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 chemical substances is described here.

II. Definitions

Objective

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

1. **Test Methods**

- **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 batch 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 batch while draining the old solutions.

2. **End-points**

- **LC_{50}**: The test substance concentration in the test solution estimated to kill 50% of the test organism within a certain period (must be mentioned).
- **EC_x**: The test substance concentration in the test solution estimated to inhibit the growth, mobility, reproduction, etc., of the test organism by x% within a certain period (must be mentioned).
- **LOEC**: The lowest tested concentration of the test substance found to have a statistically significant ($p < 0.05$) influence on the reproduction, etc., of the test organism compared to the control group during the exposure period.
  All tested concentrations higher than LOEC should result in adverse effects comparable to or more severe than those observed at LOEC. If these conditions are not satisfied, provide a sufficient explanation on how the LOEC and NOEC have been determined.
- **NOEC**: A tested concentration one step lower than LOEC, i.e., the highest tested concentration that has been found to have no statistically significant ($p < 0.05$) influence compared to the control group during the exposure period.

III. General rules

1. Basic idea of the test

Toxicity tests using algae, *Daphnia* spp. or fish are intended for determining the toxicity of test substances by exposing the test organisms to the test substances dissolved in medium or material 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 80% of the initial concentration (i.e., the set concentration or measured concentration at the beginning of the exposure) or higher. For selecting the test conditions for the test substance, gather as much information as possible on the structural formula, purity, stability against water or light, dissociation constant (pKa), octanol/water partition coefficient (Pow), vapor pressure, and biodegradability. 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 auxiliary, 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 auxiliary (which hereinafter refers to a solvent or dispersant) such as an organic solvent. However, as a rule, do not use any surface-active dispersant. 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 an auxiliary, additionally establish an auxiliary control containing the auxiliary at the same concentration as that used for preparing the test concentrations. In principle, the concentration of the auxiliary 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 combined action with the auxiliary.

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. The present study defines growth as an increase in the cell density (the number of cells per 1 mL of the medium) during the exposure period.

1. **Test organism**

   The recommended species is *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*), but other species such 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 vessel and equipment**

   The following test vessel and equipment are used for the study.

   2-1. Test vessel

   The test vessel or other instrument that contacts the test solution must be made of glass or other chemically inert materials. 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. Culture apparatus

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

2-3. Cell counter

Count the cells using a particle counter, a hemocytometer under a microscope, a fluorophotometer, a spectrophotometer or a colorimeter. When measuring a low cell density using a spectrophotometer, use a cell with an optical length of at least 4 cm.

3. Medium

The recommended composition of the medium is as follows. Media with similar compositions can also be used.

- Ammonium chloride 15 mg/L
- Magnesium chloride hexahydrate 12 mg/L
- Calcium chloride dehydrate 18 mg/L
- Magnesium sulfate heptahydrate 15 mg/L
- Dipotassium hydrogenphosphate 1.6 mg/L
- Ferric chloride (III) hexahydrate 0.08 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

After mixing these ingredients, adjust the pH to 8.3 using HCl or NaOH and sterilize the mixture.

4. Preculture

To acclimatize the algae to the test condition and to prepare a preculture of exponentially growing algae for the test, preculture the algae under the test condition for at least 2 - 3

days. Inoculate the preculture with the algae at an adjusted biomass so that the exponential growth is reached at the beginning of the exposure period.

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

Perform the exposure period for 72 hr or longer.

6-2. Initial cell density

Adjust the initial cell density to a sufficiently low level so that the exponential growth of the algae can be maintained throughout the exposure period. Do not exceed 0.5 mg/L in dry weight. For *Pseudokirchneriella subcapitata*, the recommended initial cell density is from 5×10^3 to 1×10^4 cells/mL. For other species, adjust the initial cell density to achieve an equivalent biomass.

6-3. Test concentration

Adopt a concentration range comprising at least 5 concentrations that are setup in a geometric progression. Preferably, the concentration range induces 0 – 90 % growth inhibition. Concentrations of 100 mg/L or higher do not need to be tested. Perform a control, and additionally an auxiliary control if using any auxiliary.

6-4. Number of replicates (repeats)

Perform each test concentration in 3 replicates. It is recommended to perform the control in 6 replicates.

6-5. Culture method

- Temperature: Within the range of 21°C - 24°C, with a variation within the incubator or incubation chamber of ± 2°C.
- Illumination: 60 - 120μE/m^2/s (Continuous and uniform exposure with fluorescent lamps of white or daylight color.)
- Culture method: Shake culture (Except for volatile test substances, cover the culture vessels with breathable caps. The algae must be maintained in a suspended state during the exposure period.)

7. **Beginning of the exposure to the test substance**

   Start the exposure by inoculating each test vessel with the algae that have been precultured to the initial cell density established according to 6-2.

8. **Cell density measurement**

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

9. **Measurements of test substance concentration**

   9-1. Test substance concentration measurement

   At the beginning and end of the exposure, measure the test substance concentrations at least in the lowest and highest test concentration groups and the test concentration groups around the predicted EC_{50}. If it is predicted that the test substance concentration decreases from the initial concentration by 20% 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.

   9-2. Test condition measurement

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

10. **Limiting test**

    If the test substance concentration of 100 mg/L or at the water solubility limit, whichever is lower, is predicted to show no toxicity, a limiting test can be performed at this concentration to demonstrate that NOEC, etc., is higher than this concentration. The test conditions and validity standards mentioned earlier are also applied to the limiting test, except that the number of replicates is increased to 2. Perform a t-test or other statistical analyses to compare the average growth rates between the control and each test concentration.

11. **Validity of the test**

    The test is considered valid if it satisfies the following conditions.

    - For *Pseudokirchneriella subcapitata*, the cell count in the control group shows an at least 16-fold increase during the exposure period.
    - The coefficient of daily variation for the growth rate in the control group does not exceed 35% throughout the exposure period.

- The coefficient of variation among replicates for the growth rate in the control group does not exceed 15%.

12. Calculation of the results

12-1. Processing of the results

In principle, perform the calculation of the results based on appropriate averages of the test substance concentration measurements. If it can be demonstrated that the test substance concentration was kept within ± 20% of the initial concentration throughout the exposure period, the calculation of the results can be performed based on the initial concentration.

Tabulate the cell densities of the individual test concentrations and the control together with the exposure period and test substance concentrations. Draw growth curves for the individual test concentrations and the control by plotting the average cell densities against time. Make sure that the growth curve for the control shows an exponential growth throughout the exposure period.

It is recommended to calculate the dose-effect relation of the test substance using both methods described in 12-2. and 12-3..

12-2. Comparison of growth rates

The growth rate during the exponential growth phase is calculated by the following equation:

$$\mu_{i-j} = \frac{\ln N_j - \ln N_i}{t_j - t_i}$$

provided that,

μ_{i-j} = the growth rate between time points t_i and t_j. Usually shown as per day (d^1).

N_i = the measured cell density (cells/mL) at t_i. A set value is used for the cell density at the beginning of the test (t_0).

N_j = the measured cell density (cells/mL) at t_j

t_i = the time (d) of i^{th} cell density measurement after beginning the exposure

t_j = the time (d) of j^{th} cell density measurement after beginning the exposure

For calculating EC_{50}, determine the growth rate over the entire exposure period, i.e., from the beginning of the exposure to 72 hr. For checking the validity of the test, determine the daily growth rate for the control to confirm that the coefficient of daily

variation for the growth rate does not exceed 35% throughout the exposure period. The growth rate can also be obtained from the slope of the regression line drawn by plotting the logarithmic values of the cell density measurements.
The growth (rate) inhibition rate (I_μ) for each test concentration is calculated as the difference in the average growth rate between the control (μ_c) and each test concentration (μ_T) using the following equation:

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

12-3. Comparison of the area under the growth curve

The area under the growth curve is calculated by the following equation:

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 + 2N_0}{2} \times (t_2 - t_1) + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1})$$

provided that,
A = the area
N_0 = the cell density (cells/mL) set at the beginning of the exposure (t_0)
N_1 = the measured cell density (cells/mL) at t_1
N_n = the measured cell density (cells/mL) at t_n
t_1 = the time of first cell density measurement after beginning the exposure
t_n = the time of n^{th} cell density measurement after beginning the exposure

The growth inhibition rate (I_A) for each test concentration is calculated as the difference in the area under the growth curve between the control (A_c) and each test concentration (A_t) using the following equation:

$$I_A = \frac{A_c - A_t}{A_c} \times 100$$

12-4. Calculation of toxicity values

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

EbC_{50}, respectively.

Note that the values EbC_{50} and ErC_{50} cannot be compared with each other, as a small change in the growth rate represents a large change in the biomass.

The values $\mu_{0\text{-}3d}$ or A for the control and each test concentration are further subjected to ANOVA and multiple comparison to determine the NOEC values.

13. **Summary of the results**

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

V. *Daphnia* Acute Immobilization Test

Objective

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

1. **Test organism**

   The recommended species is *Daphnia magna*, but other *Daphnia* species such as *Daphnia pulexs* may also be used.

   Use organisms that are 24 hr old or younger at the beginning of the exposure. To reduce variation, do not use the first offsprings of the parents. The test organisms must be obtained from healthy parents (i.e., showing no sign of stress under the culture conditions such as a high mortality, the appearance of males or oostegites, a prolonged period before the first offsprings and discoloration) of the same strain.

   The parent organisms must be cultured under conditions (light, temperature and water) as same as those employed in the test. When performing the test using water different from that usually used for culturing *Daphnia* spp., establish an acclimatization period before beginning the exposure. The acclimatization can be performed by culturing the organisms in the material water at the test temperature for at least 48 hr before beginning the exposure. Use the offsprings obtained from acclimatized parents for the test.

2. **Test vessel and equipment**

   The following test vessel and equipment are used for the study.

2-1. Test vessel

The test vessel or other instrument that contacts the test solution must be made of glass or other chemically inert materials. Cap the vessel loosely to prevent evaporation and dust contamination.

If the test substance is volatile, perform the test in a sealed system. Use a sufficiently large vessel to prevent shortage of dissolved oxygen.

2-2. Instrument

Use a dissolved oxygen meter (a microelectrode or other instrument suitable for measuring the dissolved oxygen concentration with a small amount of sample), a pH meter, an appropriate instrument for controlling temperature, etc., for the test.

3. **Material water**

Use water suitable for culturing and testing *Daphnia* spp. It can be natural water (surface water or groundwater), dechlorinated tap water or artificially prepared water (e.g., Appendix Table 1), but must satisfy the conditions listed in Appendix Table 2.

Do not use Elendt M4 or M7 media or water containing chelating agents for testing metal-containing substances. The water hardness should be 250 mg/L or smaller in terms of calcium carbonate concentration, and the pH should be 6-9.

Aerate the material water before using it for the test.

4. **Test solution**

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

Perform the test without adjusting the pH. If the pH of the material water is not within the range of 6-9, it is recommended to perform an additional test after adjusting the pH to that observed prior to the addition of the test substance. Perform the pH adjustment through a method causing no change in the concentration, chemical reaction or precipitation of the test substance. Preferably, use HCl or NaOH for the pH adjustment.

5. **Test conditions**

5-1. Test method

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. Exposure period

Perform the exposure period for 48 hr.

5-3. Volume and number of test organisms

- Volume: Use at least 2 mL or the test solution per organism.
- Number of test organisms: Use at least 20 organisms for each of the test concentrations and the control. Preferably, divide the organisms into 4 groups of 5 organisms.

5-4. Test concentrations

Adopt a concentration range comprising at least 5 concentrations that are setup in a geometric progression, preferably at a geometric ratio of within 2.2. The highest test concentration preferably induces 100% immobilization, but concentrations of 100 mg/L or higher do not need to be tested. Preferably, no effect is observed at the lowest concentration.

Perform a control, and additionally an auxiliary control if using any auxiliary.

5-5. Culture method

- Illumination: The photoperiod is preferably set to 16 hr light and 8 hr dark. The test can be conducted in dark if the test substance is unstable against light.
- Temperature: The temperature is set within the range of 18°C - 22°C, with variations among the test vessels of ± 1.0°C.
- Dissolved oxygen concentration: It must be kept at 3 mg/L or higher. In principle, do not perform aeration during the exposure period.
- Feeding: Do not feed the organisms.

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**

Observe the mobility of the organisms at least twice, i.e., at 24 and 48 hr after the beginning of the exposure. The organisms are considered as being immobilized when they do not move for 15 sec after the test vessel is gently shaken. During observation, record any anomaly in behavior or appearance besides immobilization.

8. **Measurements of test substance concentration, etc.**

8-1. Test substance concentration measurement

At the beginning and end of the exposure, measure the test substance concentrations at least in the lowest and highest test concentration groups. If it has been predicted that the test substance concentration decreases from the initial concentration by 20% 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. Test condition measurement

Measure the dissolved oxygen concentration and the pH in the control and at the highest test concentration at the beginning and end of the exposure period. The water temperature in the control should be measured at least twice, i.e., at the beginning and end of the exposure period, but to monitor the variation in the water temperature, it is recommended to continuously measure the atmospheric temperatures in the control or in the surrounding atmosphere, etc., and record their variations. Generally, the variation of the pH must not exceed 1.5 during the exposure period.

9. **Limiting test**

If the test substance concentration of 100 mg/L or at the water solubility limit, whichever is lower, is predicted to show no toxicity, a limiting test can be performed at this concentration to demonstrate that EC_{50} is higher than this concentration. Use 20 organisms (preferably divided into 4 groups of 5 organisms) for the limiting test and also for the control. If the immobilization rate has exceeded 10% at the end of the exposure, perform a regular test. Record any abnormal behavior observed.

10. **Validity of the test**

The test is considered valid if it satisfies the following conditions.

- The control group shows an immobilization rate of 10% or less and no organism floating on the surface.
- The dissolved oxygen concentration is 3 mg/L or higher at the end of the exposure.

11. **Calculation of the results**

In principle, perform the calculation of the results based on appropriate averages of the test substance concentration measurements. If it can be demonstrated that the test substance concentration was kept within ± 20% of the initial concentration throughout the exposure period, the calculation of the results can be performed based on the initial concentration.

Tabulate the immobilization rate for the individual test concentrations and the control together with the exposure period and test substance concentrations. Plot the immobilization rate at 24 hr and 48 hr against the test concentrations. Then, using the probit method or other appropriate statistical method, determine the slope of the regression line at 95% confidence limit and the EC_{50} for 48 hr exposure.
If the obtained data are insufficient for performing statistical calculations, estimate EC_{50} as the geometric average of the highest test concentration inducing no immobilization and the lowest test concentration inducing 100% immobilization.

12. **Summary of the results**

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

Appendix Table 1. Artificially prepared water

(1) ISO test water

(a) Calcium chloride solution
Dissolve 11.76 g calcium chloride dihydrate in dilution water and add dilution water up to 1 L.

(b) Magnesium sulfate solution
Dissolve 4.93 g magnesium sulfate heptahydrate in dilution water and add dilution water up to 1 L.

(c) Sodium hydrogencarbonate solution
Dissolve 2.59 g sodium hydrogencarbonate in dilution water and add dilution water up to 1 L.

(d) Potassium chloride solution
Dissolve 0.23 g potassium chloride in dilution water and add dilution water up to 1 L.

Mix 25 mL each of solutions (a)-(d) and make up to 1 L with dilution water.
Use appropriate purified water (e.g., ion-exchanged water, distilled water or reverse osmosis water) with an electric conductivity of 10 μS/cm or lower as the dilution water. All reagents must be of analytical grade.

(2) Elendt M4 and M7 media

Prepare each medium by adding the medium stock solutions I (microelements) and medium stock solutions II (principal components) to dilution water (use appropriate purified water, e.g., deionized water, distilled water or reverse osmosis water).

(a) Preparation of the medium stock solutions I

Prepare the medium stock solution I of each substance shown in the left column in Table 1 by dissolving the amount shown in the second column in Table 1 in 1 L of dilution water. Prepare the ferrous ethylene diamine tetraacetate solution (II) by mixing the sodium ethylene diamine tetraacetate dihydrate and ferrous sulfate heptahydrate, which have been separately prepared, and immediately autoclaving them.

After preparing the medium stock solution I of each substance, take the amount shown in the third column in Table 1 from each solution, mix them and add dilution water up to 1 L to achieve "medium stock solution I mixture."

Table 1. Components of the medium stock solutions I and their amounts, etc.

Medium stock solution I (single substance)	Amount added to water (mg/L)	Amount added for preparing medium stock solution I mixture			
		Elendt M4		Elendt M7	
	mg/L	Amount added (mL/L)	Final dilution ratio*	Amount added (mL/L)	Final dilution ratio*
Boric acid	57,190	1.0	20,000-fold	0.25	80,000-fold
Manganese chloride tetrahydrate	7,210	1.0	20,000-fold	0.25	80,000-fold
Lithium chloride	6,120	1.0	20,000-fold	0.25	80,000-fold
Rubidium chloride	1,420	1.0	20,000-fold	0.25	80,000-fold
Strontium chloride hexahydrate	3,040	1.0	20,000-fold	0.25	80,000-fold
Sodium bromide	320	1.0	20,000-fold	0.25	80,000-fold
Disodium bolybdate dihydrate	1,260	1.0	20,000-fold	0.25	80,000-fold
Copper chloride dihydrate	335	1.0	20,000-fold	0.25	80,000-fold

Zinc chloride	260	1.0	20,000-fold	1.0	20,000-fold
Cobalt chloride hexahydrate	200	1.0	20,000-fold	1.0	20,000-fold
Potassium iodide	65	1.0	20,000-fold	1.0	20,000-fold
Sodium selenite	43.8	1.0	20,000-fold	1.0	20,000-fold
Ammonium metavanadate	11.5	1.0	20,000-fold	1.0	20,000-fold
Ethylene diamine tetraacetic acid (II) solution		20.0	1,000-fold	5.0	4,000-fold
Disodium ethylene diamine tetraacetate dihydrate	5,000	-		-	
Ferrous sulfate heptahydrate	1,991	-		-	

*Final dilution ratio: The final dilution ratio of the medium stock solution I in Elendt M4 or M7 medium.

(b) Preparation of the medium stock solutions II

Except for the medium stock solution I mixture, prepare the medium stock solution II of each substance shown in the left column in Table 2 by dissolving the amount shown in the second column in Table 2 in 1 L of dilution water. After preparing the vitamin mixture stock solution, freeze them in small portions and add them to the medium immediately before use.

(c) Preparation of each medium

Prepare each medium by taking the amount shown in the third column in Table 2 from each medium stock solution II, mixing them and adding dilution water up to 1 L. When preparing each medium, add these amounts of medium stock solutions to about 500 - 800 mL dilution water and further add dilution water up to 1 L to avoid precipitation of salts.

Table 2. Components of the medium stock solutions II and their amounts (For both Elendt M4 and M7)

Medium stock solution II (principal component stock solution)	Amount added to water (mg/L)	Amount added for preparing medium (artificially prepared water)	
		Elendt M4 and M7	
		Amount added[*1]	Final dilution

		(mL/L)	ratio[*2]
Medium stock solution I mixture[*] *[*]Note that the component ratio is different between Elendt M4 and M7*	-	50	20-fold
Calcium chloride dihydrate	293,800	1.0	1,000-fold
Magnesium sulfate heptahydrate	246,600	0.5	2,000-fold
Potassium chloride	58,000	0.1	10,000-fold
Sodium hydrogencarbonate	64,800	1.0	1,000-fold
Disodium silicate nonahydrate	50,000	0.2	5,000-fold
Sodium nitrate	2,740	0.1	10,000-fold
Potassium primary phosphate	1,430	0.1	10,000-fold
Potassium secondary phosphate	1,840	0.1	10,000-fold
Vitamin mixture stock solution	-	0.1	10,000-fold
Thiamin hydrochloride	750		10,000-fold
Cyanocobalamin (B12)	10		10,000-fold
Biotin	7.5		10,000-fold

[*1]Amount added: The amount added for preparing Elendt M4 and M7 media (mL/L).

[*2]Final dilution ratio: The final dilution ratio of the medium stock solution II in Elendt M4 or M7 medium.

Appendix Table 2. Chemical conditions of the material water

Substances	Concentration requirements
Particulate matter	below 20 mg/L
Total organic carbon	below 2 mg/L
Un-ionized ammonia	below 1 μg/L
Chlorine	below 10 μg/L
Total oranophosphorus pesticide	below 50 ng/L
Total organochlorine pesticide and PCB	below 50 ng/L

Total organic chlorine	below 25 ng/L

VI. Fish acute toxicity test

Objective

The purpose of the present study 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. **Test organism**

   The recommended species is Ricefish (*Oryzias latipes*), but other fish species listed in Appendix Table 1 may also be used. Use healthy fish showing no abnormal appearance. In each test, the size of the organisms should be as uniform as possible.

Appendix Table 1.

Fish species	Recommended test temperature (°C)	Recommended 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 equipment

The following test vessel and equipment are used for the study.

2-1. Test vessel

The test vessel or other instrument that contacts the test solution must be made of glass or other chemically inert materials. Use a test vessel of an appropriate size in regard to the recommended volume. Cap the vessel loosely to prevent evaporation and dust contamination.

If the test substance is volatile, perform the test in a sealed system. Use a sufficiently large vessel to prevent shortage of dissolved oxygen.

2-2. Instrument

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

3. Material water

Use water suitable for culturing and testing the fish. It can be natural water (surface water or groundwater), dechlorinated tap water or artificially prepared water (see the note). The recommended total hardness is 10 - 250 mg/L in terms of calcium carbonate concentration, and the recommended pH is 6.0 - 8.5. Prepare the artificially prepared water using reagents of analytical grade and deionized or distilled water having an electric conductivity of 10 μS/cm or lower.

4. Acclimatization

All test fish must be acquired at least 12 days before the test and acclimatized. After monitoring the fish for 48 hr, acclimatize them in water used for the test under the following conditions for at least 7 days before beginning the exposure. If possible, avoid using medicated bath after the monitoring period.

- Illumination: 12 - 16 hr light per day
- Temperature: Appropriate temperature for the species tested (see Appendix Table 1 under VI.)
- Oxygen concentration: At least 80% of the saturated oxygen concentration
- Feeding: Daily or three times per week until 24 hr before beginning the exposure.

Record the mortality during the acclimatization period and apply the following conditions to the test fish.

- If the overall mortality for seven consecutive days during the acclimatization period exceeds 10%, do not use them for the test.
- If the overall mortality for seven consecutive days during the acclimatization period falls within 5 - 10%, extend the acclimatization period for 7 days.
- If the overall mortality for seven consecutive days during the acclimatization period is below 5%, use them for the test.

5. **Test solution**

To prepare a test solution of each concentration, directly dissolve the required amount of the test substance in the material water, or prepare a stock solution of the test substance at an appropriate concentration and dilute it with the material water. Follow the descriptions in "Preparation of the test solution" under "III. General rules."
Perform the test without adjusting the pH. If the pH of the material water is greatly changed by the addition of the test substance, it is recommended to perform an additional test after adjusting the pH to that observed prior to the addition of the test substance. Perform the pH adjustment through a method causing no change in the concentration, chemical reaction or precipitation of the test substance. Preferably, use HCl or NaOH for the pH adjustment.

6. **Test conditions**

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. Exposure period

Perform the exposure period for 96 hr.

6-3. Volume and number of test fish

- Volume: A maximum density of 1.0 g fish weight/L is recommended for a semi-static test. The volume can be larger in a flow-through test.
- Number of test fish: Use at least 7 fish for each of the test concentrations and the control.

6-4. Test concentrations

Adopt a concentration range comprising at least 5 concentrations that are setup in a geometric progression, preferably at a geometric ratio of within 2.2. The highest test concentration preferably gives fatal effects on all individuals tested, but concentrations of 100 mg/L or higher do not need to be tested. Preferably, no effect is observed at the

lowest concentration.

Perform a control, and additionally an auxiliary control if using any auxiliary.

6-5. Culture method

- Temperature: The temperature is set within the appropriate range for the species tested (see Appendix Table 1 under VI.), and kept constant with a variation of ± 2°C.
- Illumination: The photoperiod is set to 12 - 16 hr light per day.
- Dissolved oxygen concentration: It must be kept at 60% of the saturated oxygen concentration or higher. Aeration may be performed unless it causes a great loss of the test substance.
- Feeding: Do not feed the organisms.
- Disturbing factor: Avoid any factor that should disturb the fish behavior.

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**

   Observe the appearance and behavior of the fish at least at 24, 48, 72 and 96 hr after the beginning of the exposure. Organisms showing no motion (e.g., opercular movements) and no response when touched at their peduncles are considered as being dead. Remove dead organisms and record the mortality. Additional observations at 3 and 6 hr after the beginning of the exposure are also recommended. Record any anomaly in balance, swimming behavior, respiratory function or body color.

9. **Measurements of test substance concentration, etc.**

   9-1. Test substance concentration measurement

   At the beginning and end of the exposure, measure the test substance concentrations at least in the lowest and highest test concentration groups. If it has been predicted that the test substance concentration decreases from the initial concentration by 20% 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. Test condition measurement

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

10. **Limiting test**

If the test substance concentration of 100 mg/L or at the water solubility limit, whichever is lower, is predicted to show no fatality, etc., a limiting test can be performed at this concentration to demonstrate that LC_{50} is higher than this concentration. Use at least 7 organisms for the limiting test and also for the control. If any dead fish is observed by the end of the exposure, perform a regular test. Record any sublethal effects observed.

11. **Validity of the test**

The test is considered valid if it satisfies the following conditions.

- The mortality in the control group does not exceed 10% (no more than one fish dead when using less than 10 individuals).
- The dissolved oxygen concentration is kept at 60% of the saturated oxygen concentration or higher throughout the exposure period.
- A sufficient level of the test substance concentration is kept throughout the exposure period.

12. **Calculation of the results**

In principle, perform the calculation of the results based on appropriate averages of the test substance concentration measurements. If it can be demonstrated that the test substance concentration was kept within ± 20% of the initial concentration throughout the exposure period, the calculation of the results can be performed based on the initial concentration.

Tabulate the cumulative mortalities for the individual test concentrations and the control together with the exposure period and test substance concentrations. On a logarithmic-normal probability paper, plot the cumulative mortality during the exposure period against the test concentrations. Then, using the probit method or other appropriate statistical method, determine the slope of the regression line at 95% confidence limit and the LC_{50} for 96 hr exposure.

If the obtained data are insufficient for performing statistical calculations, estimate LC_{50} as the geometric average of the highest test concentration inducing no mortality and the lowest test concentration inducing 100% mortality.

13. **Summary of the results**

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

Note: Artificially prepared water
Composition designated in OECD (ISO6341-1982)

(a) Calcium chloride solution

Dissolve 11.76 g calcium chloride dihydrate in deionized water and add deionized water up to 1 L.

(b) Magnesium sulfate solution

Dissolve 4.93 g magnesium sulfate heptahydrate in deionized water and add deionized water up to 1 L.

(c) Sodium hydrogencarbonate solution

Dissolve 2.59 g sodium hydrogencarbonate in deionized water and add deionized water up to 1 L.

(d) Potassium chloride solution

Dissolve 0.23 g potassium chloride in deionized water and add deionized water up to 1 L.
Mix 25 mL each of solutions (a)-(d) and add deionized water up to 1 L. The total amount of calcium and magnesium ions in this solution is 2.5 mmol/L. The ionic ratio of calcium to magnesium is 4:1, and the ionic ratio of sodium to potassium is 10:1.
The electric conductivity of the deionized water must not exceed 10 μS/cm. All reagents must be of analytical grade.
Aerate the artificially prepared water until the dissolved oxygen concentration reaches saturation. Store the water without aeration for about 2 days until use.