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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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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.

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.

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

This third edition cancels and replaces the second edition (ISO 6341:1989), of which it constitutes a technical revision.

Annex A forms an integral part of this International Standard. Annex B is for information only.

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

1 Scope

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

a) 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;

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

c) surface or ground waters.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 5667-2:1991, *Water quality — Sampling — Part 2: Guidance on sampling techniques.*

ISO 5725-2:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method.*

ISO 5813:1983, *Water quality — Determination of dissolved oxygen — Iodometric method.*

ISO 5814:1990, *Water quality — Determination of dissolved oxygen — Electrochemical probe method.*

3 Principle

Determination of the initial concentration (that is, the concentration present at the beginning of the test) which, in 24 h, immobilizes 50 % of exposed *Daphnia magna*, under the conditions defined in this International Standard.

This concentration, known as the effective initial inhibitory concentration, is designated 24 h — $EC50_i$.

If possible and if necessary, the initial concentration which immobilizes 50 % of the exposed *Daphnia magna* in 48 h can also be determined. This concentration is designated 48 h — $EC50_i$.

An indication of the lowest concentration tested which immobilizes all the *Daphnia magna* and the highest concentration tested which does not immobilize any of the *Daphnia magna* is desirable and provides useful information in cases where the 24 h — $EC50_i$ and, where appropriate, the 48 h — $EC50_i$ 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 final toxicity test and gives an approximate value of the 24 h — $EC50_i$ and, where appropriate, the 48 h — $EC50_i$;

— a definitive test, conducted when the approximate value given by the preliminary test is not sufficient, which permits calculation of the 24 h — $EC50_i$ and 48 h — $EC50_i$ and determines concentrations corresponding to 0 % and 100 % immobilization.

If the method described in this International Standard is used for 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 8.5).

Analysis of these substances should be conducted as soon as possible. If these analyses show that, for each concentration tested, the standard deviation of the concentrations measured during the test, randomly varying, is not greater than 20 % of the mean, the median inhibitory concentrations may be calculated from the means of the measurements rather than from the initial concentrations; they are then designated 24 h — EC50 and, where appropriate, 48 h — EC50.

4 Test environment

The storage of the solutions and the exposure of organisms as described in this International Standard shall be carried out either in the dark or under a 16 h/8 h light/dark photoperiod. The testing atmosphere shall be at 20 °C ± 2 °C and free from vapours or dusts toxic to *Daphnia magna*. Photo-degradable chemicals shall be tested in the dark.

5 Reagents and materials

5.1 Test organism: *Daphnia magna* Straus (*Cladocera*, *Crustacea*), at least third generation, obtained by acyclical parthenogenesis under specified breeding conditions.

The animals used for the test shall be less than 24 h old. Transfer gravid females to vessels with fresh dilution water and collect newly released neonates within 24 h.

Since the sensitivity of *Daphnia magna* to toxicants is age-dependent and source-dependent, the age and source of the *Daphnia magna* culture shall be indicated in the test report.

5.2 Dilution water

Dissolve known quantities of reagents of recognized analytical grade in ground water, distilled water or deionized water of at least equivalent purity, having a maximum conductance of 10 µS/cm.

The dilution water thus prepared shall have a pH of 7,8 ± 0,2, a hardness of 250 mg/l ± 25 mg/l (expressed as $CaCO_3$), a molar Ca/Mg ratio close to 4 : 1 and a dissolved oxygen concentration above 7 mg/l.

Natural waters with pH and hardness characteristics similar to dilution water may be used for culturing.

As an example, the preparation of water meeting these requirements is described below:

a) Prepare the following solutions:

1) Calcium chloride solution

Dissolve 11,76 g of calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) in distilled or deionized water and dilute to 1 litre.

2) Magnesium sulfate solution

Dissolve 4,93 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) in distilled or deionized water and dilute to 1 litre.

3) Sodium bicarbonate solution

Dissolve 2,59 g of sodium bicarbonate ($NaHCO_3$) in distilled or deionized water and dilute to 1 litre.

4) Potassium chloride solution

Dissolve 0,23 g of potassium chloride (KCl) in distilled or deionized water and dilute to 1 litre.

b) Mix 25 ml of each of the four solutions 1) to 4) and complete the total volume to 1 litre with distilled or deionized water.

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 ± 0,2 by adding sodium hydroxide (NaOH) solution or hydrochloric acid (HCl) solution. The dilution water prepared in this way shall not be further aerated before use.

NOTE 1 If the test is performed for purposes necessitating the use of a dilution water with characteristics differing from those described above, mention should be made in the test report of the main characteristics of the synthetic dilution water used.

5.3 Potassium dichromate ($K_2Cr_2O_7$), of recognized analytical grade

6 Apparatus

Ordinary laboratory apparatus, and in particular the following.

6.1 Dissolved-oxygen measuring apparatus.

6.2 Test containers, of chemically inert material and of sufficient capacity (for example glass test tubes or beakers).

Before use, the test containers shall be carefully washed then rinsed, first with distilled water and then with the dilution water (5.2). At the end of the test, the containers shall be emptied, rinsed with water to eliminate any trace of the test solution and then dried.

7 Treatment and preparation of samples

7.1 Special precautions for sampling and transportation of samples of water or effluent

For the sampling of water or effluent, refer to the general procedure specified in ISO 5667-2. Bottles shall be completely filled to exclude air.

The toxicity test shall be carried out as soon as possible, within 6 h of collection. If this time interval cannot be observed, cool the sample (+ 4 °C) at the place of collection. In this case, the sample shall be analysed within 48 h after collection.

NOTE 2 If testing cannot be carried out within 48 h, the sample may be frozen for testing within 2 months of collection.

7.2 Preparation of solutions of substances to be tested

7.2.1 Preparation of stock solutions

The stock solutions of the substances to be tested shall be prepared by dissolving a known quantity of the substance to be tested in a specified volume of dilution water (5.2), deionized water or distilled water in a glass container. They shall be prepared at the moment of use unless the substance in solution is known to be stable, in which case the stock solution may be prepared up to 2 days in advance of testing.

Stock solutions of substances which are poorly soluble in water may be solubilized or dispersed directly in the medium by suitable means using ultrasonic devices or solvents of low toxicity to *Daphnia magna*. Solvents should be used only when the EC50 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.

No single procedure for the preparation of stock solutions of poorly soluble substances can be recommended due to the differing nature of chemicals.

7.2.2 Preparation of test solutions

The test solutions shall be prepared (see 8.1) by adding the stock solutions or effluent samples (7.2.1) to the dilution water (5.2) in specified quantities.

If the stock solutions are prepared in deionized or distilled water, it is recommended not to add more than 10 ml of stock solution per litre of dilution water.

8 Procedure

8.1 General

Place increasing volumes of the test solution (7.2.2), or effluent or water sample (7.1), in a series of test containers (6.2) and add the dilution water (5.2) so as to obtain the desired concentrations for the test. Place the *Daphnia magna* in the test containers so that the total number of *Daphnia magna* per container does not exceed 20, and the density of *Daphnia magna* per container does not exceed 5 *Daphnia magna* per 10 ml of solution.

For each series of tests, prepare a control having a volume of dilution water (5.2) equal to the volume of the test solutions and introduce the same number of *Daphnia 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 must not be fed during the test and vessels must be maintained at a temperature of 20 °C ± 2 °C.

At the end of the test period of 24 h (and where appropriate 48 h), count the mobile *Daphnia 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 in the behaviour of the *Daphnia magna*.

8.2 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 effluent.

An example is given in annex A.

8.3 Definitive test

This test enables determination of the percentage of *Daphnia magna* which are immobilized by different concentrations and determination of the 24 h — $EC50_i$ and, where appropriate, the 48 h — $EC50_i$.

It is desirable that the range of concentrations chosen result in at least three percentages of immobilization between 10 % and 90 %.

Examples of choices of ranges of concentrations are given in annex A.

For each concentration and each control, use a minimum of 20 *Daphnia magna*.

Immediately after counting the immobilized *Daphnia magna*, measure the dissolved oxygen concentration (see ISO 5813 and ISO 5814) in the test container with the solution of lowest concentration at which all the *Daphnia magna* have been immobilized (if necessary, pour into one container the contents of the containers corresponding to this concentration, taking suitable precautions so as not to modify the dissolved oxygen content).

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

Periodically, determine the 24 h — $EC50_i$ of potassium dichromate (5.3) using the dilution water (5.2) in order to verify the sensitivity of the *Daphnia magna*. Report the 24 h — $EC50_i$ in the test report (bearing in mind that it represents the toxicity of this compound only and is not representative of the sensitivity of *Daphnia magna* to other products).

Carry out the check as described in 8.3. If the 24 h — $EC50_i$ of the potassium dichromate falls outside the range 0,6 mg/l to 1,7 mg/l, verify the strict application of the test procedure and the manner of breeding the *Daphnia magna*, and, if necessary, use another strain of *Daphnia magna*.

8.5 Limit test

The limit test (see clause 3) is carried out with 20 *Daphnia magna* using the procedure described in 8.1.

9 Interpretation and validity of the results

9.1 Estimation of the $EC50_i$

At the end of the 24 h test, calculate the percentage immobilization for each concentration in relation to the total number of *Daphnia magna* used. Determine the 24 h — $EC50_i$ by an appropriate statistical method (probit analysis, moving average, binominal methods or graphical estimation on a Gaussian logarithmic diagram).

If chemicals are tested using this International Standard, and analyses of each concentration at the beginning of the test and during it show that the relative standard deviation of the individual concentrations measured is not greater than 20 %, it is possible to use these measured values to calculate the 24 h — EC50 rather than the 24 h — $EC50_i$ based on the initial concentrations. If the measured concentration is lower than 80 % of the initial concentration, it is not possible to calculate the EC50. In this case use the data obtained with care. If no reasonable estimation of the 24 h — $EC50_i$ (or the 24 h — EC50) is possible, the reasons shall be investigated and the test repeated.

In cases where the data are insufficient or not required to calculate the 24 h — $EC50_i$ (or the 24 h — EC50), quote the minimum concentration corresponding to 100 % immobilization and the maximum concentration corresponding to 0 % immobilization. Proceed in the same manner as above to calculate the 48 h — $EC50_i$ (or the 48 h — EC50) or, if there are sufficient data, report the minimum concentration producing 100 % immobilization and the maximum concentration producing 0 % immobilization.

9.2 Validity of results

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

a) the dissolved oxygen concentration at the end of the test (measured as indicated in 8.3) is greater than or equal to 2 mg/l;

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

c) the 24 h — $EC50_i$ of the potassium dichromate is within the range 0,6 mg/l to 1,7 mg/l.

10 Expression of results

Express the 24 h — $EC50_i$, the 48 h — $EC50_i$ (or the 24 h — EC50 and the 48 h — EC50) and the limits corresponding to 0 % and 100 % immobilization:

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

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

NOTE 3 The data can be reported in other units.

11 Precision

Under the authority of the Commission of the European Communities, an interlaboratory test was carried out during 1978. This test consisted of using the method described in this International Standard for the following substances in particular:

— tetrapropylbenzene sulfonic acid (T.P.B.S. No. 1);

— tetrapropylbenzene sodium sulfonate (T.P.B.S. No. 2);

— potassium 2,4,5-trichlorophenoxyacetate (potassium salt of 2,4,5-T).

This last substance, although of low toxicity and low solubility in water, was included in order to have results relating to a substance considered to be at the limit of the scope of the method described in this International Standard.

In 1994, participating members were requested to submit the 24 h — $EC50_i$ data on potassium dichromate from tests conducted since 1978. These data were used to calculate the acceptable range of reference toxicant sensitivity.

As a guide, the results of these interlaboratory tests (evaluated in accordance with ISO 5725-2) are summarized in table 1.

12 Test report

The test report shall include the following information:

a) a reference to this International Standard;

b) all the data necessary for the identification of the sample or the substance tested;

c) the methods of preparation of the samples:

1) for effluents or waters, the manner and duration of storage of the samples and, if necessary, the conditions in which decantation and filtration of the sample and thawing were carried out,

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

d) all biological, chemical and physical information relating to the test and not specified in this International Standard, including the origin and age of the *Daphnia magna* used;

Table 1

Product tested	Number of participating laboratories	Number of laboratories eliminated	Number of results used	Mean 24 h — $EC50_i$ mg/l	Standard deviation			
					Repeatability		Reproducibility	
					Absolute	Coefficient of variation %	Absolute	Coefficient of variation %
$K_2Cr_2O_7$	36	2	1697	1,12	0,06	5	0,56	50
T.P.B.S. No. 1	36	4	108	27,45	3,95	14,4	8,32	30
T.P.B.S. No. 2	31	4	84	27,02	3,24	12	9,51	35
Potassium salt of 2,4,5-T	32	4	72	772,25	64,5	8,3	277,8	35,9

e) the results of the test in the form of the 24 h — $EC50_i$ and, where appropriate, the 48 h — $EC50_i$ (or 24 h — EC50 and 48 h — EC50), the method of calculation, and, where appropriate, 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 concentration corresponding to 100 % immobilization and the maximum concentration corresponding to 0 % immobilization in 24 h and, where appropriate, in 48 h;

h) any abnormal behaviour of the *Daphnia magna* under the test conditions;

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

Annex A
(normative)

Example of 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

The example relates to the procedure using test tubes.

A.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 %.

A.2 Definitive test

A.2.1 Results

The results are shown in table A.1.

A.2.2 Determination of 24 h — EC50

By interpolation on the graph (see figure A.1), the 24 h — EC50 is 0,55 %.

For effluents, this is expressed as:

24 h — EC50 = 0,55 % or 5,5 ml/l

For a chemical substance, this is expressed as:

$$24\text{ h} - \text{EC50} = \frac{0{,}55 \times 1\,000}{100} = 5{,}5\text{ mg/l}$$

Table A.1

Concentration, %	Number of mobile *Daphnia magna* in tube No.				T	P
	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

T is the number of mobile *Daphnia magna* at each concentration at the end of the test.

P is the percentage of immobilized *Daphnia magna* at each concentration.