

# INTERNATIONAL STANDARD

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

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

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## Foreword

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Draft International Standards adopted by the technical committees are circulated to the member bodies for approval before their acceptance as International Standards by the ISO Council. They are approved in accordance with ISO procedures requiring at least 75 % approval by the member bodies voting.

International Standard ISO 6341 was prepared by Technical Committee ISO/TC 147, *Water quality*.

This second edition cancels and replaces the first edition (ISO 6341 : 1982), of which it constitutes a minor revision.

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

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International Organization for Standardization  
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## Introduction

The sensitivity of organisms to the toxic properties of a substance may vary considerably from one species to another, owing to differences in their metabolisms and the natures of their habitats. Thus, the proposed method enables the effect of an effluent, or a substance which has been rendered soluble in water, on *Daphnia magna* to be studied, but the results obtained should not be extrapolated to other species.

Under these circumstances, this toxicity test alone cannot be sufficient for an accurate prediction of the environmental toxicity of a substance or effluent, and a series of tests, with species belonging to different taxonomic groups and responding to different environmental conditions, is necessary.

Toxicity tests are conducted in the laboratory under arbitrarily defined conditions, which are incapable of perfectly simulating environmental conditions. They enable inter-laboratory comparisons to be made of the possible toxic effects of a substance or an effluent, but they have only a limited value in assessing the effect of this substance or effluent in actual environmental conditions in which many other factors may have an influence, for example the presence of organic and inorganic materials, hardness, pH, buffering capacity.

For an accurate prediction in a specific environmental situation, the results obtained by strict application of standardized methods should therefore be complemented by data obtained under conditions which better simulate aspects of the environment and by data obtained under field conditions.

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

## 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;
- b) industrial effluents, treated or untreated, after decantation or filtration if necessary;
- c) sewage effluents, treated or untreated, after decantation or filtration if necessary;
- d) surface or ground waters.

## 2 Normative reference

The following standard contains provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the edition indicated was 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 edition of the standard indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 5667-2 : 1982, *Water quality — Sampling — Part 2: Guidance on sampling techniques*.

## 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 — EC (I) 50.

NOTE — If possible and if necessary, the initial concentration which immobilizes 50 % of the exposed *Daphnia magna* in 48 h may also be determined. This concentration is designated 48 h — EC (I) 50.

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 — EC (I) 50 [and, where appropriate, the 48 h — EC (I) 50] cannot be determined.

The test is carried out in one or two stages:

- a preliminary test which gives an approximate value of the 24 h — EC (I) 50 [and, where appropriate, the 48 h — EC (I) 50] and determines the range of concentrations to be tested in the final toxicity test;
- if necessary (in cases where the approximate value given by the preliminary test is not sufficient), a definitive test, the result of this test alone being used [24 h — EC (I) 50, 48 h — EC (I) 50 or concentrations corresponding to 0 and 100 % immobilization].

If the method described in this International Standard is used for chemical substances, analyses of these substances may be carried out during the test. 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 — EC 50 (and, where appropriate, 48 h — EC 50).

## 4 Test environment

The preparation and storage of the solutions and all stages of the procedure described in this International Standard shall be carried out in an atmosphere controlled at 20 °C ± 2 °C and which is free from vapours or dusts toxic to *Daphnia magna*.

## 5 Reagents and materials

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

The animals used for the test, obtained by filtration through a nest of sieves of specified aperture sizes, shall be less than 24 h old.

Since the sensitivity of *Daphnia magna* to toxicants is age-dependent, the age of the *Daphnia magna* used 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 conductivity of 10  $\mu\text{S}/\text{cm}$ .

The dilution water thus prepared shall have a pH of  $7,8 \pm 0,2$ , a hardness of  $250 \text{ mg/l} \pm 25 \text{ mg/l}$  (expressed as  $\text{CaCO}_3$ ), a Ca/Mg ratio close to 4 : 1 and a dissolved oxygen concentration above 80 % of the air saturation value.

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 ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) in water and make up to 1 litre with distilled or deionized water.

2) Magnesium sulfate solution

Dissolve 4,93 g of magnesium sulfate heptahydrate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) in water and make up to 1 litre with distilled or deionized water.

3) Sodium bicarbonate solution

Dissolve 2,59 g of sodium bicarbonate ( $\text{NaHCO}_3$ ) in water and make up to 1 litre with distilled or deionized water.

4) Potassium chloride solution

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

b) Mix 25 ml of each of the four solutions 1) to 4) and bring 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  $7,8 \pm 0,2$  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 — 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 dilution water used.

The dilution water shall permit survival of the *Daphnia magna* for at least 48 h. As far as possible, check that the dilution water does not contain any substances which are known to be toxic to *Daphnia magna*, such as chlorine, heavy metals, pesticides, ammonia or polychlorinated biphenyls.

**5.3 Potassium dichromate** ( $\text{K}_2\text{Cr}_2\text{O}_7$ ), of recognized analytical grade.

## 6 Apparatus

Ordinary laboratory apparatus, and in particular

### 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 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, the sample may be cooled ( $+4 \text{ }^\circ\text{C}$ ) or frozen ( $-20 \text{ }^\circ\text{C}$ ) at the place of collection. If the sample is to be frozen, do not fill the bottles completely, in order to avoid breakage on freezing. The conditions in which the sample is frozen and the method of preliminary filtration or decantation shall be indicated in the test report. Never use a chemical preservative.

### 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, deionized water or distilled water in a glass container. They shall be prepared at the moment of use unless the stability of the substance in solution is known, in which case the stock solution may be prepared in a sufficient quantity for 2 days.

For the preparation of the stock solutions, substances which are poorly soluble in water may be solubilized or dispersed by suitable means, including ultrasonic devices or solvents of low toxicity to *Daphnia magna*. If a solvent is used, the concentration of the solvent in the final test solution shall not exceed 0,1 ml/l, and two series of control tests, one with no solvent, the other with the maximum concentration of solvent, shall be carried out at the same time as the test.

NOTE — As this method of test relates to the aquatic environment, the use of stronger concentrations of solvent is not considered acceptable within the conditions of this International Standard.

Owing to problems which may arise as a result of the transformation or loss of substances to be tested, no single procedure can be recommended for the preparation of stock solutions of substances of poor solubility in water.

#### 7.2.2 Preparation of test solutions

The test solutions shall be prepared by adding the stock solutions (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

Into a series of test containers (6.2), place increasing volumes of the test solution (7.2.2) or effluent (7.1) 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 container having a volume of dilution water (5.2) equal to the volume of the test solutions and the same number of *Daphnia magna* as the test solutions. If a solvent is used to solubilize or disperse substances, prepare a second control container with the dilution water (5.2) containing the solvent at the maximum concentration used.

During the test, keep the vessels at a temperature of  $20\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ .

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 in the 15 s which follow gentle agitation of the liquid 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 any 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 percentages of *Daphnia magna* which are immobilized by different concentrations and determination of the 24 h — EC (I) 50 [and, where appropriate, the 48 h — EC (I) 50].

Choose the range of concentrations in such a way that three or four percentages of immobilization between 10 % and 90 % are obtained.

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 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 — EC (I) 50 of the potassium dichromate (5.3) in order to verify the sensitivity of the *Daphnia magna*. Report the 24 h — EC (I) 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 *Daphnia magna* to other products).

Carry out the check as described in 8.3. If the 24 h — EC (I) 50 of the potassium dichromate falls outside the range 0,9 mg/l to 2,0 mg/l, verify the strict application of the test procedure, the manner of breeding the *Daphnia magna*, and, if necessary, use a new strain of *Daphnia magna*.

## 9 Interpretation and validity of the results

### 9.1 Estimation of the EC (I) 50

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 — EC (I) 50 by an appropriate statistical method (Probit analysis, moving average, binominal methods or graphical estimation on a Gaussian logarithmic diagram).

If the method described in this International Standard is used for chemical substances, and analyses of these substances have been carried out at the beginning of the test and during the test, showing that, for each concentration tested, the standard deviation of the concentrations measured does not deviate by more than  $\pm 20\%$  from the mean, it is possible to use these measured values to calculate the 24 h — EC 50 rather than the 24 h — EC (I) 50 based on the initial concentrations.

If no reasonable estimation of the 24 h — EC (I) 50 (or the 24 h — EC 50) is possible, the reasons shall be investigated and the test repeated.

In cases where the data are insufficient to calculate the 24 h — EC (I) 50 (or the 24 h — EC 50), it may then suffice to quote the minimum concentration corresponding to 100 % immobilization and the maximum concentration corresponding to 0 % immobilization.

NOTE — Proceed in the same manner as above to calculate also the 48 h — EC (I) 50 (or the 48 h — EC 50) or to determine the minimum concentration corresponding to 100 % immobilization and the maximum concentration corresponding to 0 % immobilization.

### 9.2 Validity of results

Consider the results as valid if the following conditions are satisfied:

- 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 — EC (I) 50 of the potassium dichromate is within the range 0,9 mg/l to 2,4 mg/l.

Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests) are summarized in table 1.

## 12 Test report

The test report shall make reference to this International Standard and shall include in particular :

## 10 Expression of results

Express the 24 h — EC (I) 50, the 48 h — EC (I) 50 (or the 24 h — EC 50 and the 48 h — EC 50) and the limits corresponding to 0 and 100 % immobilization :

- as a percentage, or in millilitres per litre, in the case of effluents;
- in milligrams per litre in the case of chemical substances.

## 11 Precision

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

- potassium dichromate ( $K_2Cr_2O_7$ );
- 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 field of application of the method described in this International Standard.

As a guide, the results of this inter-laboratory test (evaluated in accordance with ISO 5725 : 1986, Precision of test methods —

- a) all the data necessary for the identification of the sample or the substance tested;
- b) the methods of preparation of the samples :
  - 1) for effluents, 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;
- c) all biological, chemical and physical information relating to the test and not specified in this International Standard, including the age of the *Daphnia magna* used and the breeding conditions;
- d) the results of the test in the form of the 24 h — EC (I) 50 and, where appropriate, the 48 h — EC (I) 50 (or 24 h — EC 50 and 48 h — EC 50), the method of calculation, and, where appropriate, the 95 % confidence limit; in the case of chemical analysis of the substances, the method used;
- e) the minimum concentration corresponding to 100 % immobilization and the maximum concentration corresponding to 0 % immobilization in 24 h (and, where appropriate, in 48 h);
- f) any abnormal behaviour of the *Daphnia magna* under the test conditions;
- g) any operating details not specified in this International Standard and incidents which may have affected the results.

Table 1

Product tested	Number of participating laboratories	Number of laboratories eliminated	Number of results used	Mean 24h — EC (I) 50 mg/l	Standard deviation			
					Repeatability		Reproducibility	
					Absolute	Coefficient of variation %	Absolute	Coefficient of variation %
$K_2Cr_2O_7$	46	4	129	1,47	0,21	14	0,57	39
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	14	72	772,25	64,5	8,3	277,8	35,9

## 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.

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

#### A.1 Result of the preliminary test

Concentration %	Mobile <i>Daphnia magna</i>
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

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

Concentration, %	Number of mobile <i>Daphnia magna</i> in tube				$T$	$P$
	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

##### A.2.2 Determination of 24 h — EC 50

By interpolation on the graph (see figure A.1), the 24 h — EC 50 is 0,55 %.

For effluents, this is expressed as :

$$24 \text{ h — EC } 50 = 0,55 \% \text{ or } 5,5 \text{ ml/l}$$

For a chemical substance, this is expressed as :

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