. Proceed accordingly for the measurement of the pH of the most concentrated test solution.

10 Expression of results

10.1 Determination of the population growth inhibition

On completion of the count, determine the number of live female rotifers for each concentration, N_e, by adding the values of the counts of each replicate. Also determine the total number of live female rotifers in the control batch, N_t, by adding the values of the counts of each replicate.

Determine the mean number of live female rotifers for each concentration, $\overline{N}_e$, by dividing N_e by the number of replicates. Determine the average number of live female rotifers in the control batch, $\overline{N}_t$, by dividing N_t by the number of replicates.

Calculate population growth inhibition, expressed as a percentage, I, for each concentration using Equation (1):

$$I = \frac{\overline{N}_t - \overline{N}_e}{\overline{N}_t} \times 100 \quad (1)$$

where

$\overline{N}_e$ is the mean number of live female rotifers for a concentration;

$\overline{N}_t$ is the mean number of live female rotifers in the control batch.

10.2 Determination of the toxicity parameters

In order to estimate the concentration which would cause x % of growth inhibition, EC_x (e.g. EC_{10}, EC_{20} or EC_{50}), it is possible to adjust a model to the test results. For this, the values of EC_x and the parameters characterising this model should be estimated with their confidence limit (e.g. 95 %).

Assess the adjustment of the model to the data either by using a statistical test or by graphic representation.

It is possible to use the logistic model that is suitable for the statistical analysis of the data produced.

NOTE This model has been used within the framework of the analysis of the results of the interlaboratory test (see Annex B).

This model, in which N_Y is the total number of live female rotifers per replicate (variable), is characterised by Equation (2):

$$N_Y = \frac{\overline{N}_a}{1 + \left(C/EC_{50}\right)^b} \quad (2)$$

where C is the concentration being tested (test variable).

The following parameters, characterising the model, are estimated from the obtained data (e.g. by the least squares method):

EC_{50} is the concentration which causes 50 % of population growth inhibition;

$\overline{N}_a$ is the average number of live female rotifers expected in the control;

b is the slope of the curve.

The EC_x can then be estimated by Equation (3):

$$EC_x = EC_{50}\left(\frac{x}{100-x}\right)^{1/b} \quad (3)$$

where x is the effect, expressed as a percentage, that has been chosen (10, 20, 50, etc.) for calculating the EC_x (initially defined parameter).

Other models can be used. In this case, describe the model being used in the test report (Clause 12).

Check the sensitivity of the biological reagent and the conformity of application of the procedure. Periodically (at least every 2 to 3 months) or at the same time as the test, determine the EC_{50} at 48 h value for NaPCP and/or of the copper sulfate pentahydrate (6.5) by applying the protocol described in this International Standard. Indicate the EC_{50} at 48 h value and the date obtained in the test report (see Annex B for results of a ring test).

11 Validity criteria

The test is considered valid if the following conditions are met:

a) reproduction of *Brachionus calyciflorus* is observed in at least 87,5 % of the replicates (equivalent to 7 replicates out of 8) of the control batch at the end of the test;

b) the average number of live female *Brachionus calyciflorus* counted per well in the control batch is greater than or equal to 3 at the end of the test.

12 Test report

The test report shall include at least the following information:

a) all information required for the complete identification of the sample or of the substance under test;

b) the methods of preparation of the samples;

   1) for effluents, waters, and aqueous extracts, the method and the storage time of the samples, the pH and the dissolved oxygen concentration of the initial sample, if need be, the conditions in which the decantation, filtration or centrifugation of the sample and a possible adjustment of the pH were carried out,

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

c) a reference to the test method used, with reference to this International Standard;

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

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

f) all information relative to the test (light intensity, species and quantity of algae used as food, if need be the origin and number of the batch of algae beads used, sample concentrations, pH of the test and control solutions, etc.);

g) the results of the test according to Clause 10, the method with which they were calculated, and the concentration-response curves;

h) the results obtained with the reference substance(s) according to Clause 11, as well as the date of test;

i) any abnormal behaviour of the *Brachionus calyciflorus* under the test conditions;

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.

Annex A
(informative)

Preparation of the LC OLIGO medium

A.1 Reagents

A.1.1 Solution 1

Dissolve 4 g of calcium nitrate tetrahydrate [$Ca(NO_3)_2 \cdot 4H_2O$] in 100 ml pure water (6.2).

This solution may be stored for 3 months at (4 ± 3) °C.

A.1.2 Solution 2

Dissolve 10 g of potassium nitrate (KNO_3) in 100 ml pure water (6.2).

This solution may be stored for 3 months at (4 ± 3) °C.

A.1.3 Solution 3

Dissolve 3 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) in 100 ml pure water (6.2).

This solution may be stored for 3 months at (4 ± 3) °C.

A.1.4 Solution 4

Dissolve 4 g of anhydrous potassium hydrogen phosphate (K_2HPO_4) in 100 ml pure water (6.2).

This solution may be stored for 3 months at (4 ± 3) °C.

A.1.5 Solution 5

Dissolve the following compounds in approximately 800 ml pure water (6.2).

copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$)	30 mg
ammonium heptamolybdate tetrahydrate [$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$]	60 mg
zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$)	60 mg
cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$)	60 mg
manganese nitrate tetrahydrate ($Mn(NO_3)_2 \cdot 4H_2O$)	60 mg
citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$)	60 mg
boric acid (H_3BO_3)	60 mg

Dilute to 1 l with pure water (6.2).

This solution can be stored for 3 months at a temperature of (4 ± 3) °C.

A.1.6 Solution 6

Dissolve in approximately 800 ml of preheated water, while shaking, 1 625 mg of iron(III) citrate pentahydrate ($C_6H_5O_7Fe{\cdot}5H_2O$). The dissolution may require shaking overnight at a temperature of around 50 °C.

After cooling down, dissolve, while shaking, the following compounds:

iron(II) sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$)	625 mg
iron(III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$)	625 mg

Dilute to 1 l with pure water (6.2).

This solution can be stored for 3 months at a temperature of (4 ± 3) °C.

A.2 Procedure

Transfer to approximately 800 ml of pure water (6.2), shaking each time, 1 ml of solutions 1 to 4 (A.1.1, A.1.2, A.1.3, A.1.4) and 0,5 ml of solutions 5 and 6 (A.1.5 and A.1.6). Dilute to 1 l with pure water (6.2).

Check that the pH of the solution obtained is between 6,8 and 7,2. Adjust it, if need be, with hydrochloric acid or sodium hydroxide.

Sterilise the solution either in an autoclave (20 min at 120 °C) or by passing through a 0,22 µm mesh size filter. This solution may be stored for a maximum period of 1 month at a temperature of (4 ± 3) °C.

Annex B
(informative)

Precision data

An interlaboratory test concerning two substances, NaPCP and copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) was carried out in 1999. The results are given in Table B.1.

Table B.1 — Results of the interlaboratory test

Substance	No. laboratories	EC_{50} at 48 h µg/l			
		Mean	Range	Standard deviation	Coefficient of variation
NaPCP	12	548	136 to 982	232	42 %
$CuSO_4 \cdot 5H_2O$ [expressed as Cu(II)]	11	53,5	20 to 86,9	17,7	33 %

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