Algal Growth Inhibition Test, *Daphnia* Acute Immobilization Test, and Fish Acute Toxicity Test

I. Scope of application

A standard method for the algal growth inhibition test, *Daphnia* acute immobilization test or fish acute toxicity test of chemicals is described here.

II. Definitions

The definitions of the terms used in this test method are given below.

1. Test system

- **Static test**: A test performed without replacing the test solution in the test vessel throughout the exposure period.
- **Semi-static test**: A test performed by replacing the test solution in the test vessel with a new test solution like a batch system at certain intervals (e.g., 24 hr).
- **Flow-through test**: A test performed by constantly and automatically replacing the test solution in the test vessel with a new test solution while draining the old solutions.

2. Endpoints

- **LC_{50}** in this Test Guideline is the median lethal concentration, i.e. that concentration of the test substance in water which kills 50 per cent of a test batch of fish within a particular period of exposure (which must be stated).
- **EC_X** is the concentration of the test substance dissolved in test medium that results in an x % (e.g. 50%) reduction in growth, mobility, reproduction, etc., of the test organism within a stated exposure period.
- **LOEC** is the lowest tested concentration at which the substance is observed to have a statistically significant harmful effect on growth (at $p < 0.05$) when compared with the control, within a given exposure time. However, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC. When these two conditions cannot be satisfied, a full explanation must be given for how the LOEC (and hence the NOEC) has been selected.
- **NOEC** is the test concentration immediately below the LOEC and the highest tested concentration at which the substance is not observed to have a statistically significant harmful effect (at $p < 0.05$) when compared with the control, within a given exposure time.

III. General rules

1. Basic idea of the test

Toxicity tests using algae, *Daphnia* sp. or fish are intended for determining the toxicity of test substances by exposing the test organisms to the test substances dissolved in medium or dilution water (hereinafter referred to as "medium, etc."). Therefore, the solubility of the test substance to the medium, etc. under the test condition must be checked before conducting the test. Moreover, a reliable method for quantifying the test substance in the test solution must be available.

Wherever possible, the test conditions should be kept constant throughout the exposure period. For example, the test substance concentration is preferably maintained at least 80% of the initial concentration (i.e., the nominal concentration or measured concentration at the beginning of the exposure). Information on the test substance which may be useful in establishing the test conditions includes structural formula, purity, stability in light, stability under the conditions of the test, pKa, octanol water partition coefficient (Pow), vapor pressure and results of studies of transformation including biodegradability in water. Test substances with large vapor pressures may be lost during the exposure period, so it is suggested to obtain the Henry constant that serves as the index of the loss. The Henry constant can be calculated from the solubility and the vapor pressure.

2. Preparation of the test solution

To prepare a test solution of each concentration, directly dissolve the required amount of the test substance in the medium, etc., or prepare a stock solution of the test substance at an appropriate concentration and dilute it with the medium, etc. Prepare the stock solution without using any vehicle, but if it is difficult to dissolve the test substance in water or the medium, etc., perform mechanical dispersion by means of ultrasonic wave, etc., or use a low-toxicity vehicle (which hereinafter refers to a solvent or dispersant) such as an organic solvent. However, as a rule, do not use any surfactant. Set the test concentrations so that they do not exceed the solubility (hereinafter referred to as "solubility limit") of the test substance to the medium, etc. under the test conditions.

When using a vehicle, additionally establish a vehicle control containing the vehicle at the same concentration as that used for preparing the test concentrations. In principle, the concentration of the vehicle must not exceed 100 mg/L and must be constant among all the test concentrations. Note that the obtained test results may be attributed to the test substance itself or its interactive effect with the vehicle.

3. Handling of water-insoluble substances

Even if the test substance is insoluble in water, basically avoid using any dispersant. Set the test concentrations so that they do not exceed the solubility limit of the test substance. However, if the test substance has an extremely low solubility in the medium, etc. and its solubility limit cannot be determined by usual methods, and if test concentrations above the solubility limit are inevitable for determining the toxicity values such as LC_{50}, perform the test using a dispersed system. If such test substances are intended to be used with dispersants or emulsifiers, perform the test using a dispersant.

Before concluding that the toxicity values such as LC_{50} cannot be determined at concentrations within the soluble or dispersible limit of the test substance in the medium, etc., take every possible measure for dissolving or dispersing the test substance in the medium, etc. and determine the upper limit of the concentration at which the test substance can be dissolved or dispersed in the medium, etc.

IV. Algal growth inhibition test

Objective

The purpose of the present study is to determine the toxicity of the test substance against algal growth by exposing algae at the exponential growth phase to the test substance and measuring the growth inhibition rate against the control. Growth is quantified from measurements of the algal biomass as a function of time.

1. Test organism

The recommended species is *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*), but other species such as *Desmodesmus subspicatus* (formerly known as *Scenedesmus subspicatus*) may also be used. If species other than these two are used, it must be confirmed that their exponential growth can be maintained throughout the exposure phase.

2. Test vessels and other apparatus

The following test vessels and apparatus are used for the study.

2-1. Test vessels

Test vessels and other apparatus which will come into contact with the test solutions should be made entirely of glass or other chemically inert material. The test vessel has a sufficiently large surface exposed to air. For example, a 250 mL conical flask is suitable for holding a 100 mL test solution. If the test substance is volatile, take appropriate measures such as using sealable

flasks.

2-2. Culturing apparatus

Culture the test organisms in an incubator or incubation chamber where constant temperature and lighting conditions can be maintained.

2-3. Apparatus to determine algal biomass

Cell count, which is the most frequently used surrogate parameter for algal biomass, may be made using an electronic particle counter, a microscope with counting chamber. Other biomass surrogates can be measured using a fluorimeter, spectrophotometer or colorimeter. In order to provide useful measurements at low biomass concentrations when using a spectrophotometer, it may be necessary to use cuvettes with a light path of at least 4 cm.

3. Growth media

The recommended composition of the medium is as follows.

- Ammonium chloride 15 mg/L
- Magnesium chloride hexahydrate 12 mg/L
- Calcium chloride dehydrate 18 mg/L
- Magnesium sulfate heptahydrate 15 mg/L
- Potassium dihydrogen phosphate 1.6 mg/L
- Ferric chloride (III) hexahydrate 0.064 mg/L
- Disodium ethylene diamine tetraacetiate dihydrate 0.1 mg/L
- Boric acid 0.185 mg/L
- Manganese chloride tetrahydrate 0.415 mg/L
- Zinc chloride 0.003 mg/L
- Coblat chloride hexahydrate 0.0015 mg/L
- Copper chloride dihydrate 0.00001 mg/L
- Disodium molybdate dihydrate 0.007 mg/L
- Sodium hydrogen carbonate 50 mg/L

  The pH of this Media will be 8.1 in the equilibrium condition with atmosphere.

4. Preculture

In order to adapt the test alga to the test conditions and ensure that the algae are in the exponential growth phase when used to inoculate the test solutions, an inoculum culture in the test medium is prepared 2-4 days before start of the test. The algal biomass should be adjusted

in order to allow exponential growth to prevail in the inoculum culture until the test starts.

5. Test solution

To prepare a test solution of each concentration, directly dissolve the required amount of the test substance in the medium, or prepare a stock solution of the test substance at an appropriate concentration and dilute it with the medium. Follow the descriptions in "Preparation of the test solution" under "III. General rules."

6. Test condition

6-1. Exposure period

Exposure period is normally 72 hours.

6-2. Initial biomass concentration

The initial biomass in the test cultures must be the same in all test cultures and sufficiently low to allow exponential growth throughout the incubation period. The initial biomass should not exceed 0.5 mg/L as dry weight. The following initial cell concentrations are recommended:

Pseudokirchneriella subcapitata: 5×10^3 - 10^4 cells/mL

Desmodesmus subspicatus 2-5 x 10^3 cells/mL

For other species, adjust the initial biomass concentration to achieve an equivalent dry weight.

6-3. Test concentration

For the final definitive test at least five concentrations, arranged in a geometric series, should be selected. The concentration series should preferably cover the range causing 0-75 % inhibition of algal growth rate. Concentrations of 100 mg/L or higher do not need to be tested. Perform a control, and additionally a vehicle control if using any vehicle.

6-4. Number of replicates

Perform a test with at least three replicates at each test concentration including the control(s). The number of control replicates of six is recommended (e.g. three control replicates and vehicle control in six replicates when using any vehicle).

6-5. Incubation

- Temperature: The cultures should be maintained at a temperature in the range of 21 to 24 °C, controlled at ± 2 °C.

- Illumination: 60 - 120μE/m^2/s (The surface where the cultures are incubated should receive continuous, uniform fluorescent illumination e.g. of «cool-white» or «daylight» type.)
- Incubation: Cap the test vessels with air-permeable stoppers. The vessels are shaken and placed in the culturing apparatus. During the test it is necessary to keep the algae in suspension and to facilitate transfer of CO_2. To this end constant shaking or stirring should be used.

7. Beginning of the exposure to the test substance

Start the exposure by inoculating each test vessels with the algae that have been precultured to the initial biomass concentration established according to 6-2.

8. Measurement of the biomass

Measure the biomass in each test vessels at least at 24, 48 and 72 hr after the beginning of the exposure. Use sterilized medium as a background of the particle counter or a blank of the spectrophotometer, etc.

9. Analysis of the concentration of the test substance

9-1. Analysis of the concentration of the test substance

At the beginning and end of the test, analyze the concentrations of the test substance at least in the lowest and highest test concentration and the concentration around the predicted EC_{50}. If it is predicted that the concentrations of the test substance decreases from the nominal concentration by 20% or more during the exposure period, it is recommended to analyze all test concentration at the beginning and end of the test. Furthermore, for volatile or adsorbing test substances or those that are likely to be greatly decreased during the exposure period, additional sampling for analysis at 24 hr intervals during the exposure period are recommended.

9-2. Measurement of the test condition

Measure the pH of the test solution at the beginning and end of the test. Generally, the variation of the pH in the control must not exceed 1.5 units during the test.

10. Limit test

Under some circumstances, e.g. when a preliminary test indicates that the test substance has no toxic effects at concentrations up to 100 mg/L or up to its limit of solubility in water (whichever is the lower), a limit test can be performed at this concentration to demonstrate that NOEC, etc.,

is higher than this concentration. All previously described test conditions and validity criteria apply to a limit test, with the exception that the number of treatment replicates must be at least six. The average specific growth rate in the control (in vehicle control if using any vehicle) and treatment group may be analyzed using a statistical test to compare means, e.g. a Student's t-test.

11. Validity of the test

For the test to be valid, the following performance criteria should be met for *Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus*.

- The biomass in the control (also in the vehicle control) cultures should have increased exponentially by a factor of at least 16 within the exposure period.
- The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2 and 2-3, for 72-hour tests) in the control (also in the vehicle control) cultures must not exceed 35%.
- The coefficient of variation of average specific growth rates during the whole test period in replicate control (also in vehicle control) cultures must not exceed 7% in tests.

12. Analysis of the results

12-1. Processing of the results

In principle, analysis of the results must be based on appropriate averages of measured values. If there is evidence that the concentration of the substance being tested has been satisfactorily maintained within ± 20 % of the nominal or measured initial concentration throughout the test, analysis of the results can be based on nominal or measured initial values.

Tabulate the estimated biomass concentration in test cultures and controls (including vehicle control) together with the concentrations of test material and the times of measurement to produce plots of growth curves. Using the plots, examine whether control (including vehicle control) cultures grow exponentially at the expected rate throughout the test.

It is recommended to obtain a quantitative concentration-response relationship of the test substance using the methods described in 12-2.

12-2. Comparison of growth rates

The average specific growth rate for a specific period is calculated as the logarithmic increase in the biomass from the equation for each single vessel of controls and treatments:

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i}$$

where:

μ_{i-j} is the average specific growth rate from time i to j;

X_i is the biomass at time i;

X_j is the biomass at time j;

t_i is the time (*d*) of i^{th} biomass measurement after beginning the exposure;

t_j is the time (*d*) of j^{th} biomass measurement after beginning the exposure

For calculation of EC_{50}, determine the average specific growth rate over the entire test duration (normally days 0-3).

The growth rate can also be obtained from the slope of the regression line drawn by plotting the logarithmic values of the biomass measurements.

Calculate the percent inhibition of growth rate for each treatment replicate from equation:

$$I_\mu = \frac{\mu_c - \mu_T}{\mu_c} \times 100$$

where:

I_μ is the percent inhibition in average specific growth rate;

μ_C is the mean value for average specific growth rate (μ) in the control (in vehicle control if using any vehicle) group;

μ_T is the average specific growth rate for the treatment replicate

12-3. Calculation of the EC_{50} and the NOEC

Plot the values for I_μ against the logarithmic value of the test substance concentration. Using the regression equation, etc., determine the 50% inhibition concentration. The EC_{50} values obtained using I_μ is referred to as ErC_{50}.

The values $\mu_{0\text{-}3d}$ of the control (vehicle control if using any vehicle) and each test concentration are further subjected to an appropriate method for analysis of variance (ANOVA) and a multiple comparison method to determine the NOEC.

13. Summary of the results

Summarize the test results in Form 7 and attach it to the final report.

[Form 7] Report on the Results of the Algal Growth Inhibition Test

1. General information

Name of new chemical substance (based on the IUPAC nomenclature system)			
Other name			
CAS No.			
Structural or rational formula (if neither is available, summarize its formulation method)			
Molecular weight			
Purity of the new chemical substance used for the test (%)			
Lot number of the new chemical substance used for the test			
Names and contents of impurities			
Vapor pressure			
Solubility in water			
1-Octanol/water partition coefficient			
Melting point			
Boiling point			
Properties at room temperature			
Stability			
Solubility in solvents, etc.	Solvent	Solubility	Stability in solvent

[Notes] Provide the physicochemical properties wherever possible.

1. Fill in the “Vapor pressure” column with the vapor pressure of the test substance.

2. Fill in the “Stability” column with the stability of the test substance against temperature, light, etc.

3. Fill in the “Solubility in solvents, etc.” column with the solubility and stability of the test substance in a solvent.

2. Method for analyzing the test substance concentration in the test solution

Items	Methods
Analytical method	
Pretreatment	
Quantification conditions	

[Notes]

1. Specify the analytical method used for the measurement in “Analytical method”.
2. Summarize the treatment performed prior to the analysis in “Pretreatment”. Specify the means used for isolating the algal cells.
3. Write the apparatuses and conditions such as temperature and eluate used for the analysis in “Quantification conditions”.

3. Test materials and methods

Items			Contents
Applied test guideline			
Test organism	Species (scientific name, strain)		
	Source		
	Susceptibility to the reference substance (EC_{50}) (name of the reference substance)		
Preculture	Duration of preculture		
	Name of medium		
	Environmental conditions (water temperature, light intensity)		
Test conditions	Test vessel		
	Name of medium		
	Date of exposure		(Month) (Day) (Year) - (Month) (Day) (Year)
	Test concentrations (nominal values)		(Geometric ratio)
	Initial biomass		cells/mL
	Number of replicates	Exposure group	
		Control group	
	Test solution volume		
	Vehicle	Use or not	
		Kind	
		Concentration(s)	
		Number of replicates for vehicle control group	
	Culture method (shaking, stationary, continuous, etc.)		
	Water temperature or culture temperature		
	Illumination (light intensity, photoperiod, etc.)		
Calculation of results	Statistical method		

[Notes]

1. Write the results (specify the reference substance and write the EC_{50}) of the susceptibility test of the test organism in “Susceptibility to the reference substance”.
2. List all test substance concentrations used for the test and the geometric ratio in “Test concentrations (nominal values)”.
3. Write the material and volume of the test vessel in “Test vessel” in “Test conditions”. For a volatile test substance, write whether the vessel was sealed or unsealed.
4. Specify the statistical analysis method (e.g., probit, ANOVA, etc.) used for calculating the toxicity values (EC_{50} and NOEC) in “Statistical method”.

4. Test results and discussion

Items	Contents
Toxicity values	0 - 72hErC_{50} = mg/L NOEC (based on growth rate) = mg/L
Exposure concentrations used for calculation	1. Nominal values 2. Measured values
Remarks	

[Notes]

1. Specify whether the concentrations used for calculating the toxicity value (EC_{50} or NOEC) were nominal or measured values in “Exposure concentrations used for calculation”.

2. Discuss the characteristics of the toxicity values and the validity of the test based on the physicochemical properties of the test substance in “Remarks”. Write the influence on the test results, etc., of any anomaly observed in the test or any deviations from the test method.

5. Algal growth curve and concentration-inhibition (growth rate) curve

Attach 1) a growth curve (Figure example 1) and 2) a figure showing the growth inhibition rates at individual test concentrations (Figure example 2) during the exposure period.

Figure example 1; Algal growth curve

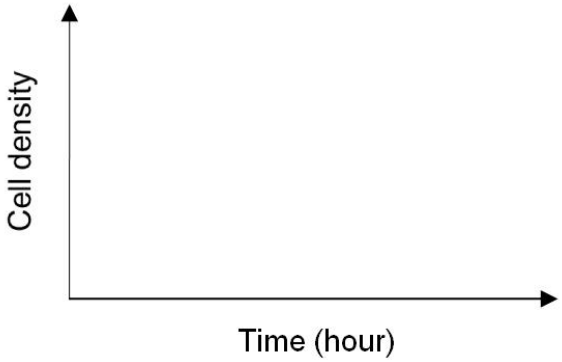
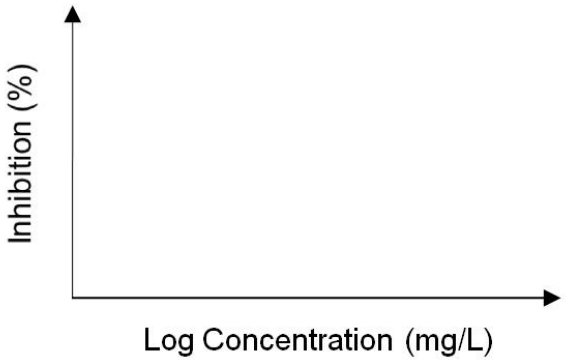


Figure example 2; Algal concentration-inhibition (growth rate) curve



6. Others

Test agency	Name	
	Address	Tel: Fax:
Test director	Name and status	
	Years of experience	
Test ID number		
Test period	From (month) (day) (year) to (month) (day) (year)	

[Notes]

1. Fill in the present form by transcribing from the final report.
2. Fill in the test ID number reported in the final report.
3. In the margin of this form, provide the name and affiliation of the person in charge of filling in this form.

V. *Daphnia* sp., Acute Immobilization Test

Objective

The purpose of the present test is to determine the toxicity of the test substance against the mobility of *Daphnia* sp. by exposing them to the test substance for 48 hr and measuring the immobilization rate against the control. The present test defines an organism as being immobilized when it does not move for 15 sec. after the test vessel is gently shaken.

1. Test organism

Daphnia magna Straus is the preferred test species although other suitable *Daphnia* species can be used in this test (e.g. *Daphnia pulex*).

At the start of the test, the animals should be less than 24 hours old, and to reduce variability, it is strongly recommended they are not first brood progeny. They should be derived from a healthy stock (i.e. showing no signs of stress such as high mortality, presence of males and ephippia, delay in the production of the first brood, discoloured animals, etc.). All organisms used for a particular test should have originated from cultures established from the same stock of daphnids.

The stock animals must be maintained in culture conditions (light, temperature, medium) similar to those to be used in the test. If the daphnids culture medium to be used in the test is different from that used for routine daphnids culture, it is good practice to include a pre-test acclimation period. For that, brood daphnids should be maintained in dilution water at the test temperature for at least 48 hours prior to the start of the test.

2. Test vessel and apparatus

The following test vessel and apparatus are used for the test.

2-1. Test vessel

Test vessels and other apparatus that will come into contact with the test solutions should be made entirely of glass or other chemically inert material. Test vessels should be loosely covered to reduce the loss of water due to evaporation and to avoid the entry of dust into the solutions. Volatile substances should be tested in completely filled closed vessels large enough to prevent oxygen becoming limiting or too low.

2-2. Apparatus

In addition some or all of the following apparatus will be used: oxygen-meter (with microelectrode or other suitable equipment for measuring dissolved oxygen in low volumes samples); pH meter; adequate apparatus for temperature control.

3. Test water

Natural water (surface or ground water), dechlorinated tap water or reconstituted water (e.g., Appendix Table 1) are acceptable as holding *daphnia* and testing water. Any water which conforms to the chemical characteristics of an acceptable dilution water as listed in Appendix Table 2 is suitable as the test water.

Note that media containing known chelating agents, such as Elendt M4 and M7 media, should be avoided for testing substances containing metals. Waters with total hardness of less than 250 mg $CaCO_3$ per liter, and with a pH 6 to 9 are preferable. The dilution water may be aerated prior to use for the test.

4. Test solution

Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. Stock solutions should preferably be prepared by dissolving the test substance in the dilution water. Follow the descriptions in "Preparation of the test solution" under "III. General rules".

The test should be carried out without the adjustment of pH. If the pH does not remain in the range 6-9, then a second test could be carried out, adjusting the pH of the stock solution to that of the dilution water before addition of the test substance. The pH adjustment should be made in such a way that the stock solution concentration is not changed to any significant extent and that no chemical reaction or precipitation of the test substance is caused. HCl and NaOH are preferred.

5. Procedure

5-1. Conditions of exposure

The test can be performed under a static, semi-static or flow-through condition. If the test substance concentration is unstable, a semi-static or flow-through test is recommended.

5-2. Duration

The test duration is 48 hr.

5-3. Test groups and controls

Volume: At least 2 ml of test solution should be provided for each animal.

Number of test organisms: At least 20 animals, preferably divided into four groups of five animals each, should be used at each test concentration and for the controls.

5-4. Test concentration

At least five test concentrations should be used. They should be arranged in a geometric series with a separation factor preferably not exceeding 2.2. The highest concentration tested should preferably result in 100 per cent immobilization, but concentrations of 100 mg/L or higher do not need to be tested, and the lowest concentration tested should preferably give no observable effect.

Perform a control, and additionally a vehicle control if using any vehicle.

5-5. Incubation conditions

- Illumination: A 16-hour light and 8-hour dark cycle is recommended. Complete darkness is also acceptable, especially for test substances unstable in light.
- Temperature: The temperature should be within the range of 18 °C – 22 °C, and for each single test it should be constant within ±1.0 °C.
- Dissolved oxygen concentration: It must be kept at 3 mg/L or higher. In principle, the test vessels must not be aerated during the test.
- Feeding: The daphnids should not be fed during the test.

6. Beginning of the exposure to the test substance

Start the exposure by transferring a specified number of organisms established in 5-3. to each test vessel.

7. Observation

Each test vessel should be checked for immobilized daphnids at 24 and 48 hours after the beginning of the test. The organisms are considered as being immobilized when it does not move for 15 sec. after the test vessel is gently shaken. In addition to immobility, any abnormal behaviour or appearance should be reported.

8. Analysis of the concentration of the test substance

8-1. Analysis of the concentration of the test substance

The concentration of the test substance should be measured, as a minimum, at the highest and lowest test concentration, at the beginning and end of the test.

If it has been predicted that the test substance concentration decreases from the initial concentration by 20 per cent or more during the exposure period, it is recommended to take measurements for all test concentration groups at the beginning and end of the exposure. Furthermore, for volatile or adsorptive substances or those that are likely to be greatly decreased

during the exposure period, additional measurements are recommended at 24 hr intervals during the exposure period.

In a semi-static test, perform at least two sets of measurements, provided that the measurements taken immediately after the water renewal and immediately before the next renewal are counted as one set.

8-2. Measurement of the test condition

The dissolved oxygen and pH are measured at the beginning and end of the test in the control(s) and in the highest test substance concentration. The temperature is usually measured in control vessels or in ambient air and it should be recorded preferably continuously during the test or, as a minimum, at the beginning and end of the test. The pH should normally not vary by more than 1.5 units in any one test.

9. Limit test

A limit test may be performed at 100 mg/l of test substance or up to its limit of solubility in the test medium (whichever is the lower) in order to demonstrate that the EC_{50} is greater than this concentration. The limit test should be performed using 20 daphnids (preferably divided into four groups of five), with the same number in the control(s). If the percentage of immobilization exceeds 10 per cent at the end of the test, a full study should be conducted. Any observed abnormal behaviour should be recorded.

10. Validity of the test

For a test to be valid, the following performance criteria apply:

- In the control, including the control containing the solubilising agent, not more that 10 per cent of the daphnids should have been immobilized;
- The dissolved oxygen concentration at the end of the test should be 3 mg/l or higher in control and test vessels.

11. Calculation of the results

In principle, perform the calculation of the results based on appropriate averages of the test substance concentration measurements. If evidence is available to demonstrate that the concentration of the test substance has been satisfactorily maintained within ± 20 per cent of the initial concentration throughout the test, then the results can be based on initial values.

Data should be summarized in tabular form, showing for each treatment group and control, the number of daphnids used, and immobilization at each observation. The percentages immobilized at 24 hours and 48 hours are plotted against test concentrations. Data are analyzed by

appropriate statistical methods (e.g. probit analysis, etc.) to calculate the slopes of the curves with 95 per cent confidence limits and the EC_{50} for 48 hr exposure.

Where the standard methods of calculating the EC_{50} are not applicable to the data obtained, the highest concentration causing no immobility and the lowest concentration producing 100 per cent immobility should be used as an approximation for the EC_{50} (this being considered the geometric mean of these two concentrations).

12. Summary of the results

Summarize the test results in Form 8 and attach it to the final report.

Appendix Table 1: A reconstituted water (ISO 6341-1982)

(1) ISO Test Water

(a) Calcium chloride solution

Dissolve 11.76 g $CaCl_2 \cdot 2H_2O$ in deionised water; make up to 1 litre with deionised water

(b) Magnesium sulphate solution

Dissolve 4.93 g $MgSO_4 \cdot 7H_2O$ in deionised water; make up to 1 litre with deionised water

(c) Sodium bicarbonate solution

Dissolve 2.59 g $NaHCO_3$ in deionised water; make up to 1 litre with deionised water

(d) Potassium chloride solution

Dissolve 0.23 g KCl in deionised water; make up to 1 litre with deionised water

25 ml each of solutions (a) to (d) are mixed and the total volume made up to 1 litre with water.

Water of suitable purity, for example deionised, distilled or reverse osmosis with conductivity preferably not exceeding 10 μScm^{-1} is recommended. All chemicals must be of analytical grade.

(2) Elendt M4 and M7 medium

Preparation

Trace element

Separate stock solutions (I) of individual trace elements are first prepared in water of suitable purity, for example deionised, distilled or reverse osmosis. From these different stock solutions (I) a second single stock solution (II) is prepared, which contains all trace elements (combined solution), i.e.:

Stock solution(s) I (Single Substance)	Amount added to water (mg/l)	Concentration (related to medium M4)	To prepare the combined stock solution II, add the following amount of stock solution I to water (ml/l)	
			Elendt M4	Elendt 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	1.0
$CoCl_2 \cdot 6H_2O$	200	20,000-fold	1.0	1.0
KI	65	20,000-fold	1.0	1.0
Na_2SeO_3	43.8	20,000-fold	1.0	1.0
NH_4VO_3	11.5	20,000-fold	1.0	1.0
$Na_2EDTA \cdot 2H_2O$	5,000	2,000-fold	-	-
$FeSO_4 \cdot 7H_2O$	1,991	2,000-fold	-	-
Both Na_2EDTA and $FeSO_4$ solutions are prepared singly, poured together and autoclaved immediately.				
21 Fe-EDTA solution		1,000-fold	20.0	5.0

M4 and M7 media

M4 and M7 media are prepared using stock solution II, the macro-nutrients and vitamin as follows:

	Amount added to water (mg/l)	Concentration (related to medium)	Amount of stock solution II added to prepare medium (ml/l)
Stock solution II (combined trace elements)		20-fold	50
Macro nutrients stock solutions (single substance)			
$CaCl_2 \cdot 2H_2O$	293,800	1,000-fold	1.0
$MgSO_4 \cdot 7H_2O$	246,600	2,000-fold	0.5
KCl	58,000	10,000-fold	0.1
$NaHSO_3$	64,800	1,000-fold	1.0
$Na_2SiO_3 \cdot 9H_2O$	50,000	5,000-fold	0.2

$NaNO_3$	2,740	10,000-fold	0.1
KH_2PO_4	1,430	10,000-fold	0.1
K_2HPO_4	1,840	10,000-fold	0.1
Combined Vitamin stock	-	10,000-fold	0.1
The combine vitamin stock solution is prepared by adding the 3 vitamin to 1 litre water, as shown below:			
Thiamine hydrochroride	750	10,000-fold	
Cyanocobaltamine (B12)	10	10,000-fold	
Biotine	7.5	10,000-fold	

The combined vitamin stock is stored frozen in small aliquots. Vitamins are added 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 – 800 ml deionised water and then fill up to 1 litre.

Appendix Table 2: Some chemical characteristics of an acceptable dilution water

Substance	Concentration
Particulate matter	< 20 mg/l
Total organic carbon	< 2 mg/l
Unionised ammonia	< 1 μg/l
Residual chlorine	< 10 μg/l
Total organophosphorus pesticides	< 50 ng/l
Total organochorine pesticides plus polychlorinated biphenyls	< 50 ng/l
Total organic chlorine	< 25 ng/l

[Form 8] Report on the Results of the *Daphnia* sp., Acute Immobilization Test

1. General information

Name of new chemical substance (based on the IUPAC nomenclature system)			
Other name			
CAS No.			
Structural or rational formula (if neither is available, summarize its formulation method)			
Molecular weight			
Purity of the new chemical substance used for the test (%)			
Lot number of the new chemical substance used for the test			
Names and contents of impurities			
Vapor pressure			
Solubility in water			
1-Octanol/water partition coefficient			
Melting point			
Boiling point			
Properties at room temperature			
Stability			
Solubility in solvents, etc.	Solvent	Solubility	Stability in solvent

[Notes] Provide the physicochemical properties wherever possible.

1. Fill in the “Vapor pressure” column with the vapor pressure of the test substance.

2. Fill in the “Stability” column with the stability of the test substance against temperature, light, etc.

3. Fill in the “Solubility in solvents, etc.” column with the solubility and stability of the test substance in a solvent.

2. Method for analyzing the test substance concentration in the test solution

Items	Methods
Analytical method	
Pretreatment	
Quantification conditions	

[Notes]

1. Specify the analytical method used for the measurement in “Analytical method”.
2. Summarize the treatment performed prior to the analysis in “Pretreatment”. Specify the means used for isolating the algal cells.
3. Write the apparatuses and conditions such as temperature and eluate used for the analysis in “Quantification conditions”.

3. Test materials and methods

Items			Contents
Test organism	Species (scientific name, strain, age in hours)		
	Source		
	Susceptibility to the reference substance (EC_{50}) (name of the reference substance)		
Culture	Kind of medium		
	Environmental conditions (water temperature, photoperiod)		
Test conditions	Test vessel		
	Test water	Kind (natural water, dechlorinated tap water, artificially prepared water, etc.)	
		Hardness	
		pH	
	Date of exposure		(Month) (Day) (Year) - (Month) (Day) (Year)
	Test concentrations (nominal values)		(Geometric ratio)
	Number of organisms		organisms/test vessel
	Number of replicates	Exposure group	
		Control group	
	Test solution volume		
	Vehicle	Use or not	
		Kind	
		Concentration(s)	
		Number of replicates for vehicle control group	
	Culture method (static, semi-static, flow-through, etc.)		
	Conditions for water renewal or flow-through		
	Water temperature		°C
	Dissolved oxygen concentration (DO)		mg/L
	Photoperiod		
Calculation of results	Statistical method		

[Notes]

1. Write the results (specify the reference substance and write the EC_{50}) of the susceptibility test of the test organism in “Susceptibility to the reference substance”.
2. List all test substance concentrations used for the test and the geometric ratio in “Test concentrations (nominal values)”.
3. Write the material and volume of the test vessel in “Test vessel” in “Test conditions”. For a volatile test substance, write whether the vessel was sealed or unsealed.
4. Specify the statistical analysis method (e.g., probit, etc.) used for calculating the toxicity value (EC_{50}) in “Statistical method”.

4. Test results and discussion

Items	Contents
Toxicity value	48hEC_{50} = mg/L
Exposure concentrations used for calculation	1. Nominal values 2. Measured values
Remarks	

[Notes]

1. Write the EC_{50} for immobilization for 48 hr in “Toxicity value”.

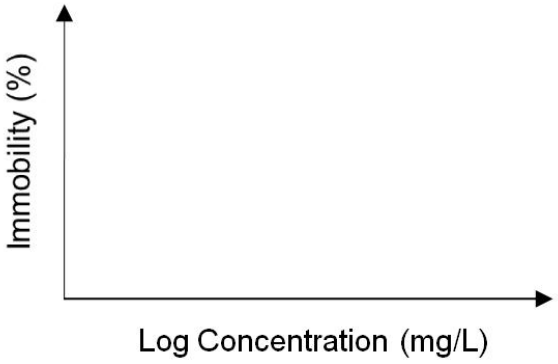
2. Specify whether the concentrations used for calculating the toxicity value (EC_{50}) were nominal or measured values in “Exposure concentrations used for calculation”.

3. Discuss the characteristics of the toxicity value and the validity of the test based on the physicochemical properties of the test substance in “Remarks”. Write the influence on the test results, etc., of any anomaly observed in the test or any deviation from the test method.

5. *Daphnia* concentration-immobilization rate curve

Attach a figure showing the *Daphnia* immobilization rates at individual test concentrations (Figure example 1) during the exposure period.

Figure example 1; *Daphnia* concentration-immobilization rate curve



6. Others

Test agency	Name	
	Address	Tel: Fax:
Test director	Name and status	
	Years of experience	
Test ID number		
Test period	From (month) (day) (year) to (month) (day) (year)	

[Notes]

1. Fill in the present form by transcribing from the final report.
2. Fill in the test ID number reported in the final report.
3. In the margin of this form, provide the name and affiliation of the person in charge of filling in this form.

VI. Fish acute toxicity test

Objective

The purpose of the present test is to determine the toxicity of the test substance against fish by exposing them to the test substance for 96 hr and measuring the mortality.

1. Selection of species

The recommended species is Ricefish (*Oryzias latipes*), but other fish species listed in Appendix Table 1 may also be used. The fish should be in good health and free from any apparent malformation. In each test, the size of the organisms should be as uniform as possible.

Appendix Table 1

Recommended species	Recommended test temperature range (°C)	Recommended total length of test fish (cm)
Danio rerio Zebra-fish	21 – 25	2.0 ± 1.0
Pimephales promelas Fathead Minnow	21 – 25	2.0 ± 1.0
Cyprinus carpio Common carp	20 – 24	4.0 ± 2.0
Oryzias latipes Ricefish	21 – 25	2.3 ± 1.2
Poecilia reticulata Guppy	21 – 25	2.0 ± 1.0
Lepomis macrochirus Bluegill	21 – 25	2.0 ± 1.0
Oncorhynchus mykiss Rainbow trout	13 – 17	5.0 ± 1.0

2. Test vessel and apparatus

The following test vessel and apparatus are used for the test.

2-1. Test Vessel

Test vessels and other apparatus that will come into contact with the test solutions should be made entirely of glass or other chemically inert material. Use a test vessel of an appropriate size

in regard to the recommended volume. Test vessels should be loosely covered to reduce the loss of water due to evaporation and to avoid the entry of dust into the solutions.

Volatile substances should be tested in completely filled closed vessels large enough to prevent oxygen becoming limiting or too low.

2-2. Apparatus

Use a dissolved oxygen meter and an appropriate equipment or apparatus for controlling the temperature for the test.

3. Test water

Natural water (surface or ground water), reconstituted water or dechlorinated tap water are acceptable as holding and dilution water (see the note). Waters with total hardness of between 10 and 250 mg $CaCO_3$ per liter, and with a pH 6.0 to 8.5 are preferable. The reagents used for the preparation of reconstituted water should be of analytical grade and the deionised or distilled water should be of conductivity less than 10 μScm^{-1}.

4. Holding of fish

All fish must be obtained and held in the laboratory for at least 12 days before they are used for testing. After monitoring the fish for 48 hr, they must be held in water of the quality to be used in the test for at least seven days immediately before testing and under the following conditions. Avoid medicated treatment after the monitoring period.

- Light: 12 to 16 hours photoperiod daily;
- Temperature: appropriate to the species (see Appendix Table 1.);
- Oxygen concentration: at least 80 per cent of air saturation value;
- Feeding: three times per week or daily until 24 hours before the test is started
- Following a 48-hour settling-in period, mortalities are recorded and the following criteria applied.
- Mortalities of greater than 10 per cent of population in seven days: rejection of entire batch;
- Mortalities of between 5 and 10 per cent of population: acclimatization continued for seven additional days;
- Mortalities of less than 5 per cent of population: acceptance of batch

5. Test solution

Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. Stock solutions should preferably be prepared by dissolving the test substance in the dilution

water. Follow the descriptions in "Preparation of the test solution" under "III. General rules".

The test should be carried out without adjustment of pH. If there is evidence of marked change in the pH of the tank water after addition of the test substance, it is advisable that the test be repeated, adjusting the pH of the stock solution to that of the tank water before addition of the test substance. This pH adjustment should be made in such a way that the stock solution concentration is not changed to any significant extent and that no chemical reaction or precipitation of the test substance is caused. HCl and NaOH are preferred.

6. Conditions of exposure

6-1. Test method

The test should be performed under a flow-through or semi-static condition. If the test substance concentration is unstable, a flow-through test is recommended.

6-2. Duration

Preferably 96 hours.

6-3. Volume and number of test fish

Loading: maximum loading of 1.0 g fish/litre for static and semi-static tests is recommended; for flow-through systems higher loading can be accepted.

Number of fish: At least 7 fishes must be used at each test concentration and in the controls.

6-4. Test concentrations

At least five concentrations in a geometric series with a factor is preferably not exceeding 2.2. The highest concentration tested should preferably result in 100 per cent immobilization, but concentrations of 100 mg/L or higher do not need to be tested, and the lowest concentration tested should preferably give no observable effect.

Perform a control, and additionally a vehicle control if using any vehicle.

6-5. Culture method

Temperature: appropriate to the species (see Table) and constant within a range of 2 °C.

Light: 12 to 16 hours photoperiod daily.

Oxygen concentration: not less than 60 per cent of the air saturation value. Aeration can be used provided that it does not lead to a significant loss of test substance.

Feeding: none.

Disturbance: disturbances that may change the behaviour of the fish should be avoided.

7. Beginning of the exposure to the test substance

Start the exposure by transferring a specified number of acclimatized fish established in 6-3 to each test vessel.

8. Observation

The fish are inspected at least after 24, 48, 72 and 96 hours. Fish are considered dead if there is no visible movement (e.g. gill movements) and if touching of the caudal peduncle produces no reaction. Dead fish are removed when observed and mortalities are recorded. Observations at three and six hours after the start of the test are desirable. Records are kept of visible abnormalities (e.g. loss of equilibrium, swimming behaviour, respiratory function, pigmentation, etc.).

9. Analysis of the concentration of the test substance

9-1. Analysis of the concentration of the test substance

The concentration of the test substance should be measured, as a minimum, at the highest and lowest test concentration, at the beginning and end of the test. If it has been predicted that the test substance concentration decreases from the initial concentration by 20 per cent or more during the exposure period, it is recommended to take measurements for all test concentration groups at the beginning and end of the exposure. Furthermore, for volatile or adsorptive substances or those that are likely to be greatly decreased during the exposure period, additional measurements are recommended at 24 hr intervals during the exposure period.

In a semi-static test, perform at least two sets of measurements, provided that the measurements taken immediately after the water renewal and immediately before the next renewal are counted as one set.

9-2. Measurement of the test condition

Measure the pH, dissolved oxygen concentration and water temperature at least once daily.

10. Limit test

A limit test may be performed at 100 mg (active ingredient)/l in order to demonstrate that the LC_{50} is greater than this concentration. The limit test should be performed using a minimum of 7 fish, with the same number in the control(s). If any mortalities occur, a full study should be conducted. If sublethal effects are observed, these should be recorded.

11. Validity of the test

For a test to be valid the following conditions should be fulfilled:

- the mortality in the control(s) should not exceed 10 per cent (or one fish if less than ten are used) at the end of the test;
- the dissolved oxygen concentration must have been at least 60 per cent of the air saturation value throughout the test;
- there must be evidence that the concentration of the substance being tested has been satisfactorily maintained throughout the test.

12. Calculation of the results

If evidence is available to demonstrate that the concentration of the test substance has been satisfactorily maintained within ± 20 per cent of the nominal or measured initial concentration throughout the test, then the results can be based on nominal or measured initial values.

Data should be summarized in tabular form, the cumulative mortalities, for the individual test concentrations and the control together with the exposure period and test substance concentrations at each observation. On a logarithmic-normal probability paper, plot the cumulative mortality during the exposure period against the test concentrations.

Then, using the probit analysis or other appropriate statistical method, determine the slope of the curve with 95 per cent confidence limit and the LC_{50} for 96 hr exposure.

Where the standard methods of calculating the LC_{50} are not applicable to the data obtained, the highest concentration causing no mortality and the lowest concentration producing 100 per cent mortality should be used as an approximation for the LC_{50}.

13. Summary of the results

Summarize the test results in Form 9 and attach it to the final report.

Note: A RECONSTITUTED WATER (ISO 6341-1982)

(a) Calcium chloride solution

Dissolve 11.76 g $CaCl_2 \cdot 2H_2O$ in deionised water; make up to 1 litre with deionised water

(b) Magnesium sulphate solution

Dissolve 4.93 g $MgSO_4 \cdot 7H_2O$ in deionised water; make up to 1 litre with deionised water

(c) Sodium bicarbonate solution

Dissolve 2.59 g $NaHCO_3$ in deionised water; make up to 1 litre with deionised water

(d) Potassium chloride solution

Dissolve 0.23 g KCl in deionised water; make up to 1 litre with deionised water

25 ml each of solutions (a) to (d) are mixed and the total volume made up to 1 litre with deionised water. The sum of the calcium and magnesium ions in the solutions is 2.5 mmol/l. The proportion Ca:Mg ions is 4:1 and Na:K ions 10:1.

The conductivity of the deionised water should not exceed 10 μScm^{-1}. All chemicals must be of analytical grade.

Aerate the dilution water until oxygen saturation is achieved, then store it for about two days without further aeration before use.

[Form 9] Report on the Results of the Fish Acute Toxicity Test

1. General information

Name of new chemical substance (based on the IUPAC nomenclature system)			
Other name			
CAS No.			
Structural or rational formula (if neither is available, summarize its formulation method)			
Molecular weight			
Purity of the new chemical substance used for the test (%)			
Lot number of the new chemical substance used for the test			
Names and contents of impurities			
Vapor pressure			
Solubility in water			
1-Octanol/water partition coefficient			
Melting point			
Boiling point			
Properties at room temperature			
Stability			
Solubility in solvents, etc.	Solvent	Solubility	Stability in solvent

[Notes] Provide the physicochemical properties wherever possible.

1. Fill in the “Vapor pressure” column with the vapor pressure of the test substance.

2. Fill in the “Stability” column with the stability of the test substance against temperature, light, etc.

3. Fill in the “Solubility in solvents, etc.” column with the solubility and stability of the test substance in a solvent.

2. Method for analyzing the test substance concentration in the test solution

Items	Methods
Analytical method	
Pretreatment	
Quantification conditions	

[Notes]

1. Specify the analytical method used for the measurement in “Analytical method”.
2. Summarize the treatment performed prior to the analysis in “Pretreatment”. Specify the means used for isolating the algal cells.
3. Write the apparatuses and conditions such as temperature and eluate used for the analysis in “Quantification conditions”.

3. Test materials and methods

Items			Contents
Test organism	Species (Japanese name, scientific name, strain)		
	Source		
	Size (body length, body weight), age in months		
	Susceptibility to the reference substance (LC_{50}) (name of the reference substance)		
Acclimatization	Duration of acclimatization		
	Kind of medium		
	Presence or absence of medicated bath before acclimatization		
	Acclimatization method (static, semi-static, flow-through, etc.)		
	Environmental conditions (water temperature, photoperiod)		
	Feed (kind, amount, frequency, etc.)		
Test conditions	Test vessel		
	Test water	Kind (natural water, dechlorinated tap water, artificially prepared water, etc.)	
		Hardness	
		pH	
	Date of exposure		(Month) (Day) (Year) - (Month) (Day) (Year)
	Test concentrations (nominal values)		(Geometric ratio)
	Number of organisms		organisms/test vessel
	Test solution volume		
	Vehicle	Use or not	
		Kind	
		Concentration(s)	
	Culture method (static, semi-static, flow-through, etc.)		
	Conditions for water renewal or flow-through		
	Water temperature		°C
	Dissolved oxygen concentration (DO)		mg/L
	Photoperiod		
Calculation of results	Statistical method		

[Notes]
1. Write the results (specify the reference substance and write the LC_{50}) of the susceptibility test of the test organism in “Susceptibility to the reference substance”.
2. Write whether medicated bath was used before the acclimatization and if so, specify the kind of medication used in “Presence or absence of medicated bath before acclimatization”.
3. List all test substance concentrations used for the test and the geometric ratio in “Test concentrations (nominal values)”.
4. Write the material and volume of the test vessel in “Test vessel” in “Test conditions”. For a volatile test substance, write whether the vessel was sealed or unsealed.
5. Specify the statistical analysis method (e.g., probit, etc.) used for calculating the toxicity value (LC_{50}) in “Statistical method”.

4. Test results and discussion

Items	Contents
Toxicity value	96hErC_{50} = mg/L
Exposure concentrations used for calculation	1. Nominal values 2. Measured values
Remarks	

[Notes]

1. Write the LC_{50} for 96 hr in “Toxicity value”.

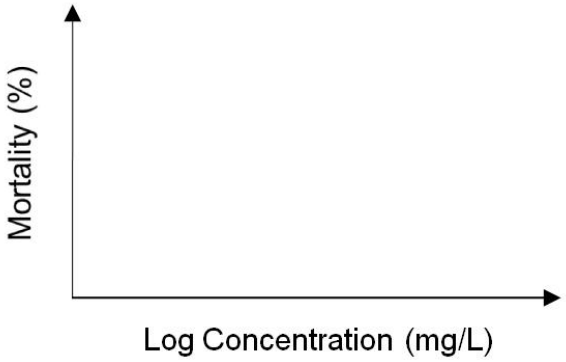
2. Specify whether the concentrations used for calculating the toxicity value (LC_{50}) were nominal or measured values in “Exposure concentrations used for calculation”.

3. Discuss the characteristics of the toxicity values and the validity of the test based on the physicochemical properties of the test substance in “Remarks”. Write the influence on the test results, etc., of any anomaly observed in the test or any deviations from the test method.

5. Fish concentration-mortality curve

Attach a figure showing the fish mortality at individual test concentrations (Figure example 1) during the exposure period.

Figure example 1; Fish concentration-mortality curve



6. Others

Test agency	Name	
	Address	Tel: Fax:
Test director	Name and status	
	Years of experience	
Test ID number		
Test period	From (month) (day) (year) to (month) (day) (year)	

[Notes]

1. Fill in the present form by transcribing from the final report.
2. Fill in the test ID number reported in the final report.
3. In the margin of this form, provide the name and affiliation of the person in charge of filling in this form.