INTERNATIONAL STANDARD

ISO 6341

Fourth edition
2012-10-15

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*

Reference number
ISO 6341:2012(E)

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Published in Switzerland

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 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.