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Water quality — Determination of chronic toxicity to *Ceriodaphnia dubia*

Qualité de l'eau — Détermination de la toxicité chronique vis-à-vis de Ceriodaphnia dubia

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Contents

Page

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 20665 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Introduction

The highlighting of harmful effects for water quality has for several years involved the carrying out of biological tests. The Cladocera, *Ceriodaphnia dubia,* is recognised as being representative of the zooplankton species widely used in aquatic toxicity tests.

The shortness of the chronic toxicity test, (7 ± 1) d, and the low volumes used are major assets for obtaining relevant results on samples that may be subject to changes during the storage period.

The user should be aware that particular problems could require the specifications of additional marginal conditions.

Water quality — Determination of chronic toxicity to *Ceriodaphnia dubia*

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 *Ceriodaphnia dubia* (Cladocera, Crustacea), based on reproduction inhibition after (7 ± 1) d.

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, if appropriate after decantation, filtration or centrifugation;

c) fresh waters;

d) aqueous extracts.

This International Standard is not applicable 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 6059, *Water quality — Determination of the sum of calcium and magnesium — EDTA titrimetric method*

ISO 10523, *Water quality — Determination of pH*

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
brood
group or cohort of sibling offspring, consisting of two or more neonates in any test container, during any given day of the test, released from the adult female during an inter-moult period (i.e. before the carapace is shed by that female during moulting)

3.2
brood organism
healthy adult female daphnid that produces and releases multiple broods of live neonates

3.3
control batch
series of replicates containing **control solution** (3.4)

NOTE In this International Standard, 10 replicates constitute the control batch.

3.4
control solution
mixture of test medium and of food without sample under test

3.5
effective concentration producing x % reproduction inhibition
EC_x
estimated concentration of a test sample giving rise to x % **reproduction inhibition** with respect to the **control batch** (3.3), which represents a point of the test sample concentration that is estimated to cause a designated percent impairment in a quantitative biological function

3.6
neonate
newly born or newly hatched individual

NOTE In this International Standard, a neonate is a first-instar daphnid, < 24 h old.

3.7
reproduction inhibition
comparison between the number of living offspring born from all adults at the end of the test between the **control batch** (3.3) and the **test batch** (3.8)

3.8
test batch
series of replicates containing the same **test solution** (3.9)

NOTE In this International Standard, 10 replicates constitute a test batch.

3.9
test solution
mixture of test medium, of food and of sample under test

4 Principle

Ceriodaphnia dubia, less than 24 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 (7 ± 1) d. The test typically ends after 7 d when 60 % of the control organisms have produced their third brood. The mortality of the adult females and their reproduction are monitored throughout the exposure time. All other relevant biological parameters can also be studied.

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

5 Test environment

Carry out the test in a temperature-controlled room or chamber at (25 ± 2) °C in the test containers. Ensure that, within one test, the temperature does not vary by more than 2 °C.

Adjust the day/night test cycle (photoperiod) to 16 h of daylight and 8 h of darkness. In the test containers (7.2), a range of lighting intensity at the air/water interface of 100 lx to 600 lx (7.8) is recommended. Do not shake or aerate 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 being performed in an atmosphere free from toxic dusts and vapours.

6 Reagents, test organisms and media

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

6.1 Test organisms

Ceriodaphnia dubia neonates are obtained by parthenogenesis from adult females for at least three generations under the conditions of temperature, photoperiod and food identical to those in the test.

The *Ceriodaphnia dubia* used for the test shall be less than 24 h old and shall have been taken from a brood comprising at least eight newly born animals.

The day before the test, isolate from the culture a dozen or more adults that are over 6 d and less than 14 d old. Isolate each one in a separate container containing food (6.4.1 or 6.4.2) and test medium (6.3.2 or 6.3.3). Before the test, remove the adults from their containers and count the offspring. Discard all vessels containing less than eight live offspring.

The *Ceriodaphnia dubia* may also derive from the hatching of ephippia purchased from a specialised company[1)]. These organisms may be directly used as test organisms.

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

6.3 Test media

6.3.1 General

Two test media are recommended: ELENDT M4 (6.3.2) or moderately hard water (6.3.3). Alternative test media may be used as long as validity criteria (Clause 11) are met.

For alternative test media, supply either a reference to a publication, or for natural waters (in case of effluent testing) the date of collection, details of storage, handling, and additions, as well as physical chemistry data relating to major ions [Na(I), K(I), Ca(II), Mg(II), carbonates, chloride, sulfate], pH and dissolved organic carbon.

1) Microbiotest, Deinze, Belgium, is an example of a supplier able to provide suitable ephippia commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this supplier.

2) 1 mS/m.

6.3.2 ELENDT M4 medium option

Prepare ELENDT M4 test medium in accordance with Annex A. The test medium thus prepared shall have a pH of 8,0 ± 0,3 (measured as specified in ISO 10523), a total hardness of (250 ± 20) mg/l (expressed as $CaCO_3$ and measured as specified in ISO 6059). 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 8,0 ± 0,3 using a diluted solution of sodium hydroxide or hydrochloric acid.

NOTE On account of the high hardness of the test medium and of the presence of EDTA within this medium, the bioavailability of the bivalent metal ions can be reduced, thus resulting in a decrease in the apparent toxicity of these ions.

6.3.3 Moderately hard water medium option

Prepare moderately hard water in accordance with Annex B. The test medium thus prepared shall have a pH of 7,4 to 7,8 (measured as specified in ISO 10523) and a total hardness of (90 ± 10) mg/l (expressed as $CaCO_3$ and measured as specified in ISO 6059).

6.4 Food

6.4.1 Option 1: Fish food and two algae diet

The food is composed of fish food, *Chlorella vulgaris* algae and *Pseudokirchneriella subcapitata*[3] algae (formerly known as *Selenastrum capricornutum* and *Raphidocelis subcapitata*) (see NF T90-376[1] and Reference [2]).

Prepare a 5 g/l suspension of fish food[4] in the test medium (6.3.2), homogenised with a crusher or any other means allowing particles of a few micrometers to be obtained. Prepare this suspension each day for the daily feeding of cultures or during testing.

Grow the algae separately in any suitable medium (e.g. LC OLIGO, Annex C). Use them when the culture is in the exponential growth phase and has reached a density greater than 5×10^6 cells per millilitre. These cultures may be stored at (4 ± 3) °C, in darkness, for a maximum period of 10 d.

The following constituents should be added to each test solution (9.3) before the transfer of organisms:

a) 12×10^6 cells per litre of *Chlorella vulgaris*;

b) 6×10^6 cells per litre of *Pseudokirchneriella subcapitata*;

c) 500 µl per litre of the fish food suspension.

In any case, the quantity of food shall not constitute more than 10 % of the final volume of each container (9.3).

The use of *Chlorella vulgaris* and/or *Pseudokirchneriella subcapitata* algae immobilised in an inert matrix (gelose), in the form of algae beads[5] 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 (6.3.2 or 6.3.3). Repeat this operation a second time. The algal concentration in the test medium shall be the same as described above.

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) Sera Micron is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

5) Microbiotest, Deinze, 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.

6.4.2 Option 2: Yeast/Cerophyll[6]/trout chow and one algae diet

A second food combination based on the US EPA, method 1002.0 (Reference [11], p. 141) and Environment Canada (Reference [5]) test methods is recommended (see also References [3] and [4]). Daily feeding with Yeast/Cerophyll/trout chow (YCT) and a single algal species is required for culturing and testing of *Ceriodaphnia*. The algal species most commonly used is *Pseudokirchneriella subcapitata*.

The formula for preparing YCT is given in Annex D. If the YCT/one algae diet is used, mass cultures should be fed at a rate of:

— 7 ml algae concentrate per litre culture;

— 7 ml YCT concentrate per litre culture.

Individual cultures should be fed at the rate of:

— 0,1 ml algae concentrate per 15 ml culture;

— 0,1 ml YCT concentrate per 15 ml culture.

Food should be added to fresh culture medium immediately before or after the transfer of organisms.

Thoroughly mix algal concentrate and YCT by shaking before dispensing. If the YCT is stored frozen, store thawed aliquots at (4 ± 3) °C. Discard unused portions of unfrozen or thawed YCT after 2 weeks. Store unused portions of algal concentrate at (4 ± 3) °C, in darkness, and discard after 10 d.

6.5 Reference substance

Sodium pentachlorophenolate (C_6Cl_5ONa), copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), sodium chloride (NaCl), or zinc sulfate ($ZnSO_4$) are acceptable.

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 apparatus and in particular the following.

7.1 Temperature-controlled room, chamber or **water bath**.

7.2 Test containers, made from a chemically inert material.

If closed containers are used, make sure that the capacity is sufficient to allow for a gas phase/aqueous phase volume ratio of 1:1.

Prior to use, rinse the containers with the test medium (6.3.2 or 6.3.3) or with pure water (6.2).

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

6) Cereal Grass Media – Cerophyll is a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

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

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 ceriodaphnids.

7.7 Membrane filtration device, with filters, 0,45 µm, 0,22 µm.

7.8 Light source[7)], providing a range of light intensity at the air/water interface in the test containers (7.2) of 100 lx to 600 lx.

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

7.10 Sieve, of nominal size of openings <100 µm.

8 Sampling and samples

Carry out sampling, transportation and storage of samples in accordance with the general procedures specified in ISO 5667-16.

Collect samples in bottles made from chemically inert materials (7.9).

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 it 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 (use 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 micro-crustaceans, residual suspended matter, protozoa, micro-organisms, etc.), filter through a 0,45 µm membrane filter (7.7) or centrifuge the raw or decanted sample.

The sample obtained by either of these methods is the sample submitted to testing. It is also used for the renewal of test solutions. Store this sample in full containers at (4 ± 3) °C in the dark throughout the test period.

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 13).

If the aim of the test is to assess the chronic toxicity without considering extreme pH effects, a second test may also be carried out after adjustment of the pH value to 8,0 ± 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 13).

7) Grolux is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

9 Procedure

9.1 Preparation of the stock solutions of substances to be tested

Prepare the stock solution of the test substance by dissolving a known quantity of substance in a specified volume of test medium (6.3.2 or 6.3.3) 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 poorly soluble substances in the test medium, refer to the specifications of ISO 5667-16.

9.2 Selection of concentrations

The test shall comprise at least five concentrations of the sample to be tested (Clause 8 or 9.1), selected within a geometric series with a separation factor not exceeding 3,2.

Take the following criterion 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 10 replicates for each concentration. These replicates constitute a test batch (3.8).

Include in each test a control batch (3.3) without any sample to be tested. A control batch is also made up of at least 10 replicates.

When using a solvent in order to dissolve or disperse the substances, the solvent concentration shall be the same in all containers. Include a second control containing the solvent at the concentration being used in test concentrations.

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 corresponding to a small percent of the volume of sample being used.

For the purpose of toxicant range-finding or single concentration screening purposes, the test may also be carried out with a lesser number of concentrations, but in this case, an EC_x cannot always be estimated.

9.3 Preparation of the test and control solutions

Prepare the test solutions by mixing the appropriate volumes of the sample to be tested (Clause 8 or 9.1) or of its initial dilution with test medium (6.3.2 or 6.3.3) and food (6.4.1 or 6.4.2).

If the fish food/two algae diet (6.4.1) is used for feeding during a test, the quantity of food contained in each test container shall not be greater than one tenth of the total volume (i.e. 50 ml for a 500 ml test or control solution).

In the case where the YCT/one algae diet (6.4.2) is used for feeding during a test, the quantity of food contained in each test container shall be equal to 1,5 % of the total volume in the test container (i.e. 0,1 ml of YCT and 0,1 ml of algae concentrate added per 15 ml volume).

In both cases, the volume of test and control solutions should be at least (15 ± 2) ml and not exceed (50 ± 2) ml.

Split the test solution for the replicate containers of each concentration into volumes of (15 ± 2) ml to (50 ± 2) ml. Prepare the control solutions by mixing the food (6.4.1 or 6.4.2) with the test medium (6.3.2 or 6.3.3). Split the control solution into volumes of approximately (15 ± 2) ml to (50 ± 2) ml in each control container.

9.4 Introduction of the organisms

Place the containers in a temperature-controlled room or chamber (7.1) to obtain a test and control solutions temperature of (25 ± 2) °C.

As soon as this temperature is attained, introduce one *Ceriodaphnia dubia* aged less than 24 h (6.1) into each container (7.2). Use a pipette (7.4) for the transfer, and release the crustaceans under the water surface.

9.5 Renewal of the test and control solutions

When the fish food/two algae diet (6.4.1) is used for feeding during a test, carry out renewal of the test and control solutions (9.3) according to one of the timetables given in Table 1.

Table 1 — Test timetable for solution renewal (fish food/2 algae diet)

Timetable									
Day	0	1 [a]	2	3 [b]	4	5	6	7 [c]	8
Renewal	—	yes or no	no	no or yes	yes	yes	yes	yes or no	no

[a] If the test starts on Thursday, renew test and control solutions; if it starts on Friday, do not renew solutions

[b] If the test starts on Thursday, do not renew test and control solutions; if it starts on Friday renew test and control solutions

[c] If the test is to continue to an 8th day, renew test and control solutions.

On the days when the timetable requires a renewal of the test and control solutions, transfer the adult females from the old containers into new containers containing test and control solutions freshly prepared according to 9.3 (fresh food and medium) and bearing the same identification.

It is recommended that the transfer of the adult females take place at the beginning of the illuminated photoperiod, the quantities of food need to be absorbed prior to the reduction of light. If a degradable substance or sample is tested, the renewal should be daily, as per the timetable outlined in Table 2.

In the case where the YCT/one algae diet (6.4.2) is used for feeding during a test, renewal of the test and control solutions (9.3) is carried out according to the timetable given in Table 2.

Table 2 — Test timetable for solution renewal (YCT/one algae diet)

Timetable									
Day	0	1	2	3	4	5	6	7 [a]	8
Renewal	—	yes	yes	yes	yes	yes	yes	yes or no	no

[a] If the test is to continue to an 8th day, renew test and control solutions.

On days 1 to 6 where a renewal of the test and control solutions is required, transfer the adult females from the old containers into new containers containing test and control solutions freshly prepared according to 9.3 (fresh food and medium) and bearing the same identification.

It is recommended that the transfer of the adult females take place at the beginning of the illuminated photoperiod, the quantities of food need to be absorbed prior to the reduction of light, because of the diurnal mode of feeding of this species.

9.6 Observations and measurements

Record the mortality of the female adults when renewing the test and control solutions. If an adult dies before the end of the test, check the physical chemistry parameters (pH, conductivity, dissolved oxygen concentration) of the test container. Record all test observations and measurements on a data collection sheet (see Annex E).

Before the renewal of the test and control solutions (Table 1 or Table 2 timetables), conduct measurements of the temperature, pH and dissolved oxygen concentration on the old solutions of at least one container for each test batch and control batch. Prior to transferring the test organisms to renewed test and control solutions, conduct measurements of the temperature, pH (as specified in ISO 10523) and dissolved oxygen concentration (as specified in ISO 5814) on the freshly prepared solutions. Make sure that the pH of the freshly prepared test solution does not vary by more than 0,3 of a pH unit compared to the pH of the old test solution. Temperature and dissolved oxygen concentration shall not exceed (25 ± 2) °C and 100 % saturation, respectively.

NOTE Temperature can be controlled continuously in the test chamber. In this case, it is not necessary to measure it in each container.

At the time of renewal, all live adult females are transferred to new containers and live offspring counted. The presence of a single neonate in a test container can occur if the release of a brood is inadvertently interrupted during the daily transfer of the adult brood organism to a fresh test solution, resulting in a split in the brood count between two consecutive days. This can also occur if the brood organism has just started to release its young when it is transferred to a fresh test solution. Prior to changes of the test solutions, the test containers should be observed to see if any brood organisms are in the process of releasing its young. If this is the case, sufficient time is to be given for the release of all young prior to the change of test solutions. The presence of two or more neonates in any test container, during any given day of the test, constitutes a brood.

Perform the count, either by transferring the contents of the container to an assay dish, or by filtering contents through a sieve with nominal size of openings <100 µm (7.10) over a container. Observe the number of live offspring under a binocular magnifying glass (7.5), if necessary. An image analysis system (7.6) may also be used to count the offspring.

At the point where 60 % of the adult females in the control solutions have produced neonates during their third brood, the test shall end. Typically, this is achieved on day 7 but can happen as early as day 6 or as late as day 8. On the last day of the test, it may be interesting to record the size of the live adult females. In this case, place the adults individually on a microscope slide together with a drop of test medium (6.3.2 or 6.3.3). It is recommended that the *Ceriodaphnia dubia* be fixed with an anaesthetic solution in order to immobilise them (e.g. 2 min exposure to a volume fraction of 5 % of glycerol or ethanol can be used before freezing on a microscope slide). Measure the size between the head at the level of the eye and the base of the caudal spine.

10 Expression of results

10.1 Determination of the reproduction inhibition

Determine the total number of live *Ceriodaphnia dubia* for each concentration, N_e, by adding the values of the counts of each replicate. Also determine the total number of live young *Ceriodaphnia dubia* in the control batch, N_t, by adding the values of the counts of each replicate.

Determine the mean number of live young *Ceriodaphnia dubia* for each concentration, $\overline{N}_e$, by dividing N_e by the number of replicates. Determine the mean number of live young *Ceriodaphnia dubia* in the control, $\overline{N}_t$, by dividing N_t by the number of replicates.

Calculate the reproduction 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 *Ceriodaphnia dubia* for a concentration;

$\overline{N}_t$ is the mean number of live *Ceriodaphnia dubia* in the control.

In order to estimate the concentration which would cause x % of reproduction 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 95 % confidence limits.

When 95 % confidence limits are calculated, it should be taken into account that this statistical result is complicated by the fact that the effective concentration estimate is calculated inversely from the effect levels. 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 which is generally 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 European interlaboratory test (see Reference [2]).

This model, in which N_Y is the total number of live *Ceriodaphnia dubia* aged less than 24 h per replicate (variable), is characterised by Equation (2):

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

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

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

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

$\overline{N}_a$ is the mean number of live *Ceriodaphnia dubia* aged less than 24 h expected in the control;

b is the slope of the curve.

EC_x can then be estimated by Equation (3):

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

where x is the effect level, expressed as a percentage, sought to calculate the EC_x (initially defined parameter).

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.

10.2 Sensitivity of test organisms

Check the sensitivity of the biological reagent and conformity of application of the procedure. Periodically or at the same time as the test, determine the EC_{50} value for NaPCP, copper sulfate pentahydrate, sodium chloride or zinc sulfate (6.5) by applying the protocol described in this International Standard. Indicate the EC_{50} value and the date on which it was obtained in the test report.

A control chart to monitor within laboratory precision should be used to ensure that sensitivity of individuals in the laboratory's *Ceriodaphnia* culture does not change over time. Typically, 10 or more reference toxicant data points are needed before the geometric mean EC_{50} can be calculated, together with its respective upper and lower warning limits of $\pm 2s$, where s is standard deviation (see Reference [6]).

11 Validity criteria

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

a) the mean mortality rate of the adult females at the end of the test does not exceed 20 %;

b) the proportion of adult males does not exceed 10 %;

c) 60 % or more of the adult females produce three broods by the end of the test;

d) the mean number of offspring born per alive adult female at the end of the test is greater than or equal to 15.

12 Precision

Eight European laboratories participated in 1999 in an interlaboratory test concerning two substances, sodium pentachlorophenolate (NaPCP) and copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) (Reference [2]). The results are given in Table 3 and were obtained in ELENDT M4 medium.

Table 3 — Results of the European interlaboratory test

Substance	Effective concentration producing 50 % reproduction inhibition after 7 days EC_{50} at 7 d µg/l				
	Mean $\overline{C}$	Range C	Standard deviation s_R	Coefficient of variation of reproducibility CV(R)	No. of laboratories N
NaPCP	280	170 to 330	68	24	8
$CuSO_4$ $5H_2O$ [as Cu(II)]	232	135 to 311	59	25	8

Table 4 gives the results obtained by Environment Canada from 1996 to 2001 for zinc sulfate, and from 1992 to 2001 for sodium chloride. These results were obtained in a moderately hard water medium.

Table 4 — Precision data (Environment Canada)

Substance	Effective concentration producing 50 % reproduction inhibition after 8 days EC_{50} at 8 d mg/l				
	Mean $\overline{C}$	Range C	Standard deviation s_R	Coefficient of variation of reproducibility CV (R)	No. of laboratories N
$ZnSO_4$	0,117	0,053 to 0,168	0,037	32,0	58
NaCl	1 110	580 to 1 560	336	30,3	106

13 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 *Ceriodaphnia dubia* ephippia used;

f) all information relative to the test (light intensity, species and quantity and origin of algae used as food, sample concentration, pH and dissolved oxygen concentration of the test and control solutions, etc.);

g) the test results according to 10.1, the method according to which they were calculated, and the concentration-response curves;

h) the results obtained with the reference substance(s) according to 10.2, as well as the date of these tests;

i) data to prove that all test validity criteria according to Clause 11 are met, including any abnormal behaviour of the *Ceriodaphnia dubia* under the test conditions;

j) the possible presence of males;

k) 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;

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

Annex A
(normative)

Preparation of the ELENDT M4 medium

A.1 Trace elements

Prepare separate stock solutions (stock solutions I) of individual trace elements in pure water (6.2). From these different stock solutions (stock solutions I), prepare a second single stock solution (stock solution II) containing the 13 trace elements listed below (combined stock solution II). See Table A.1.

A.2 M4 medium

Prepare the M4 medium using stock solution II, the macronutrients and vitamins in accordance with Table A.2.

Prepare the combined vitamin stock solution by adding the three vitamins to 1 l of ultrapure water, as described in Table A.3.

Store the combined vitamin stock frozen in small aliquots. Add the vitamins to the medium shortly before use.

To avoid any precipitation of salts when preparing the complete medium, add the aliquots of the stock solutions to about 500 ml to 800 ml of pure water, then dilute to 1 l.

Table A.1 — Stock solutions for the Elendt M4 medium

Stock solution(s) I (single substance)	**Concentration in pure water** mg/l	**Concentration** (in relation to the M4 medium)	**To prepare the combined stock solution II, add the following volume of stock solution I to the pure water** ml/l
H_3BO_3	57 190	20 000 fold	1,0
$MnCl_2 \cdot 4H_2O$	7 210	20 000 fold	1,0
LiCl	6 120	20 000 fold	1.0
RbCl	1 420	20 000 fold	1,0
$SrCl_2 \cdot 6H_2O$	3 040	20 000 fold	1,0
NaBr	320	20 000 fold	1,0
$Na_2MoO_4 \cdot 2H_2O$	1 260	20 000 fold	1,0
$CuCl_2 \cdot 2H_2O$	335	20 000 fold	1,0
$ZnCl_2$	260	20 000 fold	1,0
$CoCl_2 \cdot 6H_2O$	200	20 000 fold	1,0
KI	65	20 000 fold	1,0
Na_2SeO_3	43,8	20 000 fold	1,0
NH_4VO_3	11,5	20 000 fold	1,0
$Na_2EDTA \cdot 2H_2O$[a]	5 000	2 000 fold	
$FeSO_4 \cdot 7H_2O$[a]	1 991	2 000 fold	
[a] Both Na_2EDTA and $FeSO_4$ solutions are prepared individually, then poured together and immediately autoclaved. This gives:			
Fe-EDTA solution		1 000 fold	20,0

Table A.2 — Preparation of the M4 medium using stock solution II, the macro-nutrients and vitamins

Stock solutions	**Concentration in pure water** mg/l	**Concentration** (in relation to the M4 medium)	**Volume of stock solution added to prepare the M4 medium** ml/l
Stock solution II (combined trace elements)		20 fold	50
Macro-nutrient stock solutions (single substance)			
$CaCl_2 \cdot 2H_2O$	293 800	1 000 fold	1,0
$MgSO_4 \cdot 7H_2O$	246 600	2 000 fold	0,5
KCl	58 000	10 000 fold	0,1
$NaHCO_3$	64 800	1 000 fold	1,0
$Na_2SiO_3 \cdot 9H_2O$	50 000	5 000 fold	0,2
$NaNO_3$	2 740	10 000 fold	0,1
KH_2PO_4	1 430	10 000 fold	0,1
K_2HPO_4	1 840	10 000 fold	0,1
Combined vitamin stock	—	10 000 fold	0,1

Table A.3 — Composition of the vitamin stock solution

Vitamin	**Concentration** mg/l	**Concentration** (in relation to the M4 medium)
Thiamine hydrochloride (B_1)	750	10 000 fold
Cyanocobalamin (B_{12})	10	10 000 fold
Biotin (B_7)	7,5	10 000 fold

Annex B
(normative)

Preparation of the moderately hard water medium

B.1 For 20 l of synthetic, moderately hard reconstituted water, use reagent grade chemicals and prepare as follows.

B.1.1 Place 19 l of pure water in a properly cleaned carboy.

B.1.2 Add 1,20 g of $MgSO_4$, 1,92 g of $NaHCO_3$, and 0,08 g of KCl to the carboy.

B.1.3 Aerate overnight.

B.1.4 Add 1,20 g of $CaSO_4 \cdot 2H_2O$ to 1 l of pure water in a separate flask. Stir on magnetic stirrer until calcium sulfate dihydrate is dissolved, add to 19 l above and mix well.

B.1.5 For *Ceriodaphnia* culture and testing, add sufficient sodium selenate (Na_2SeO_4) to provide 2 µg/l selenium in the final dilution of the water.

B.1.6 Aerate the combined solution vigorously for an additional 24 h to dissolve the added chemicals and stabilise the medium.

B.1.7 Add the vitamins (1 µg to 2 µg of crystalline vitamin B_{12} per litre of water) to the medium shortly before use (see Table A.3).

B.2 The final water quality is:

a) pH 7,4 to 7,8;

b) hardness 80 mg/l to 100 mg/l as $CaCO_3$;

c) alkalinity 60 mg/l to 70 mg/l as $CaCO_3$.

Annex C
(informative)

Preparation of the LC OLIGO medium

C.1 Reagents

C.1.1 Solution 1. Dissolve 4 g of calcium nitrate tetrahydrate [$Ca(NO_3)_2 \cdot 4H_2O$) in 100 ml of pure water (6.2).

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

C.1.2 Solution 2. Dissolve 10 g of potassium nitrate (KNO_3) in 100 ml of pure water (6.2).

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

C.1.3 Solution 3. Dissolve 3 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) in 100 ml of pure water (6.2).

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

C.1.4 Solution 4. Dissolve 4 g of anhydrous dipotassium hydrogen phosphate (K_2HPO_4) in 100 ml of pure water (6.2).

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

C.1.5 Solution 5. Dissolve the following compounds in approximately 800 ml of 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 may be stored for 3 months at (4 ± 3) °C.

C.1.6 Solution 6. Dissolve in approximately 800 ml of preheated water, while shaking, 1,625 mg of iron(III) citrate pentahydrate [$C_6H_5O_7Fe(III) \cdot 5H_2O$). The dissolution may require shaking overnight at about 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 may be stored for 3 months at (4 ± 3) °C.