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Water quality — Determination of the inhibition of the mobility of *Daphnia magna* Straus (*Cladocera*, *Crustacea*) — Acute toxicity test

Qualité de l'eau — Détermination de l'inhibition de la mobilité de Daphnia magna *Straus (*Cladocera, Crustacea*) — Essai de toxicité aiguë*

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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 6341 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

This fourth edition cancels and replaces the third edition (ISO 6341:1996), which has been technically revised. It also incorporates the Technical Corrigendum ISO 6341:1996/Cor. 1:1998.

Introduction

This International Standard specifies a procedure for the determination of the acute toxicity of chemicals, waters and waste waters to the water flea *Daphnia magna* Straus.

The evaluation of harmful effects on water quality has for several years involved the performance of biological tests. Crustaceans are of interest from the ecotoxicological point of view because they are primary consumers and a major component of the zooplankton in aquatic ecosystems.

The test specified in this International Standard involves the determination of the immobilization of the water flea *Daphnia magna* Straus after 24 h or 48 h exposure (depending on the requirement of users or national authorities) to the test sample under the conditions specified in this International Standard.

Water quality — Determination of the inhibition of the mobility of *Daphnia magna* Straus (*Cladocera*, *Crustacea*) — Acute toxicity test

WARNING — Persons using this International Standard 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 International Standard be carried out by suitably qualified staff.

1 Scope

This International Standard specifies a method for the determination of the acute toxicity to *Daphnia magna* Straus (*Cladocera, Crustacea*).

This method is applicable to:

— chemical substances which are soluble under the conditions of the test, or can be maintained as a stable suspension or dispersion under the conditions of the test;

— industrial or sewage effluents;

— treated or untreated waste water;

— aqueous extracts and leachates;

— fresh water (surface and ground water);

— eluates of fresh water sediment;

— pore water of fresh water sediment.

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 5667-16:1998, *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

[ISO 20665:2008,[3] 3.3]

3.2
control solution
test medium without sample under test

3.3
immobilization
inability of the organisms to swim during the 15 s which follow gentle agitation of the test and control solutions, even if they can still move their antennae

3.4
EC_{50}
concentration at which there is an effect on 50 % of the organisms in line with the test criterion

[ISO 15088:2007,[1] 3.3]

3.5
neonate
newly born or newly hatched individual

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

[ISO 20665:2008,[3] 3.6]

3.6
test batch
series of replicates filled with the same test solution

[ISO 20665:2008,[3] 3.8]

4 Principle

Determination of the initial concentration (i.e. the concentration present at the beginning of the test) which, in 24 h or 48 h, immobilizes 50 % of exposed *D. magna*, under the conditions specified in this International Standard. This concentration, known as the effective initial inhibitory concentration, is designated 24 h EC_{50} or 48 h EC_{50}.

An indication of the lowest concentration tested which immobilizes all the *D. magna* and the highest concentration tested which does not immobilize any of the *D. magna* is desirable and provides useful information in cases where the EC_{50} cannot be determined.

The test is carried out in one or two stages:

— a preliminary test which determines the range of concentrations to be tested in the definitive toxicity test and gives an approximate value of the 24 h EC_{50} or 48 h EC_{50};

— a definitive test, conducted when the approximate value given by the preliminary test is not sufficient, which permits calculation of the 24 h EC_{50} or 48 h EC_{50}, and determines concentrations corresponding to 0 % and 100 % immobilization.

If the method specified in this International Standard is used for biotesting of chemical substances, a limit test can be performed at 100 mg/l or at a lower concentration, at which the substance is soluble or is in stable dispersion under the conditions of the test (see 9.5). If it provides useful information, a limit test may also be performed at concentrations above 100 mg/l as long as the substance is soluble or in stable dispersion.

5 Test environment

The exposure of organisms as specified in this International Standard shall be carried out either in the dark or under a 16 h + 8 h light + dark photoperiod, in a temperature-controlled room or incubator at (20 ± 2) °C in the test containers.

The testing atmosphere shall be free from vapours or dusts toxic to *D. magna*. Photodegradable chemicals shall be tested in the dark, or using minimal lighting with the specified photoperiod, or minimal red lighting, as appropriate.

The use of controls (3.1) also allows checking 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 test organisms are neonates of *D. magna* Straus (*Cladocera*, *Crustacea*), obtained by acyclical parthenogenesis under specified breeding conditions (see Annex C).

The animals used for the test shall be less than 24 h old and should not be first brood progeny. The *D. magna* shall be from a healthy stock, showing no signs of stress such as mortality >20 % in 2 d, presence of males, ephippia, or discoloured animals, and there shall be no delay in the production of the first brood. Isolate gravid females and collect newly released neonates within 24 h.

If the culture conditions differ significantly from test conditions, it is recommended that one generation be acclimated under the test conditions for about one week to avoid stressing the parent animals and the offspring.

The age of the stock culture and the source (including clone, if possible) of the *D. magna* culture shall be indicated in the test report, since the sensitivity of *D. magna* to toxicants can be affected by the source of the culture.

The *D. magna* may also derive from the hatching of ephippia obtained from laboratory cultures of the crustacean as described in Annex D or can be purchased from a specialized company.[1)] The neonates hatched from the ephippia may be used directly as test organisms if they comply with all validity criteria given in this International Standard.

6.2 Pure water, conductivity below 10 µS/cm.

6.3 Dilution and culturing water.

6.3.1 General. Natural water (surface or ground water), reconstituted water or dechlorinated tap water are acceptable as culturing and dilution water if *D. magna* survives in it for the duration of the culturing, acclimation and testing without showing signs of stress. These waters may be used if they comply with all criteria and conditions specified in this International Standard. Waters in the range pH 6 to pH 9, with hardness between 140 mg/l and 275 mg/l (as $CaCO_3$) are recommended.

For stock culture of *D. magna* in the laboratory, the M4 medium (see Annex A) may also be used.

M4 medium (Annex A) should not be used as dilution water for samples containing bivalent metal ions. The EDTA in this medium can reduce the bioavailability of such ions, resulting in a decrease in apparent toxicity. In addition, for the same reason, M4 medium should not be used as the dilution water for samples of unknown composition.

NOTE If the test is performed for purposes necessitating the use of a dilution water with characteristics differing from those described in the preceding three paragraphs, state the main characteristics of the synthetic dilution water used in the test report.

As an example, the preparation of dilution water meeting the requirements is described below.

Dissolve known quantities of reagents in pure water (6.2) The dilution water prepared shall have a pH of 7,8 ± 0,5, a hardness of (225 ± 50) mg/l (expressed as $CaCO_3$), a molar Ca + Mg ratio close to 4 + 1 and a dissolved oxygen concentration above 7 mg/l.

Prepare the solutions specified in 6.3.2 to 6.3.5.

1) MicroBioTests Inc., Mariakerke, Belgium, is an example of a suitable supplier. This information is given for the convenience of the users of this document and does not constitute an endorsement by ISO of this supplier.

6.3.2 Calcium chloride solution. Dissolve 11,76 g of calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) in pure water (6.2) and make up to 1 l with pure water (6.2).

6.3.3 Magnesium sulfate solution. Dissolve 4,93 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) in pure water (6.2) and make up to 1 l with pure water (6.2).

6.3.4 Sodium bicarbonate solution. Dissolve 2,59 g of sodium bicarbonate ($NaHCO_3$) in pure water (6.2) and make up to 1 l with pure water (6.2).

6.3.5 Potassium chloride solution. Dissolve 0,23 g of potassium chloride (KCl) in pure water (6.2) and make up to 1 l with pure water (6.2).

6.3.6 Mixing. Mix 25 ml of each of the four solutions (6.3.2 to 6.3.5) and make up to 1 l with pure water (6.2).

The dilution water shall be aerated until the dissolved oxygen concentration has reached saturation and the pH has stabilized. If necessary, adjust the pH to 7,8 ± 0,5 by adding sodium hydroxide (NaOH) solution or hydrochloric acid (HCl). The dilution water prepared in this way shall not be further aerated before use.

6.4 Reference substance. Potassium dichromate ($K_2Cr_2O_7$) is recommended.

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.

6.5 Sodium hydroxide solution, e.g. [NaOH] = 1 mol/l.

6.6 Hydrochloric acid, e.g. [HCl] = 1 mol/l.

7 Apparatus and materials

Usual laboratory apparatus and in particular the following.

7.1 Temperature-controlled room or **chamber**.

7.2 Dissolved oxygen-measuring apparatus.

7.3 Culture vessels, of chemically inert material and of sufficient capacity, e.g. 2 l glass beakers.

7.4 Test containers, of chemically inert material and of sufficient capacity, e.g. glass test tubes or beakers.

7.5 Pipette for sampling the test organisms, with a sufficient diameter for capturing the animals while allowing sampling of only a small volume of medium.

Micropipettes of inert plastic material with a bulb at the end are very suitable for the operations.

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

7.7 Sieves. Appropriate sieves (e.g. mesh 1,0 mm and 0,3 mm) to transfer the adult organisms to stock culture and to separate the young from the adults.

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.

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 as specified in ISO 5667-16.

Carry out the toxicity test as soon as possible, preferably 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 as soon as possible after sampling and maintained deep-frozen (below –18 °C) for testing within 2 months of collection (see ISO 5667-16:1998, Clause 5).

Immediately test the frozen samples after complete thawing, e.g. in a water bath at a maximum temperature of 30 °C. Do not use a microwave for thawing the samples.

At the time of testing, homogenize the sample to be analysed by shaking manually. High concentrations of suspended inorganic or organic solids in a sample can be harmful to filter-feeding *D. magna*. Compensation for this interference can be made by a sample treatment for turbidity. If necessary, allow to settle for a maximum of 2 h in a container, and sample, e.g. by drawing off the required quantity of supernatant using a pipette, maintaining the end of the pipette in the centre of the section of the test container and halfway between the surface of the deposited substances and the surface of the liquid. If the raw sample of the decanted supernatant is likely to interfere with the test (due to presence of residual suspended matter, protozoa, microorganisms, etc.), centrifuge, for example, for 10 min at 5 000*g* or filter the raw or decanted sample. Test the residual toxicity of the supernatant. The particular kind of filter to be used should be checked by a test with control medium run through the filters.

NOTE Some filters and apparatus can add measurable toxicity, sometimes because of wetting agents added to the filters. A filter paper can also absorb toxic substances and remove them from the sample filtrate.

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

Usually no aeration of sample or prepared test concentrations is necessary. If, and only if, the dissolved oxygen is <40 % saturation, a pre-aerate of the sample or all test solutions for at most 20 min by appropriate methods, e.g. aeration or stirring may be performed. Any supersaturation should be remedied.

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.

Report any pre-aeration of test solutions or sample.

Tests shall be carried out without pH adjustment of the test sample.

The pH of test batches (3.6) is measured at the beginning and at the end of the test and reported.

However, in some cases, the final pH of a test solution may significantly differ from original pH of the test sample due to the concentration range selected and as a result of the buffer capacity of the dilution water or test sample. If toxic effects are observed at concentrations where the pH is not compatible with the survival of the organisms (i.e. outside the pH 6,0 to pH 9,0 range), the test(s) can be repeated with pH adjustment of the test sample.

IMPORTANT — Adjustment of the pH can alter the nature of the sample.

If the pH is to be adjusted, the recommendation is to adjust to the pH of the dilution water (6.3) selected. Choose the concentration of the hydrochloric acid (6.6) or the sodium hydroxide (6.5) solutions to restrict the volume fraction added to not more than 5 %.

If, as a result of pH adjustment, there is an issue with suspended matter, separate the suspended matter from the remaining sample as specified in ISO 5667-16. Any pH adjustment shall be included in the test report.

Adjust the temperature of the pretreated sample to the test temperature.

8.2 Preparation of solutions of substances to be tested

8.2.1 Preparation of stock solutions

Prepare the stock solution of the substance to be tested by dissolving a known quantity of substance in a specified volume of dilution water (6.3) at the time of use. However, if the stock solution of the substance is stable under certain conditions, it can 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.

As far as possible, the use of solvents, emulsifiers or dispersants should be avoided. However, such compounds may be required in some cases in order to produce a suitably concentrated stock solution. Guidance for suitable solvents, emulsifiers and dispersants is given in Reference [4]. Special considerations concerning test design and data evaluation are necessary (ISO/TS 20281[2]).

8.2.2 Preparation of test solutions

Prepare the test solution (see 9.1) by adding the stock solutions (8.2.1) to the dilution water (6.3) in specified quantities.

The test should comprise at least five concentrations of the sample to be tested. Select the dilutions within a geometric series with a separation factor which depends on the nature of the sample to be analysed (chemical substances, effluents, waters or extracts) and on 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 treated or untreated waste water, fresh water, pore water or extracts, a separation factor of2 between dilutions is usually performed (i.e. dilution of the previous dilution by half).

The preparation of the dilution series for lowest ineffective dilution (LID) determinations is described in Annex F. Depending on the purpose of the test and the statistical requirements concerning the test results, other dilution schemes with concentrations in geometric or logarithmic series can also be appropriate.

Dilution series for the definitive test on chemical substances are prepared with a separation factor not exceeding 3,2.

If steep concentration–response curves are expected, it is recommended that a separation factor not exceeding 2,2 be used.

Each dilution is preferably carried out in four replicates with a control (3.1) also in four replicates.

Substances which are poorly soluble in water may be solubilized or dispersed directly in pure water or dilution water by suitable means using ultrasonic devices or solvents of low toxicity to *D. magna*. Solvents should be used only when the EC_{50} is greater than the solubility of the test substance. If a solvent is used, the concentration of the solvent in the final test solution shall not exceed 0,1 ml/l, and two control solutions, one with no solvent, the other with the maximum concentration of solvent, shall be included in the test. Consider special requirements concerning test design for chemicals with solvents, e.g. additional solvent-control and statistical evaluations according to ISO/TS 20281.[2]

9 Procedure

9.1 General

Prepare a dilution series with the test solution (8.2.2) and the dilution water (6.3).

Combine increasing volumes of the test solution (8.2.2) with the dilution water (6.3), so as to obtain the desired concentrations for the test and transfer to the test containers.

To obtain a test and solution temperature of (20 ± 2) °C, for example, place the containers in a temperature-controlled room.

As soon as this temperature is attained, introduce the *D. magna* into the test containers with the pipette (7.5), taking care to add as little hatching medium as possible, and release the crustaceans under the water surface.

At least 20 animals, preferably divided into four groups of five animals each, should be used at each test concentration and for the controls. At least 2 ml of test solution should be provided for each animal (i.e. a volume of 10 ml for five daphnia per test container).

For each series of tests, prepare a control having a volume of dilution water (6.3) equal to the volume of the test solutions and introduce the same number of *D. magna* as in the test solutions. If a solvent is used to solubilize or disperse substances, prepare a second control with the dilution water containing the solvent at the maximum concentration used (i.e. not greater than 0,1 ml/l).

Animals shall not be fed during the test and test containers shall be maintained in a temperature-controlled room or chamber (7.1) at a temperature of (20 ± 2) °C. Observations of test organism responses are made at the end of the exposure time.

At the end of the test period of 24 h or 48 h, count the immobile *D. magna* in each container. Those which are not able to swim after gentle agitation of the liquid for 15 s shall be considered to be immobilized, even if they can still move their antennae.

Determine the concentration range giving 0 % to 100 % immobilization and note anomalies (e.g. lethargy, floating on the surface, abnormal rotating or circling) in the behaviour of the *D. magna*.

9.2 Preliminary test

This test enables determination of the range of concentrations over which the definitive test is to be carried out. For this purpose, use only a single series of concentrations (generally chosen in geometric progression) of stock solution or sample. Five *D. magna* should be exposed to each test concentration, and no replicates are necessary. An example is given in Annex B. Depending on the purpose of the test and the statistical requirements concerning the test results, other dilution designs with concentrations in a geometric or a logarithmic series can also be appropriate.

9.3 Definitive test

This test determines the percentage of *D. magna* which are immobilized by different concentrations, the 24 h EC_{50} or 48 h EC_{50}, or a LID value (see Annex F).

For the calculation of an EC_{50} value, it is desirable that the range of concentration chosen results in at least three percentages of immobilization between 10 % and 90 %. Examples of choices of ranges of concentrations are given in Annex B.

For each concentration and each control, use a minimum of 20 *D. magna*, preferably divided into four replicates, with five animals per test container.

Immediately after counting the immobilized *D. magna*, measure the dissolved oxygen concentration (see ISO 5814) in the test containers with the control batch (3.1) and with the most concentrated test batch (3.6) (if necessary, pour the contents of all containers corresponding to this concentration into one container, taking suitable precautions so as not to modify the dissolved oxygen content).

If the dissolved oxygen concentration (measured as indicated in 9.3) in the most concentrated test batch drops below 2 mg/l, the dissolved oxygen concentration shall be measured in the other test batches to check whether they meet the required 2 mg/l minimum concentration. Any test batches with dissolved oxygen concentration below 2 mg/l shall not be considered for the final calculations.

9.4 Check of the sensitivity of the *Daphnia magna* and conformity with the procedure

Within one month of the performance of the tests, determine the 24 h EC_{50} of potassium dichromate (6.4) using the dilution water (6.3) in order to verify the sensitivity of the *D. magna*.

Record the 24 h EC_{50} in the test report (bearing in mind that it represents the toxicity of this compound only and is not representative of the sensitivity of *D. magna* to other products).

Carry out the check as described in 9.3. If the 24 h EC_{50} of the potassium dichromate falls outside the range 0,6 mg/l to 2,1 mg/l, verify that the test procedure has been strictly applied.

9.5 Limit test

The limit test (see Clause 4 and Annex F) is carried out with 20 *D. magna* using the procedure described in 9.1. If the percentage of immobilization exceeds 10 % at the end of the test, a full study shall be conducted. Any observed abnormal behaviour shall be recorded.

10 Interpretation and validity of the results

10.1 Estimation of the EC_{50}

At the end of the 24 h or 48 h test, calculate the percentage immobilization for each concentration in relation to the total number of *D. magna* used. Determine the 24 h EC_{50} or 48 h EC_{50} by an appropriate statistical method (References [4][5]) {probit analysis, moving average, binomial methods or graphical estimation (Reference [6]) on a Gaussian logarithmic diagram}.

The concentration of the test substance should be measured, as a minimum at the highest and lowest test concentration, at the beginning and end of the test. It is recommended that results be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance has been satisfactorily maintained within ±20 % of the nominal or measured initial concentration throughout the test, then the results can be based on nominal or measured initial values.

If the data are insufficient or calculation of the EC_{50} is not required, quote the minimum concentration corresponding to 100 % immobilization and the maximum concentration corresponding to 0 % immobilization.

Record the mean percentage immobilization in the control and in each test concentration.

10.2 Validity criteria

Consider the results as valid if the following conditions are satisfied at the end of the test:

a) the percentage immobilization of the controls is less than or equal to 10 %;

b) the 24 h EC_{50} of the potassium dichromate is within the range 0,6 mg/l to 2,1 mg/l.

11 Expression of results

Express the EC_{50}, and the values corresponding to 0 % and 100 % immobilization:

— as a percentage, in the case of effluents, waters, eluates or extracts;

— in milligrams per litre, in the case of chemical substances.

NOTE The data can be reported in other units.

If determined, report the LID value (see Annex F).

12 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 6341:2012);

b) all information required for the complete identification of the original sample (before treatment) or of the chemical substance under test;

c) the methods of preparation of the samples:

   1) for effluents, waters, eluates and extracts, the method and the storage time of the samples, the pH and the dissolved oxygen concentration of the original 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;

d) all biological, chemical, and physical information relative to the test set out in this International Standard, including the origin and the age of the stock culture of the *D. magna* used;

e) the results of the test in the form of the 24 h EC_{50} or 48 h EC_{50}, the method of calculation, and, if possible, the 95 % confidence limit; in the case of chemical analysis of the substances, the method used;

f) the results of the limit test, if conducted;

g) the minimum tested concentration corresponding to 100 % immobilization and the maximum tested concentration corresponding to 0 % immobilization in 24 h or 48 h;

h) any abnormal behaviour of the *D. magna* under the test conditions (e.g. lethargy, floating on the surface, abnormal rotating or circling);

i) any operating details not specified in this International Standard and incidents which may have affected the results;

j) the results obtained with the reference chemical (9.4) as well as the date of the reference test;

k) data to prove that the validity criteria (10.2) are met;

l) 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 Elendt M4 medium

A.1 General

This annex gives one option for the preparation of the M4 medium using stock solutions.

A.2 Trace elements

Prepare, for each individual trace element, a separate stock solution (stock solution I) 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 in Table A.1.

A.3 M4 medium

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

Prepare the combined vitamin stock solution by adding the three vitamins to 1 l of pure water (6.2), 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 (6.2), then dilute to 1 l.

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

Stock solution(s) I (single substance)	Concentration in pure water (6.2) 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 pure water (6.2) 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 [b]	1 000 fold	20,0	
[b] Dissolve 5 000 mg $Na_2EDTA{\cdot}2H_2O$ in pure water (6.2) and make up to 500 ml with pure water. Dissolve 1 991 mg $FeSO_4{\cdot}7H_2O$ in pure water (6.2) and make up to 500 ml with pure water. Pour together and immediately autoclave. Store this solution in the dark.			

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

Stock solutions	Concentration in pure water (6.2) 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
$MgSO_2{\cdot}7H_2O$	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	750	10 000 fold
Cyanocobalamin (B_{12})	10	10 000 fold
Biotin	7,5	10 000 fold

Annex B
(informative)

Example of graphical determination of the inhibition of mobility of *Daphnia magna* by an effluent or stock solution of a substance at a concentration of 1 000 mg/l

NOTE The example relates to the procedure using test tubes.

B.1 Results

See Tables B.1 and B.2.

Table B.1 — Result of the preliminary test

Concentration %	Mobile *Daphnia magna*
90	0
35	0
10	0
3,5	0
1	0
0,35	5
0,1	5
0,035	5
0,01	5

The range of concentrations over which the definitive test is to be carried out is therefore 0,35 % to 1 %.

Table B.2 — Result of the definitive test

Concentration %	Number of mobile Daphnia magna in tube No.				N^a	P^b
	1	2	3	4		
0 (control)	5	5	5	5	20	0
0,35	5	5	3	4	17	15
0,48	2	3	4	3	12	40
0,62	3	1	1	2	7	65
0,80	1	0	2	1	4	80
1,0	0	1	0	0	1	95

[a] Number of mobile *D. magna* at each concentration at the end of the test.

[b] Percentage of immobilized *D. magna* at each concentration.

B.2 Determination of 24 h EC_{50}

By interpolation on the graph (see Figure B.1), the 24 h EC_{50} is 0,55 %.

For effluents, this is expressed as:

24 h EC_{50} = 0,55 %

or

24 h EC_{50} = 5,5 ml/l

For a chemical substance, this is expressed as:

$$24\text{ h } EC_{50} = \frac{0{,}55 \times 1\,000}{100} = 5{,}5\text{ mg/l}$$

From the graph, the values of EC_{50}, EC_{16} and EC_{84} can be obtained by interpolation. The lower confidence limit (95 %), T_0, may then be estimated as:

$$T_0 = \frac{EC_{50}}{f_{EC_{50}}}$$

and the upper confidence limit (95 %), T_1, as:

$$T_1 = EC_{50} f_{EC_{50}}$$

where the multiplication/division factor for obtaining confidence intervals, $f_{EC_{50}}$, is given by

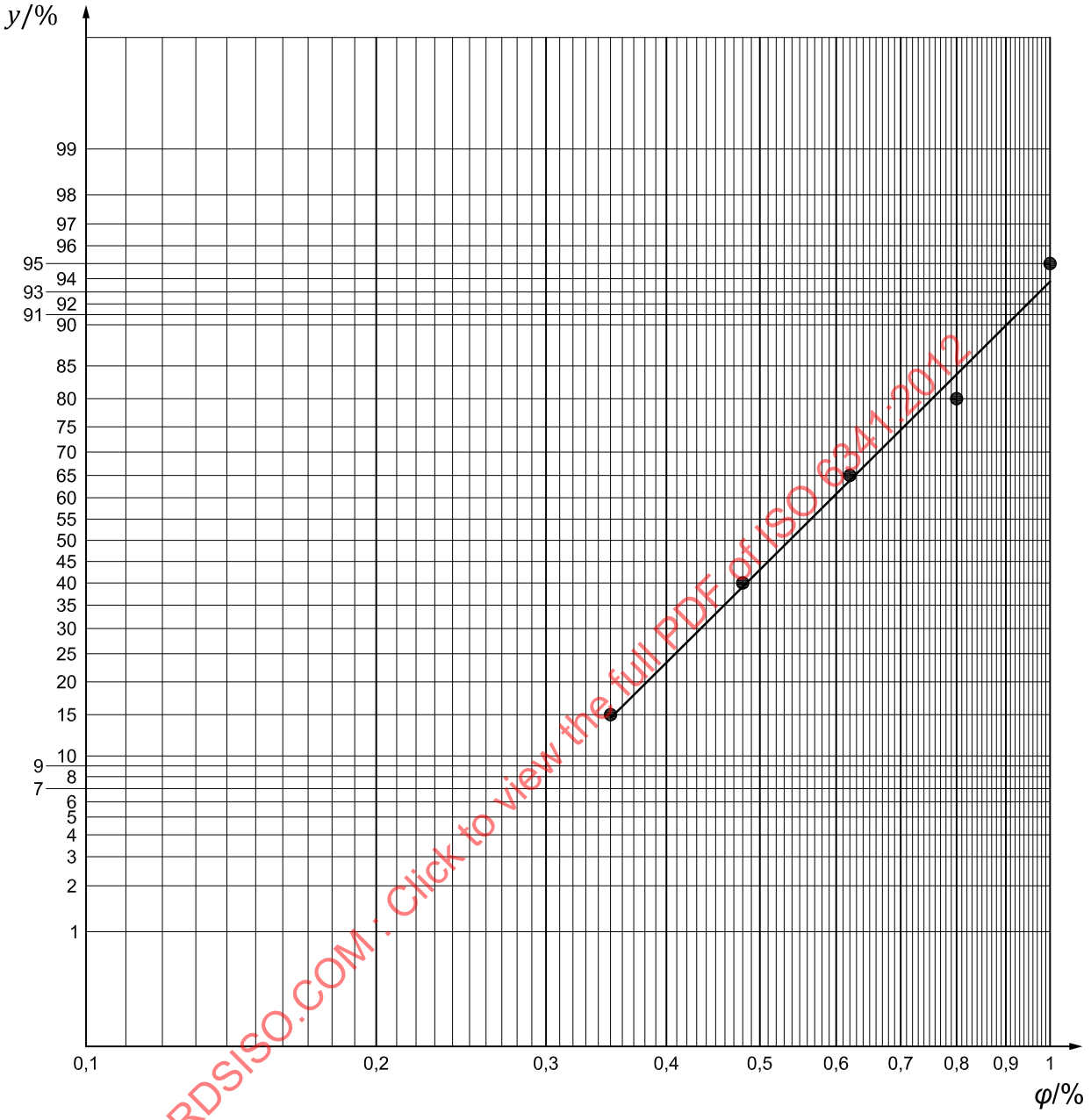
$$f_{EC_{50}} = S^{\left(2{,}77/\sqrt{N'}\right)}$$

in which

S is the slope factor, given by:

$$S = \left(\frac{EC_{84}}{EC_{50}} + \frac{EC_{50}}{EC_{16}}\right) \Big/ 2$$

N' is the total number of organisms exposed in the interval 16 % to 84 %.



Key

y immobilization

φ volume fraction

Figure B.1 — Regression curve (Gaussian logarithmic scale)

Annex C
(informative)

General recommendations for stock culturing

Loading of stock culture should be 25 to 50 animals per litre. They should be kept in mass stocks.

NOTE A loading of stock culture similar to the loading to be employed in the test is recommended. For example, a stock loading of 25 animals per litre would be suitable for test regimes employing replicates of five animals in approximately 200 ml of test solution.

Stock culture of daphnia should be fed by freshly prepared unicellular green algae from laboratory culture. Food algae should be in the exponential growth phase. Algae cultures can be used as long as they are growing without showing degradation effects. Harvested algae should not be stored at room temperature for a long time, because then degradation processes occur. They can be stored in a refrigerator in the dark or can even be frozen as long as daphnia cultures fed with these algae are still healthy and in a good reproduction status. Food should not be overdosed. It is recommended that daphnia stocks be fed with carbon levels at 0,1 mg per organism day to 0,2 mg per organism day. To avoid the transfer of algae growth medium to stock culture of daphnia it is recommended that the algae be separated from the algae growth medium and resuspended in daphnia culture medium. For stock culture a regular maintenance is necessary. Change the medium at least 2 to 3 times per week. Transfer the adults to a clean vessel with new medium and separate the neonates from the stock. Remove the exuviae, the dead and/or discoloured animals and the feeding residues.

Stock culturing of *D. magna* shall be carried out under a 16 h + 8 h light + dark photoperiod of diffuse daylight or artificial daylight. The testing atmosphere shall be at (20 ± 2) °C and free from vapours or dusts toxic to *D. magna*.

See References [8]–[12] for selected *D. magna* culture methods.

Annex D
(informative)

Culturing of *Daphnia magna* for production of dormant eggs

D.1 Life cycle of *Daphnia magna* (see Figure D.1)

In nature, *D. magna* can reproduce asexually as well as sexually.

However, in well-maintained stock cultures, reproduction only occurs asexually, with production of (diploid) females. Adult daphnia produce parthenogenetic eggs which develop in the dorsal brood chamber and give rise to live offspring.

The young daphnia grow through successive juvenile instars before they start to produce eggs.

Under specific environmental conditions (which in the scientific literature are referred to as “stressors”), daphnia shift from the asexual to the sexual mode of reproduction, whereby (haploid) males are produced, as well as (haploid) eggs which require fertilization by the males.

The females subsequently produce a protective dorsal shell (called an ephippium) in which two fertilized eggs (called dormant eggs) are deposited.

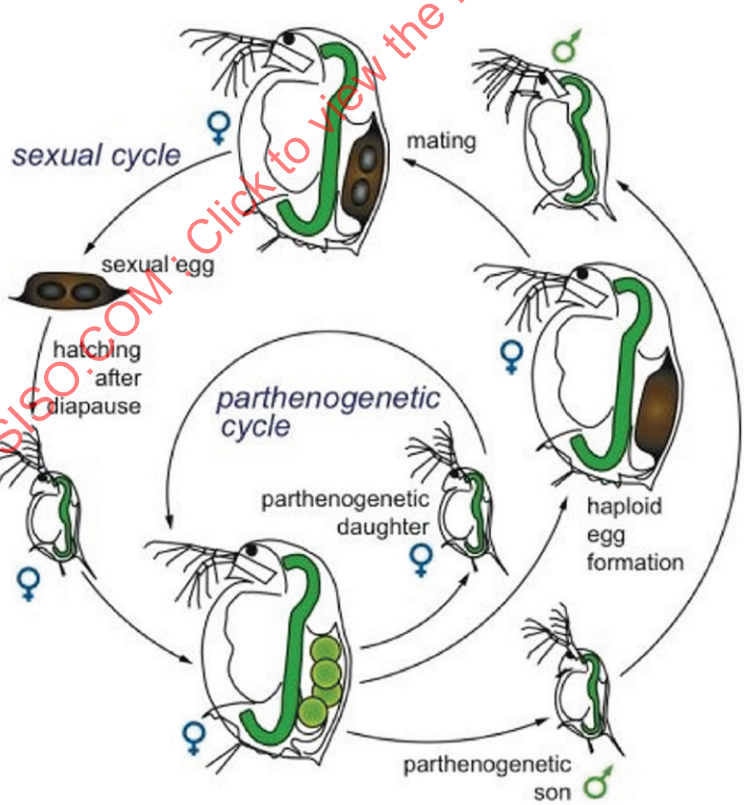


Figure D.1 — Life cycle of *Daphnia magna*
(Reference [7], reproduced with permission of the copyright holder)

The ephippium is cast off at the next moult of the female and usually sinks.

Dormant eggs usually go through a “refractory phase” (which can take several months) during which the eggs do not (cannot) respond to the stimuli which trigger the embryonic development leading to the birth of a neonate.

D.2 Culturing of *Daphnia magna* for production of dormant eggs

The specific environmental conditions triggering the sexual mode of reproduction of *D. magna* can be simulated in the laboratory to obtain dormant eggs. The ephippia can be stored and hatched at the time of performance of the assays, to obtain the live biological material for the toxicity tests.

Daphnia cultures to produce dormant eggs can be set up in any type of test container and in any volume, depending on the number of ephippia required.

The organisms are cultured and fed in exactly the same way as for laboratory stock cultures. The same daphnia strains can be used for production of dormant eggs.

Cultures can be started with a population density of 100 daphnia/l to 200 daphnia/l, with a light cycle of 12L:12D at ambient temperature.

Provided there is enough food, the population density in the cultures gradually increases and can attain 1 000 daphnia/l to 2 000 daphnia/l.

The shift from asexual to sexual reproduction is mainly triggered by two variables: population density (crowding) and shortage of food.

The exact figures for these two variables and the time needed to arrive at the shift to sexual reproduction are entirely dependent on the specific "own" culturing conditions, and have to be determined experimentally.

The onset of the sexual reproduction can be found by regular microscopic observations of the cultures, in which males will appear (which are smaller than the females) as well as females carrying an ephippium.

After the females have cast off their ephippium, the latter can be collected at the bottom of the culturing container.

The ephippia shall be stored in the refrigerator (at 4 °C) in darkness, in tubes with either tap water or a reconstituted natural water.

Taking into account the "refractory phase" (indicated in D.1.), the dormant eggs have to be stored for about 3 months after harvesting to obtain successful hatching. Viable neonates can even be obtained after 1 to 2 years if the dormant eggs are stored properly.

D.3 Hatching of the ephippia

The two main triggers in nature for the start of the embryonic development of the sexual eggs are light and temperature.

Under laboratory conditions the time for the development of the sexual eggs to neonates takes about 72 h.

To obtain neonates, ephippia shall be transferred to a Petri dish containing the same medium as prescribed for daphnia stock cultures.

The Petri dish shall be incubated for 72 h at (20 ± 1) °C under continuous illumination of ~6 000 lx.

D.4 Quality control testing

D. magna neonates hatched from dormant eggs must comply with all criteria and conditions specified in this International Standard for test organisms taken from laboratory cultures.

Quality control tests with potassium dichromate must therefore be carried out with the neonates hatched from the ephippia and the 24 h EC_{50} must be in the range 0,6 mg/l to 2,1 mg/l (see 9.4) (Reference [7]).