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Water quality — Determination of long term toxicity of substances to *Daphnia magna* Straus (*Cladocera*, *Crustacea*)

Qualité de l'eau — Détermination de la toxicité à long terme de substances vis-à-vis de Daphnia magna *Straus (*Cladocera, Crustacea*)*

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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 3.

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 International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 10706 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Annexes A, B, C and D of this International Standard are for information only.

Introduction

This International Standard defines a procedure for the determination of the long term sublethal toxicity of chemicals, waters and waste waters to the water-flea *Daphnia magna* Straus. The methodology is adapted from a guideline produced by the Organisation for Economic Co-operation and Development (OECD Guideline 211, see reference [1] in the Bibliography).

Water quality — Determination of long term toxicity of substances to *Daphnia magna* Straus (*Cladocera*, *Crustacea*)

WARNING — Activated sludge and sewage contain potentially pathogenic organisms. Therefore, appropriate precautions should be taken when handling them. Toxic test compounds and those whose properties are unknown should be handled with care.

1 Scope

This International Standard describes a method for the determination of the long term sublethal 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 stable suspensions or dispersions under the conditions of the test,

b) industrial or sewage effluents, treated or untreated, after decanting, filtration, or centrifugation,

c) surface or ground waters.

NOTE This International Standard is adapted from OECD Guideline 211 [1].

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

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

ISO 5667-16, *Water quality — Sampling — Part 16: Guidance on biotesting of samples.*

3 Principle

Daphnia magna females, less than 24 h old, are exposed for a period of 21 days to a test substance, industrial or sewage effluent or surface/ground water added to dilution water in a range of concentrations in a semi-static or flow-through system. The survival of parents is recorded together with the number of live offspring produced per live parent at the end of the test.

The survival is reported and the reproductive output of exposed parents living at the end of the test (expressed in a number of terms) is compared to those of the control parents.

4 Test environment

The test atmosphere shall be free from vapours or dusts which may be toxic to *Daphnia magna* Straus.

The exposure solutions shall not be aerated.

The dissolved oxygen concentration in the exposure solutions shall be above 3 mg/l and the pH shall be within the range of pH 6 to pH 9 and not change by more than 1,5 pH units for the duration of the test. Hardness shall be above 140 mg/l (as $CaCO_3$), which has been demonstrated to be necessary to promote reproductive performance that shall meet the validity criteria (see 8.7).

The photoperiod of the test shall be 16 h of light and 8 h of darkness. The intensity shall be in the range 600 lux to 800 lux but not exceeding 1 200 lux.

The temperature of the test exposures shall be maintained within the range of 18 °C to 22 °C and the temperature for the test shall not vary by more than 2 °C (for instance 18 °C to 20 °C, 19 °C to 21 °C or 20 °C to 22 °C) throughout the test.

5 Reagents and materials

5.1 Test organism: *Daphnia magna* Straus (*Cladocera, Crustacea*), hereafter referred to as *D. magna,* obtained by acyclical parthenogenesis for at least three generations under specified culture conditions.

The age 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 animals used for the test shall be less than 24 h old and be from the second to fifth brood. The *D. magna* shall be from a healthy stock showing no signs of stress such as mortality > 20 %, presence of males, ephippia, or discoloured animals and there shall be no delay in the production of the first brood.

The stock animals shall be maintained in culture conditions (light, temperature, medium, feeding and animals per unit volume) similar to those in the test. If the culture conditions differ from test conditions, it is recommended that one generation be acclimated under the test conditions for about three weeks so as to avoid stressing the parent animals.

5.2 Dilution water, synthetic as described in 5.3 or uncontaminated natural waters with comparable pH and hardness characteristics to these dilution waters for culturing and testing.

5.3 Synthetic dilution water, prepared using one of the following methods:

a) OECD M4 and M7 media (see informative annex A);

b) ASTM reconstituted hard freshwater (see informative annex B).

The dilution water shall be aerated until the dissolved oxygen concentration has reached 95 % saturation and the pH has stabilized. If necessary, adjust the pH to $8{,}0 \pm 0{,}5$ 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.

It is recommended that the TOC levels be < 5 mg/l before the addition of algae. The use of dilution water containing EDTA (for instance M4 and M7) is not recommended when testing compounds containing metals because chelation may reduce the toxicity of the compound.

If the test has to be performed for purposes necessitating the use of a dilution water with characteristics differing from those described above, mention is necessary in the test report of the main characteristics (for instance pH, hardness, TOC, COD) of the synthetic dilution water used.

6 Apparatus

Ordinary laboratory apparatus and in particular the following.

6.1 Environmental controls, for controlling temperature, photoperiod and light intensity.

6.2 Measuring apparatus and/or instruments, for measuring dissolved oxygen, pH, hardness, total organic carbon, chemical oxygen demand, light intensity and temperature.

6.3 Test containers, of chemically inert material and of sufficient capacity for the tests (for example 50 ml or 100 ml glass test tubes or beakers) and which are clean and uncontaminated.

7 Treatment and preparation of samples

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

Sampling of water or effluent shall be carried out in accordance with the general procedure specified in ISO 5667-2. Bottles shall be completely filled to exclude air.

The preservation and storage of water or effluent samples shall be carried out in accordance with ISO 5667-16; the following is only a summary. Carry out the toxicity test as soon as possible, ideally within 12 h of collection. If this time interval cannot be observed, cool the sample (0 °C to 4 °C) and test the sample within 24 h. If testing cannot be carried out within 48 h, the sample may be frozen (below –18 °C) for testing within 2 months of collection.

All portions (subsamples) shall be pretreated identically (i.e. if freezing of unstable water is necessary all portions, including those needed for the first day, shall be frozen prior to testing; see ISO 5667-16).

Because of the duration of this test (21 days) and the periodic renewal of solutions, a sufficient number of portions of the sample must be frozen to renew test solutions and to repeat the test, if needed (reserve samples). The minimum volume of the frozen portions is dependent upon the toxicity of the sample to be tested. The volume of sample needed for a semi-static test with ten daphnids exposed individually in 100 ml of undiluted sample is 9 l; additional sample will be required for diluted exposures.

If the test is conducted on site or close to the sampling site, fresh samples may be used to replace the test solutions. In this case the variations at the sampling site are incorporated into the test design.

7.2 Preparation of solutions of substances to be tested

7.2.1 Preparation of stock solutions

A stock solution of the substance(s) to be tested shall be prepared by dissolving a known quantity of the substance(s) in a specified volume of dilution water, deionized water or distilled water in a glass container. The stock solution shall be prepared immediately before preparing the exposure solutions unless the substance(s) is/are known to be stable in defined storage conditions, in which case the stock solution may be prepared in advance of testing and stored in these conditions.

Stock solutions or suspensions of substance(s) which are poorly soluble in water can be solubilized or dispersed directly in the medium with ultrasonic dispersion, and/or stirring or with solvents or dispersants of low toxicity to *D. magna* as discussed in ISO 5667-16. If a solvent is used, the concentration of the solvent in the stock solution shall be such that the concentration in the highest exposure solution does not exceed 0,1 ml/l.

The use of organic solvents should be avoided. If they are required organic solvents such as acetone, ethanol, methanol, dimethylformamide, triethylene glycol or dispersants such as Cremophor RH40, methylcellulose 0,1 %, and HCO-40 may be used to produce a suitably concentrated stock solution. They are not toxic to *D. magna* at 0,1 ml/l concentrations. 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 exposure solutions

The exposure solutions shall be prepared (8.2) by adding the stock solutions (7.2.1) or effluent samples (7.1) to the dilution water (5.2) in specified quantities (see 8.2).

If the stock solutions are prepared in deionized or distilled water, no more than 100 ml of stock solution shall be added to each litre of dilution water.

The chosen concentrations can also be prepared separately by the direct addition of the test substance to dilution water where the amounts to be added can be accurately weighed out or pipetted for liquids.

If the sample pH is not between 6 and 9, the test shall be carried out after adjusting the pH to the value of the nearest limit (6 or 9) using solutions of hydrochloric acid or sodium hydroxide.

NOTE Testing water samples at concentrations above 100 ml/l may reduce the reproduction and survival of *D. magna* because of deficiency in the medium (for instance hardness). Identifying effects of such deficiencies may require the addition of the same salts to the sample as in the dilution water.

8 Procedure

8.1 Controls

Every test shall include a control containing no test substance.

Two controls are required when a solvent or dispersant is used. One control shall contain no solvent or dispersant. The second control shall contain a concentration of solvent or dispersant equal to that in the highest exposure concentration.

8.2 Selection of exposure concentrations

There shall be at least five exposure concentrations arranged in a geometric series with a separation factor not exceeding 3,2.

In setting the range of concentrations, the following shall be borne in mind:

a) if the objective is to obtain the NOEC (no observed effect concentration), the range of concentrations shall include at least one concentration producing a significant effect compared to the control (LOEC: lowest observed effect concentration), preceded by a NOEC.

   If this is not the case, the test shall be repeated with a reduced lowest concentration. See footnote 1 to clause 9.

b) If the objective is to obtain the EC_p (effect concentration producing a percentage response; typically this is 20 % and/or 50 %) for the effect on reproduction and survival, it is advisable that two exposure concentrations be higher than this EC_p concentration (p is the percentage response selected). Otherwise, although it will still be possible to estimate the EC_p, the confidence interval for the EC_{50} will be very wide and it may not be possible to assess satisfactorily the adequacy of the fitted model.

NOTE Prior knowledge of the toxicity of the test substance from an acute test and/or from range-finding studies (ISO 6341) is helpful in selecting appropriate exposure concentrations.

Where a solvent or dispersant is used to aid preparation of stock solutions, the concentration of solvent or dispersant in the highest concentration shall not be greater than 0,1 ml/l. The concentration of solvent or dispersant shall, as far as possible, be the same in all vessels.

8.3 Renewal of exposure solutions

The exposure solutions shall be renewed at least three times per week. If preliminary stability tests indicate that the test substance concentration falls below 80 % of the initial measured concentration before day three, consideration shall be given to either increasing the frequency of exposure solution renewal or using a flow-through test. Changes in the frequency of solution replacement or the rate of continuous flow shall be reported.

When semi-static tests are conducted, minimize the volume of medium transferred with the *D. magna.*

8.4 Introduction of animals into the test system

Transfer young animals (< 24 h old) from the culture system to the test system at the beginning of the test. Place each individual in a single vessel containing 50 ml to 100 ml of exposure solution.

Larger volumes of exposure solution may be required for chemical analysis of exposure concentrations. Alternatively, replicates can be pooled for chemical analysis.

Semi-static testing shall require at least ten animals exposed individually to each exposure concentration and control. Furthermore, a second series of test vessels shall be prepared at the time of solution renewal and the parent animals are transferred to them by means of a glass pipette of suitable bore diameter.

Organisms shall be exposed for a total of 21 days.

If a continuous flow test is being conducted, this International Standard should be used for guidance.

8.5 Feeding of organisms

The diet of the parent animals shall be living algal cells of one of the following species: *Chlorella* spp, *Pseudokirchneriella subcapitata* (formally known as *Selenastrum capricornutum*) or *Scenedesmus subspicatus*. It is preferable to feed the animals daily but as a minimum when solutions are being replaced. The organisms shall be fed at a rate of 0,1 mg to 0,2 mg of carbon per animal per day. The ration shall be supplied either at a constant rate throughout the test period or at a gradually increasing rate consistent with the growth of the parent animals. When volumes greater than 100 ml are used in the test, the ration given shall be increased proportionately.

NOTE Supplementing the algal food with other sources of particulate carbon has proved valuable, to avoid nutritional deficiencies in pure, monospecific, cultured algae provided that the total carbon does not exceed the limits above.

Where a surrogate measure of carbon is used, such as algal cell number or light absorbance, the testing laboratory shall generate a nomograph relating the surrogate measure to carbon content. Nomographs shall be confirmed at least annually or when algal culture conditions are changed.

A concentrated algal suspension produced by centrifugation or decanting, followed by re-suspension in culture medium shall be transferred to the animals to minimize the volume of algal culture medium transferred to the test vessels.

8.6 Observations and measurements

Record all observations on a data sheet. An example of a suitable format is presented for information in annex C; other formats are acceptable.

The light intensity at the surface of the test solution shall be reported.

Measure and record the dissolved oxygen, temperature, water hardness, and the pH of the control(s) and the highest exposure concentration weekly, at the beginning and end of one of the renewal periods.

Live offspring produced by each parent shall be counted and removed at least three times per week when changing solutions. Record the presence of dead parents, offspring, males or ephippia eggs.

If relevant to the objectives of the analysis, measure and record the length and dry mass of parent animals at the end of the test.

In the case where chemical substances are being tested, exposure concentrations shall be confirmed by measurement at the beginning and the end of the test and more frequently depending on the stability of the substance or the method of exposure (for instance semi-static or flow-through).

In semi-static tests, where the concentration of test substance is expected to remain within ± 20 % (i.e. within the range of 80 % to 120 %) of the nominal concentration, the highest and lowest concentrations shall be analysed when freshly prepared and at the time of renewal on one occasion during each of the three weeks.

In semi-static tests, where the concentration of test substance is not expected to remain within ± 20 % of the nominal concentration, the concentration of all the exposure solutions shall be analysed at least weekly. If concentrations are demonstrated to be within 20 % of initial measured concentrations, then analyses shall be conducted on only the highest and lowest exposure concentrations. If the concentrations are not within 20 % of the initial measured concentrations, see clause 9.

An analysis regime similar to that for the semi-static tests shall be used for flow-through tests except that the frequency of analysis shall be increased during the first week to demonstrate the stability of the dosing system and test substance stock solution.

8.7 Validity of the test

Consider the test valid when the following criteria shall have been met:

a) the total number of control replicates exhibiting adult mortality and male development is $\leqslant$ 20 % at the end of the test;

b) the mean number of living offspring per living parent in the controls is $\geqslant$ 60;

c) the coefficient of variation for control fecundity, based on the number of offspring per parent per day, does not exceed 20 % in the controls.

The control animals shall be sexually mature and have produced their first brood within 11 days of the start of the test otherwise criterion b) may not be met.

9 Data analysis and expression of results

If the exposure concentration of the test substance is within 20 % of the nominal or initial concentration, then the results shall be expressed in terms of the nominal or initial concentration.

If the deviation of the exposure concentration of the test substance is greater than ± 20 % of the nominal or initial concentration, it is recommended that they be expressed in terms of the time-weighted mean concentration (see annex D for calculation).

If any parent is identified as a male, then exclude the replicate from the data analysis for reproduction.

Count the total number of offspring produced by each parent and calculate the mean number of live offspring (to the nearest single decimal place) produced by each live parent per exposure concentration.

The reproductive output shall be expressed as the total number of living offspring per live parent (for each replicate) at the end of the test. If parent animals are exposed individually, the reproductive output can only be expressed as the mean number of live offspring per live parent at the end of the test. When more than one organism has been exposed per vessel, reproductive output shall be expressed as "the total number of living offspring produced per live parent".

Compare the mean number of live offspring produced per parent in each exposure concentration to the control mean by Dunnett's or Williams' tests providing the variance among exposure concentrations is shown to be homogeneous by ANOVA (analysis of variance). If variances are not homogeneous, transform and re-evaluate the data.

The lowest exposure concentration that produces a response statistically different from the control response is the LOEC (lowest observed effect concentration). The highest exposure concentration below the LOEC that produces a response statistically similar to the control response is the NOEC[1)] (no observed effect concentration).

Estimation of the exposure concentration producing different levels of inhibition of offspring production plus 95 % confidence limits can be conducted using several different regression analyses[2)] for instance probit, logit, weibul, moving average, Spearman-Karber. Inhibition concentrations of 10 %, 20 % and 50 % (for instance EC_{10}, EC_{20}, EC_{50}), or lethal concentrations (LC_x), can be reported using these methods, which are also available as computer programs. The data shall also be plotted on log-probit axes to illustrate computer output results.

10 Test report

The test report shall include the following.

a) **Identity of test laboratory:**

— the name and location of the test facility and the date of the study;

— the identification of the individual(s) responsible for the test results.

b) **Description of the test substance, water or effluent:**

— the name(s) or the identity of the sample;

— the sample source;

— the Chemical Abstracts Service number if applicable;

— the physical-chemical properties (solubility, stability, pK_a, K_{ow} if known);

— the purity (and known impurities);

— the acute toxicity to *D. magna* and the biodegradability (if known).

c) **Description of test species:**

— the source and the culture identification;

— the clone (if known) and the supplier;

— the culture conditions.

1) A workshop convened by OECD 1998b (see reference [2] of the Bibliography) concluded that regression-based estimates (EC_x at time t) were preferable to the NOEC as a summary parameter of toxicity.

Hypothesis testing in general is not well suited to the type of data obtained from most toxicity tests (with the possible exception of limit tests) and the NOEC, in particular, is statistically unfounded. The OECD recommends that the NOEC be phased out as a summary of toxicity in preference to a regression-based estimation procedure in which the following should be reported: model parameters plus measures of error and goodness of fit; $EC_{x,t}$ (i.e. EC_x at time t); important biological parameters; parameters describing the time course of effects (OECD, 1998b).

2) Regression methods to calculate IC_x values can be found in references [3] to [5].

d) **Exposure conditions**

— the physical conditions (photoperiod, light intensity, temperature);

— the exposure method (exposure concentrations, semi-static, flow-through and rates of solution exchange);

— the number and the concentration of the nominal exposure solutions;

— the method of chemical analysis used for the test substance;

— the analytical sample design;

— the description of the culture medium;

— the exposure design (parents per vessel, number of replicates, number of concentrations);

— the preparation of the stock and the exposure solutions (solvents or dispersants used);

— the feeding regime (mg/l of carbon, schedule, algae used).

e) **Results**

— a description of the test substance stability (if available);

— the analyses of the exposure concentrations of the test substance;

— a description of the test conditions (dissolved oxygen, temperature, TOC/DOC hardness);

— the adult mortality and the incidence of dead offspring (number and dates);

— the average daily production of live offspring (number and dates);

— the total offspring produced in each exposure concentration and control;

— the mean live offspring produced (to a single decimal place) per live adult for each exposure concentration and each control;

— the total number of males produced in each exposure concentration and in each control;

— the NOEC and the LOEC of adult reproduction and mortality, if appropriate, plus the method of calculation (with statistical output data);

— the EC_{50} value, as appropriate, with the confidence limits and the method of calculation as well as the statistical output data concentration-response graph;

— any other biological effects observed or measured (for instance length, mass of adults);

— a description of any deviations from the method and an explanation;

— the variance of control reproduction.

Annex A
(informative)

OECD M4 and M7 media — Preparation and acclimation of Elendt M4 and M7 media

A.1 Transfer of *D. magna* to M4 and M7 media

Some laboratories have experienced difficulty in directly transferring *D. magna* to M4 (the first publication of the M4 medium can be found in reference [6] of the Bibliography) and M7 media. However, some success has been achieved with gradual acclimation by changing from the laboratory's medium to 30 % Elendt, then to 60 % Elendt and finally to 100 % Elendt. The acclimation periods may need to be as long as one month.

A.2 Preparation

A.2.1 Trace elements

Separate stock solutions (stock solutions I) of individual trace elements are first prepared in deionized water. From these different stock solutions (stock solutions I) a second single stock solution (stock solution II) is prepared, which contains all the (13) trace elements listed (combined stock solution II). See Table A.1.

A.2.2 M4 and M7 media

Prepare M4 and M7 media using stock solution II, the macro-nutrients and vitamins in accordance with Table A.2.

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

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

To avoid precipitation of salts when preparing the complete media, add the aliquots of stock solutions to about 500 ml to 800 ml deionized water and then dilute to1 l.

Table A.1

Stock solution(s) I (single substance)	Concentration in deionized water mg/l	Concentration (in relation to medium M4)	To prepare the combined stock solution II add the following volume of stock solution I to deionized water ml/l	
			M4	M7
H_3BO_3	57 190	20 000-fold	1,0	0,25
$MnCl_2 \cdot 4H_2O$	7 210	20 000-fold	1,0	0,25
LiCl	6 120	20 000-fold	1,0	0,25
RbCl	1 420	20 000-fold	1,0	0,25
$SrCl_2 \cdot 6H_2O$	3 040	20 000-fold	1,0	0,25
NaBr	320	20 000-fold	1,0	0,25
$Na_2MoO_4 \cdot 2H_2O$	1 260	20 000-fold	1,0	0,25
$CuCl_2 \cdot 2H_2O$	335	20 000-fold	1,0	0,25
$ZnCl_2$	260	20 000-fold	1,0	1,00
$CoCl_2 \cdot 6H_2O$	200	20 000-fold	1,0	1,00
KI	65	20 000-fold	1,0	1,00
Na_2SeO_3	43,8	20 000-fold	1,0	1,00
NH_4VO_3	11,5	20 000-fold	1,0	1,00
$Na_2EDTA \cdot 2H_2O$[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 autoclaved immediately. This gives:				
Fe-EDTA solution		1 000-fold	20,0	5,0

Table A.2

Stock solutions	Concentration in deionized water mg/l	Concentration (in relation to medium M4)	Volume of stock solution added to prepare medium ml/l	
			M4	M7
Stock solution II (combined trace elements)		20-fold	50	50
Macro nutrient stock solutions (single substance)				
$CaCl_2 \cdot 2H_2O$	293 800	1 000-fold	1,0	1,0
$MgSO_4 \cdot 7H_2O$	246 600	2 000-fold	0,5	0,5
KCl	58 000	10 000-fold	0,1	0,1
$NaHCO_3$	64 800	1 000-fold	1,0	1,0
$Na_2SiO_3 \cdot 9H_2O$	50 000	5 000-fold	0,2	0,2
$NaNO_3$	2 740	10 000-fold	0,1	0,1
KH_2PO_4	1 430	10 000-fold	0,1	0,1
K_2HPO_4	1 840	10 000-fold	0,1	0,1
Combined vitamin stock	—	10 000-fold	0,1	0,1

Table A.3

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

Annex B
(informative)

ASTM reconstituted hard fresh water

Quantities of reagent grade chemicals required to prepare reconstituted freshwaters and the resulting water quality are given in Table B.1.

Table B.1 [a]

Type of freshwater	Salts required mg/l				pH [b]	Hardness	Alkalinity
	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCl		mg/l of $CaCO_3$	mg/l of $CaCO_3$
Hard	192	120,0	120,0	8,0	7,8 to 8,0	160 to 180	110 to 120

[a] Values taken from reference [7] of the Bibliography.

[b] Approximate equilibrium pH after aeration.

Annex C
(informative)

Data collection sheet

An example data sheet for recording medium renewal, physical/chemical monitoring data and feeding, *D. magna* reproduction and mortality is presented in Table C.1.

Table C.1 — Example data sheet

Experiment No.:
Date started:
Clone:
Medium:
Type of food:
Test substance:
Nominal concentration:

Day	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**		
Medium renewal (tick)																								
pH [a]																							fresh	
																							old	
O_2 (mg/l) [a]																							fresh	
																							old	
Temperature (°C) [a]																							fresh	
																							old	
Food provided (tick)																								
Number of live offspring [b]																							Total	
Vessel 1																								
2																								
3																								
4																								
5																								
6																								
7																								
8																								
9																								
10																								
																							Total	
Cumulative adult mortality [c]																								

[a] Indicate which vessel was used for the experiment.
[b] Record aborted broods as ‘AB’ in relevant box.
[c] Record mortality of any adult animals as ‘M’ in relevant box.