Concentration Test of Chemical Substances in Fish

I. Scope of application

A standard method for the concentration test of chemical substances in aquatic life, especially in fish, is described.

II. Terms

The terms used in this test method are as defined in the Japanese Industrial Standards (hereinafter referred to as "JIS").

III. Test Method

1. **Setting of the test concentration of the test substance**

   Conduct an acute toxicity test in fish using the following method to determine the test concentration of the test substance to be employed in the concentration test.

   1-1. Test fish

   The fish species recommended for the acute toxicity test are rice-fish and common carp, but species used in the concentration test or other species satisfying the following basic conditions may also be used. The test fish must be adaptable to laboratory culture conditions concerning water temperature, feed, handling, as well as uniform in size, in good health, available in large numbers at once. Do not use diseased fish or those showing abnormal appearance or behavior.

   1-2. Practice of acute toxicity test (LC_{50} determination)

   The acute toxicity test is conducted according to the fish acute toxicity test prescribed in this notification, the method prescribed in JIS K0102-1998-71 or the method prescribed in OECD test guideline 203.
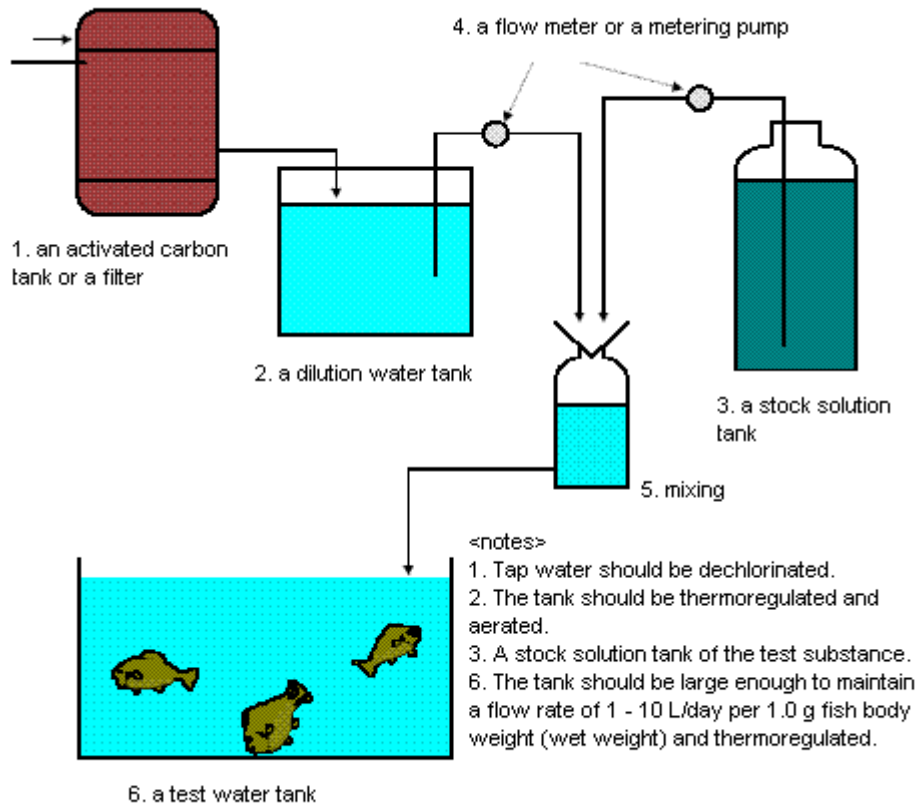
2. **Practice of concentration test**

   Conduct the test under a flow-through condition to study the degree of concentration of the test substance in fish.

   2-1. Apparatus and instrument

   2-1-1. Apparatus

   The apparatuses used in the test are summarized in the following diagram.

4. a flow meter or a metering pump

1. an activated carbon tank or a filter

2. a dilution water tank

3. a stock solution tank

5. mixing

<notes>
1. Tap water should be dechlorinated.
2. The tank should be thermoregulated and aerated.
3. A stock solution tank of the test substance.
6. The tank should be large enough to maintain a flow rate of 1 - 10 L/day per 1.0 g fish body weight (wet weight) and thermoregulated.

6. a test water tank

2-1-2. Test water tank

Use a clean, glass tank of a volume suitable for culturing the test fish.

2-1-3. Other instrument

Wherever possible, use clean instruments made of glass, Teflon® or stainless steel for passing water or diluting the test substance. Avoid the use of flexible plastic piping as much as possible; its use may be accepted only at parts such as connections, if absolutely necessary.

2-2. Test fish

The fish species recommended for the concentration test are common carp and rice-fish, but other species listed in Table 1 may also be used. Set appropriate conditions for the test and carry out the procedures following these conditions. In such a case, report the grounds for the selection of the fish species and the test method.

In each test, choose organisms of body weights as uniform as possible so that the minimum body weight does not become smaller than 2/3 of the maximum body weight. If possible, choose organisms of the same age and from the same source.

Table 1. Fish species recommended for the test

Recommended fish species	Recommended test temperature range (°C)	Recommended body length of the test organism (cm)
Zebra-fish *Danio rerio* (Cyprinidae)	20 - 25	3.0 ± 0.5
Fathead minnow *Pimephales promelas* (Cyprinidae)	20 - 25	5.0 ± 2.0
Common carp *Cyprinus carpio* (Cyprinidae)	20 - 25	8.0 ± 4.0
Rice-fish *Oryzias latipes* (Adrianichthyidae)	20 - 25	3.0 ± 2.0
Guppy *Poecilia reticulate* (Poecilidae)	20 - 25	3.0 ± 1.0
Bluegill *Lepomis macrochirus* (Centrarchidae)	20 - 25	5.0 ± 2.0
Rainbow trout *Oncorhynchus mykiss* (Salmonidae)	13 - 17	8.0 ± 4.0

2-3. Cultivation and acclimatization

Culture the test fish in an appropriate pool. Remove any diseased, weak or abnormal fish. Then, if required, exterminate external and internal parasitic pathogens by means of medicated bath or medication. After the fish have recovered their conditions and have been sterilized, transfer them to the acclimatization tank. Monitor the cultured fish school for 48 hr, and acclimatize them for at least 2 weeks. During acclimatization, feed them sufficiently with the same type of feed used during the test period (described in 2-5-5).

Following the 48 hr monitoring period, record the mortality during the acclimatization period and apply the following standards to the cultured fish school.

- 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.

  If the overall mortality for the subsequent seven days exceeds 5%, do not use them for the test.

  Do not use diseased fish for the test. It is better not to perform treatments such as medicated bath on the fish from 2 weeks before the test and throughout the test.

2-4. Feeding

During the acclimatization and test periods, feed the fish with appropriate feed with known lipid and total protein contents. Feed a sufficient amount of the feed to maintain their good health and body weight. Feed them daily with an amount of about 1 – 2 % of the body weight during the acclimatization and test periods. During the test period, remove uneaten feed and excrement about once daily to keep the tank clean and to maintain a low organic matter concentration in the tank.

2-5. Practice of test

2-5-1. Material water

As the material water, use natural water obtained from a clean water source of a homogeneous quality or dechlorinated tap water. The water quality must be suitable for the survival of the selected fish species, inducing no abnormal appearance or behavior during the acclimatization and test periods.

2-5-2. Test substance solution

Prepare a stock solution of the test substance at an appropriate concentration. The stock solution is preferably prepared by simply mixing or stirring the test substance in the material water. The use of any solvent or dispersant (auxiliary) is not recommended but is acceptable if required for preparing a stock solution at an appropriate concentration.

Examples of acceptable solvents are ethanol, methanol, ethylene glycol monomethyl ether, ethylene glycol dimethyl ether, dimethylformamide, triethylene glycol, dimethylsulfoxide.

Examples of acceptable dispersants are Cremophor®RH40, Tween®80, NIKKOL®HCO-40. Be careful for bacterial propagation when using any biodegradable reagent.

A flow-through test requires a system for continuously supplying the stock solution of the test substance to the test water tank and diluting it. Preferably, each test water tank is supplied at least five times its volume of the material water per day. A flow-through test is recommended, but if this is difficult (due to harmful effects on the test organisms), the test may be conducted under a semi-static condition by regularly replacing the test liquid with a new batch. The test substance may be labeled with a radioisotope.

2-5-3. Test concentrations

Under a flow-through condition, expose the test fish to at least two different concentrations of the test substance. Usually, the higher (or the highest) concentration is set to a value that does not exceed about 1/100 of the LC_{50} (the concentration that kills 50% of the test fish during a prescribed exposure period) of the test substance. Select the lowest two concentrations (one is 10 times higher than the other) among the concentrations that are analyzable by the analysis method[*1]. If the detection of the concentration corresponding to 1/100 of the LC_{50} value is considered difficult in terms of the detection limit of the analysis, the ratio of the two concentrations can be smaller than 10, or the test substance can be labeled with a radioisotope. Avoid employing a concentration above the aqueous solubility of the test substance.

The concentration of any auxiliary must not exceed 0.1 mL/L. The contribution of the auxiliary (and the test substance) to the total organic carbon content in the test water should be recognized. Nevertheless, the use of such substance should be avoided wherever possible.

If the auxiliary has been confirmed to have no effect on the fish species tested, perform a control by maintaining the fish in either the material water alone or the material water containing the auxiliary. If the effect of the auxiliary on the test fish has not been confirmed, perform both controls.

[*1]: *Among the concentrations corresponding to 1/100, 1/1000 and 1/10000 of the LC_{50} value obtained in 1-2, the lowest two analyzable concentrations are employed.*

2-5-4. Test temperature and illumination

The temperature must be suitable for the fish species tested (see Table 1), with a variation of less than ± 2°C. The recommended photoperiod is 12 - 16 hr light per day.

2-5-5. Test period

Establish the following periods by calculating the concentration rate based on either or both the ratio (BCF_{SS}) of the test substance concentration in fish to its concentration in water and/or the ratio (BCF_K) of the elimination rate constant to the uptake rate constant.

(1) Uptake period

The uptake period should be established for 28 days or until a steady state is reached. If the steady state is not achieved within 28 days, continue monitoring of the concentration rate and extend the uptake period until the steady state is reached, with the maximum being 60 days.

It is regarded that the steady state has been reached when the variation of the concentration rates obtained in three consecutive measurements with intervals of at least 48 hr falls within 20%. (If the concentration rate is below 100, it is considered that the steady state has been reached by day 28, independent of the variation of the concentration rate.)

(2) Elimination period

If the concentration rate is calculated based on BCF_K, start an elimination period after the uptake period by transferring the test fish to a clean tank free of the test substance.

The elimination period is preferably continued until the test substance concentration in the fish falls to below 5% of that at the steady state. If this takes too long, the elimination period can be shortened to a period required for determining the half-life.

If the concentration rate is calculated based on only BCF_{SS} and if the BCF_{SS} is 1000 or larger, it is recommended to set an elimination period.

2-5-6. Operation

Conduct the test using the apparatus and instrument described in 2-1 and fish meeting the requirements described in 2-3 under the conditions described in 2-5-1. to 2-5-5..

Add the test substance to achieve the test concentrations and sufficiently

ventilate the test solution in the tank. Then, sample the test water from the test water tank for quantifying the test substance before transferring the test fish to the test water tank (for example, 24 hr before starting the uptake test). If the concentration rate is calculated based on only BCF_{SS}, the test water can be analyzed after transferring the fish to the tank, gradually adding the test substance and sufficiently ventilating the test water in the tank. During the uptake period, sample the test water from the test water tank before feeding, at least at the same time as the sampling of the test fish, and measure the test substance concentrations to check whether the validity standards (see 2-6) are met. Test water analysis is not performed during the elimination period (when the elimination test is performed).

During the test period, remove fish excrement, tank wall stains about once daily.

2-6. Validity of the test

Satisfy the following conditions to validate the test.

- The temperature variation must be less than ± 2°C.
- Generally, the dissolved oxygen concentration must be kept above 60% of the saturated oxygen concentration.
  (If aeration cannot be performed, e.g., in tanks for volatile substances, take appropriate measures to control the dissolved oxygen concentration, e.g., by increasing the flow rate. Report the measures taken here.)
- Under either a flow-through condition or a semi-static condition, the variation of the test substance concentration in the tank must be kept within ± 20% of the average of the measurements taken during the uptake period.
  (An extremely high concentration rate may result in a large variation of the test substance concentration during the uptake period. In such a case, the variation of the test substance concentration at the steady state must be kept within ± 20% of the average of the measurements.)
  (When testing any volatile substance, take appropriate measures such as using a tank for volatile substances with a smaller gas phase.)
- The proportion of abnormal organisms such as dead and diseased fish in the control or at each test concentration must be kept below 10% at the end of the test. If the test is extended for several weeks or months, the proportion of dead and diseased organisms in the control or at each test concentration must be kept below 5% per month and must not exceed 30% over the entire period.

2-7. Analysis of the test fish and test water

2-7-1. Sampling schedules for the test fish and test water

Sample the test fish at least five times during the uptake period and four times during the elimination period if the elimination test is performed (see Description 8). If necessary, store additional samples (see 2-7-2) and analyze them only when the results have turned out inappropriate for calculating the BCF with a required precision.

2-7-2. Sampling and pretreatment of samples

Sample the test water for the analysis, e.g., by sucking the water from the center of the test tank through an inert tube.

At each sampling time, take an appropriate number of test fish (usually at least 4) from each test tank and measure their body weights.

2-7-3. Analysis of test fish samples

Measure the test substance concentration in each sample. If the samples are too small to be measured individually, measure the test substance concentration collectively for samples taken at the same sampling time. In such a case, it is recommended to divide the samples taken at each sampling time into two or more groups.

Before and after the test, measure the lipid content in a fish sample cultivated under the same condition as those employed for cultivating the test fish. If possible, perform the lipid content measurement for each sampling time. The lipid content measurement can be performed for each test or for each fish lot.

2-8. Calculation and reporting of the test results

2-8-1. Processing of the results

Plot the test substance concentration in fish during the uptake period against time and calculate the BCF_{SS} at the steady state using the following equation employing the concentrations in fish (C_f) and in water (C_w).

$$BCF_{ss} = \frac{C_f \text{ at steady state (average)}}{C_w \text{ at steady state (average)}}$$

The concentration factor (BCF_K) can be determined as a ratio of the coefficients in the two linear expressions (the uptake and elimination curves), k_1/k_2. The elimination rate constant (k_2) is generally determined from the elimination curve (i.e., a graph depicting the temporal decrease of the test substance concentration in fish). The uptake rate constant (k_1) is calculated from the corresponding k_2 and the value C_f obtained from the uptake curve.

$$BCF_K = \frac{uptake\ rate\ constant\ (k_1)}{elimination\ rate\ constant\ (k_2)}$$

2-9. Summary of the results

Summarize the test results in Form 2 and submit it with the final report.

Test method description

1. Test fish

The important criteria for selecting the fish species are their availability, appropriate size and adaptability to laboratory cultivation. Also consider the recreational, commercial and ecological values of the species as well as their susceptibility to toxins. The fish species recommended are listed in Table 1. Other species may also be used, but the test procedure and conditions must be adapted to the selected species. In such case, report the grounds for the selection of the fish species and the test method.

If the test temperature is different from the temperature in the cultivation pool, acclimatize the fish in an acclimatization tank by the following procedure (1) or (2). During the acclimatization period, remove any fish with gill or skin damage or any weak or diseased fish. Flow-through conditions are recommended for the cultivation pool and the acclimatization tank.

(1) If the test temperature is higher than the temperature in the cultivation pool, acclimatize the fish at a temperature higher than that in the cultivation pool by within 5° C for at least one day. Then, gradually raise the temperature by up to 3° C per day until it reaches the test temperature, and cultivate them for 5 − 7 days at that temperature.

(2) If the test temperature is lower than the temperature in the cultivation pool, acclimatize the fish at a temperature lower than that in the cultivation pool by

within 3°C for at least one day. Then, gradually decrease the temperature by up to 2°C per day until it reaches the test temperature, and cultivate them for 7 – 10 days at that temperature. Determine the number of fish so that at least 4 samples per concentration are available per sampling time.

Report the sex(es) of the fish when using adult fish.

In each test, choose organisms of uniform body weights so that the minimum body weight does not become smaller than 2/3 of the maximum body weight. If direct measurement of their body weights is difficult, choose them visually based on their body length. If possible, choose fish of the same age and from the same source. Body weights or ages of the fish may have significant influence on the BCF value, so be sure to make precise records on them. It is recommended to conduct a preliminary measurement of their average body weight before the test.

2. Material water

The material water is defined as the water used for treating the group free of the test substance and auxiliaries. In general, the material water is natural water and must be clean and homogeneous. Dechlorinated tap water may also be used. Maintain the pH within the range of 6.0 - 8.5, with a variation of ± 0.5 throughout the test period.

The water quality must be suitable for the survival of the selected fish species, inducing no abnormal appearance or behavior during the acclimatization and test periods. The material water can be guaranteed to have no adverse effects on the test results (such as complexing of the test substance) or on fish activities by analyzing the parameters of the material water sample listed in Table 2. If the water quality has been proved to have been stable for at least a year, the measurements can be performed at reduced frequencies and at longer intervals (e.g., every 6 months).

Refer to the OECD test guideline for the upper limits of the parameters listed in Table 2. If the required concentration cannot be reached for any of these parameters, check the suitability of the material water for the cultivation of the test fish before conducting the test.

The adsorption of the test substance by organic matters may inhibit the uptake of the test substance by the fish. To avoid this, reduce the total organic carbon (TOC) content as well as the natural particle content in the material water. If necessary, filter the material water before use. Keep the content of organic carbons derived from fish excrement or uneaten feed as low as possible.

Increase the flow rate relative to the number of fish individuals to minimize the decrease in C_w triggered by the addition of fish at the beginning of the test and to prevent the decrease in the dissolved oxygen concentration. Choose a flow rate appropriate for the fish species used. In each case, it is usually recommended to set the flow rate to 1 - 10 L/day per 1.0 g fish body weight (wet weight). The flow rate should be sufficiently high to keep the variation in the test substance concentration within ± 20% of the settled value and to prevent the dissolved oxygen concentration from falling below 60% of the saturated oxygen concentration.

Table 2. Suggested parameters used for checking the water quality of the material water

1.	Suspended solids	21.	Free chlorine
2.	Total organic carbon	22.	Bromide ion
3.	Chemical oxygen demand (COD)	23.	Fluorine compound
4.	Total phosphorus	24.	Sulfide ion
5.	pH	25.	Ammonium ion
6.	Coliform group	26.	Nitrite nitrogen
7.	Mercury	27.	Arsenic
8.	Copper	28.	Nonionic surfactant
9.	Cadmium	29.	Selenium
10.	Zinc	30.	Evaporation residue
11.	Lead	31.	Electric conductivity
12.	Aluminum	32.	Total hardness (in terms of $CaCO_3$)
13.	Nickel	33.	Alkalinity
14.	Chromium	34.	Sodium
15.	Manganese	35.	Potassium
16.	Tin	36.	Calcium
17.	Silver	37.	Magnesium
18.	Cobalt	38.	Organochlorine pesticide
19.	Iron	39.	Organophosphorus pesticide
20.	Cyanide		

3. Test substance solution

A flow-through test requires a system for continuously supplying the stock solution of the test

substance to the test water tank and diluting the stock solution. Preferably, each test water tank is supplied at least five times its volume of the material water per day. A flow-through test is recommended, but if this is difficult (due to harmful effects on the test organisms), the test may be conducted under a semi-static condition by regularly replacing the test liquid with a new batch.

Check the flow rates of the stock solution and the material water at least 48 hr before beginning the test and everyday during the test period. Measure the flow rate in each test water tank and confirm that the variation of the flow rates among and within the tanks is within 20%.

The stock solution is preferably prepared by simply mixing or stirring the test substance in the material water. The use of any solvent or dispersant (auxiliary) is not recommended but is acceptable if required for preparing a stock solution at an appropriate concentration.

Table 3. 48 hr LC$_{50}$ values for solvents and dispersants used in the concentration test (mg/L, w/v)

Solvent		Dispersant	
Methyl alcohol	16,200	HCO-10	5,300
Ethyl alcohol	12,000	HCO-20	>50,000
Acetone	11,200	HCO-40	>100,000
Dimethylformamide	9,800	HCO-50	>100,000
Dimethylsulfoxide	33,000	HCO-100	>100,000
Tetrahydrofuran	3,800	Tween-40	2,800
1,4-Dioxane	7,200	Tween-80	50,000
Ethylene glycol dimethyl ether	21,500	SPAN-85	1,000
Ethylene glycol monomethyl ether	22,000		

Fish: Ricefish

Water temperature: 25°C

HCO: Polyoxyethylene-hardened caster oil

4. Test water

The test water is defined as water containing the test substance or auxiliary. Keep the water quality of the test water constant during the test period. Measure at least the pH, dissolved oxygen concentration and temperature of the test water.

5. Illumination and temperature

The photoperiod is usually 12 - 16 hr light. Choose a temperature appropriate for the test fish species. The type and characteristics of the illumination used should be understood. Choose the light condition for the test carefully, as it may cause the photolysis of the test substance. Use appropriate illumination to avoid the exposure of fish to artificial photoreaction products. If necessary, use appropriate filters to block UV radiation at wavelengths shorter than 290 nm.

6. Estimation of the duration of the uptake period

The estimation of the elimination rate constant (k_2) and the time required for reaching a concentration corresponding to a certain proportion of the concentration at the steady state can be obtained prior to the test, based on the empirical relation between either k_2 and the octanol-water partition coefficient (Pow) or k_2 and the solubility in water(s).

For example, the k_2 (per day) can be estimated from the following empirical formula[2].

$\log_{10}k_2 = -0.414 \log_{10}(\text{Pow}) + 1.47 \ (r^2 = 0.95)$ [Formula 1]

The formula by Kristensen can be used instead[3].

If the partition coefficient (Pow) is unknown, it can be estimated from the solubility(s) of the test substance in water[4].

$\log_{10}(\text{Pow}) = -8621\log_{10}(s) + 0.710 \ (r^2 = 0.994)$ [Formula 2]

Here, s = solubility in water (mol/L):(n=36)

These relational expressions can only be applied to chemical substances with Pow values of 2 -6.5[5].

The time required for reaching a concentration corresponding to a certain proportion of the concentration at the steady state can be obtained from a general kinetic equation (a linear expression) on the uptake and elimination using the estimated k_2.

$$\frac{dC_f}{dt} = k_1 \cdot C_w - k_2 \cdot C_f$$

If C_W is constant,

$$C_f = \frac{k_1}{k_2} \cdot C_w \left(1 - e^{-k_2 t}\right)$$

[Formula 3]

When the concentration approaches the steady state (t→∞), Formula 3 can be approximated as below[6,7].

$$C_f = \frac{k_1}{k_2} \cdot C_w$$

or,

$$C_f / C_w = k_1 / k_2 = BCF$$

Here, $k_1/k_2 \cdot C_W$ approaches the concentration in fish at the steady state ($C_{f,s}$). Formula 3 can be reformulated as below.

$$C_f = C_{f,s} \cdot (1 - e^{-k_2 t})$$

or,

$$\frac{C_f}{C_{f,s}} = 1 - e^{-k_2 t}$$

[Formula 4]

By estimating k_2 using Formula 1 or 2, the time required for reaching a concentration corresponding to a certain proportion of the concentration at the steady state can be estimated using Formula 4.

The optimum duration of the uptake period for obtaining statistically satisfactory data (BCF_K) can be determined as the time required for reaching the midpoint or $1.6/k_2$ or 80% of the steady state (the time corresponding to $3.0/k_2$ or 90% of the steady state or higher is not acceptable) in a logarithmic curve obtained by plotting the test substance concentration in fish against time[8].

The time required for reaching 80% of the steady state can be obtained from Formula 4 as:

$$0.80 = 1 - e^{-k_2 t_{80}}$$

or,

$$t_{80} = \frac{1.6}{k_2}$$

[Formula 5]

Similarly, the time required for reaching 95% of the steady state can be obtained as:

$$t_{95} = \frac{3.0}{k_2}$$

<div align="center">[Formula 6]</div>

For example, the duration (up) of the uptake period for a test substance with logPow=4 can be determined using Formulas 1, 5 and 6 as:

$$log_{10} k_2 = -0.414 \cdot (4) + 1.47$$
$$k_2 = 0.652 days^{-1}$$
$$up(80pct) = 1.6 / 0.652, i.e., 2.45\ days\ (59\ hr)$$
or
$$up(95pct) = 3.0 / 0.652, i.e., 4.60\ days\ (110\ hr)$$

Similarly, the duration of the uptake period for a test substance with s=10^{-5} mol/L (log(s)=-5.0) can be determined using Formulas 1, 2, 5 and 6 as:

$$log_{10}(Pow) = -0.862 \cdot (-5.0) + 0.710 = 5.02$$
$$log_{10} k_2 = -0.414 \cdot (5.02) + 1.47$$
$$k_2 = 0.246 days^{-1}$$
$$up(80pct) = 1.6 / 0.246, i.e., 6.5\ days\ (156\ hr)$$
or
$$up(95pct) = 3.0 / 0.246, i.e., 12.2\ days\ (293\ hr)$$

Alternatively, the time required for reaching the steady state can be obtained by the following formula[8].

$$teq = 6.54 \times 10^{-3} Pow + 55.31 (hr)$$

7. Estimation of the duration of the elimination period

The elimination period is continued until the concentration falls to below 5% of that at the steady state. If this takes too long and is impractical, the duration of the elimination period can be settled as at least twice the duration of a normal uptake period (i.e., at least 56 days), or can be shortened (e.g., until the test substance concentration reaches 10% of that at the steady state). However, if the substance exhibits a complex uptake-elimination pattern rather than a simple one following linear expressions, the elimination period can be sufficiently extended for obtaining the elimination rate constant. In such a case, the duration depends on how long the test substance concentration in fish stays above the lower detection limit of the analysis.

The time required for the in vivo concentration to reduce to a value corresponding to a certain proportion of the initial concentration can be estimated from a general relational expression (a linear kinetic equation) on the uptake and elimination[3,9].

C_w is assumed as being 0 during the elimination period, so the equation can be simplified as below:

$$\frac{dC_f}{dt} = -k_2 \cdot C_f$$

or,

$$C_f C_{f,0} \cdot e^{k_2 t}$$

Here, $C_{f,0}$ is the initial concentration at the beginning of the elimination period.
The time (t_{50}) required for reaching 50% elimination can be obtained by the following formula:

$$\frac{C_f}{C_{f,0}} = \frac{1}{2} e = e^{-k_2 t_{50}}$$

or,

$$t_{50} = \frac{0.693}{k_2}$$

Similarly, the time (t_{95}) required for reaching 95% elimination can be obtained by the following formula:

$$t_{95} = \frac{3.0}{k_2}$$

If the durations of the uptake and elimination periods are settled to reach 80% uptake ($1.6/k_2$) and 95% elimination ($3.0/k_2$), respectively, the duration of the elimination period will be about twice that of the uptake period.

Note that the above calculations are based on the assumption that the uptake-elimination pattern follows linear expressions. If the pattern apparently deviates from linear expressions, a more complex model should be used[2].

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6: Branson D.R., Blau G.E., Alexander H.C. and Neely W.B.: Transactions of the American Fisheries Society, 104 (4), 785-792 (1975).

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8:Reilly P.M., Bajramovic R., Blau G.E., Branson D.R. and Sauerhoff M.W.: Guidelines for the optimal design of experiments to estimate parameters in first order kinetic models, Can. J. Chem. Eng., 55, 614-622 (1977).

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8. Sampling schedules for test fish and test water for determining BCF$_K$

Sample the test water from the test water tank before adding the test fish and during the uptake and elimination periods to quantify the test substance in the samples. Sample the test water before feeding, at least at the same time as the sampling of the test fish. During the uptake period, measure the test substance concentration to check whether the validity standards (see 2-6) are met.

Sample the test fish at least five and four times during the uptake and elimination periods, respectively. If the elimination rate cannot be expressed by a simple linear kinetic equation, a greater number of samples will be required to calculate exact BCF values. Therefore, it is recommended that sampling be performed at higher frequencies during both periods (see Table 4). As mentioned in 2-7-2, store additional samples if necessary and analyze them only when the results have turned out inappropriate for calculating the BCF with a required precision.

An example of an appropriate sampling schedule for determining BCF_K is represented in Table 4. For calculating the exposure time corresponding to up to 95% uptake, other schedules can be easily established using the calculated Pow value.

During the uptake period, sampling is continued for 28 days or until a steady state is reached. If the steady state is not reached within 28 days, continue sampling until the steady state is reached, with the maximum being 60 days.

After the uptake period, start an elimination test by transferring the test fish to a clean tank free of the test substance.

Table 4. A theoretical example of the sampling schedule for the bioconcentration test of a substance with logPow=4

| Fish sampling | Sampling time schedule | | Frequency of water sampling | Number of fish sampled per sampling |
	minimally required sampling dates (day)	additional sampling dates (day)		
Uptake period	-1 0		2^* 2	add 45 - 80 fish
First	0.3	0.4	2 (2)	4 (4)
Second	0.6	0.9	2 (2)	4 (4)
Third	1.2	1.7	2 (2)	4 (4)
Fourth	2.4	3.3	4 (4)	4 (4)
Fifth	4.7		2	6
Elimination period				Transfer fish to water free of test substance
Sixth	5.0	5.3		4 (4)
Seventh	5.9	7.0		4 (4)
Eighth	9.3			4

		11.2		(4)
Ninth	14.0	17.5		6 (4)

* Water is sampled after supplying the tank at least three times its volume of the test water.
The values in parentheses represent the numbers of water or fish samples taken at additional samplings.

Note: When logPow is 4.0, the preliminary estimation of k_2 is 0.6521/day.

The total test period is 3× uptake period = 3× 4.6 days, i.e., 14 days.

9. Sampling and pretreatment of samples

Sample the test water for the analysis, e.g., by sucking the water from the center of the test tank through an inert tube. Be careful not to remove any contaminants in the test water through filtration or centrifugation. If the test substance is highly soluble in oil (i.e., has logPow > 5), the fish may also take up the test substance adsorbed by the contaminants. In such a case, perform an alternative treatment to keep the tank as clean as possible.

At each sampling time, take an appropriate number (usually at least 4) of test fish from each test water tank. Wash the fish samples quickly with water, wipe up water and humanely kill the samples immediately through an optimum method before measuring their body weights. When collectively analyzing small individuals weighing less than 1g, measure their body weights individually whenever possible.

In order to prevent decomposition or other loss, and to perform rough calculations of the uptake and elimination rates during the test, it is recommended that the test fish and test water samples are analyzed as soon as they are sampled. Immediate analysis should also enable early detection of the equilibrium.

If the samples cannot be analyzed immediately, store them using an appropriate method. Collect information on the appropriate storage methods, shelf lives and pretreatment methods for the individual test substances.

10. Method of analysis

The overall procedure depends on the precision and sensitivity of the analytical method used, so

check the precision and reproducibility of the chemical analysis by experiment. In addition, check whether the recovery rate of the test substance from the test water and test fish is sufficiently high for conducting the particular method. Make sure that no test substance is detected from the material water. If necessary, correct the C_w and C_f values based on the recovery test and the background values in the control group. Treat the test fish and test water samples appropriately for minimizing contamination and loss (e.g., adsorption to the sampler).

If the test is performed using substances labeled by radioisotopes, analysis can be performed on all labeled matters (i.e., the parent compounds and their metabolites).

11. Analysis of test fish samples

Measure the test substance concentration in each of the fish samples weighed. If the samples are too small to be analyzed individually, measure the test substance concentration collectively for samples taken at the same sampling time. If the statistical method or power is a critical issue, include an appropriate number of fish (usually at least four) in the test for satisfying the required number of samples, statistical method and power. If the samples taken at each sampling time are analyzed collectively, it is recommended to divide the samples taken at each sampling time into two or more groups.

BCF is expressed by a function of the total wet weight. If the test substance shows a high solubility in oil, BCF may be expressed by a function of the lipid content. If possible, measure the lipid content in fish samples at each sampling time. Use an appropriate method for the measurement of the lipid content. For the time being, the chloroform/methanol extraction technique is recommended as a standard method[10]. Lipids are often removed from the extracts before being analyzed by chromatography, so it is recommended to perform the lipid analysis using the same extract as that used for analyzing the test substance. The lipid content (mg/kg wet weight) in fish at the end of the test should be ± 25% of the initial content. In the case where the lipid concentration needs to be expressed in terms of dry weight instead of wet weight, it is suggested to report the dry weight ratio (dry weight/wet weight) of the test fish.

[10]:Gardner et al. : Limol. & Oceanogr., 30, 1099-1105 (1995)

12. Calculation of BCF$_K$

If the plotting of the test substance concentration in fish during the elimination period on a

semilogarithmic paper results in a linear approximation curve, it is reasonable to employ a simple model for accurate description of the bioconcentration data. (If these points cannot be plotted linearly, a more complex model should be used.)

12-1. Method for determining the elimination rate constant (k_2) by plotting
Plot the test substance concentration in fish at each sampling time on a semilogarithmic paper. The slope of the line is k_2.

$$k_2 = \frac{\ln(C_{f1}/C_{f2})}{t_1 - t_2}$$
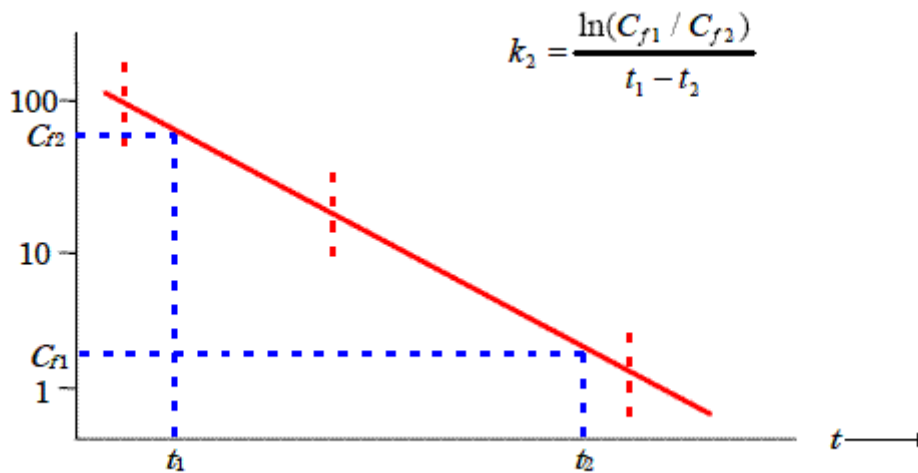
Note that the deviation of the elimination curve from a line may imply that the elimination pattern is more complex than a linear expression. The method by plotting can be used for revealing elimination patterns deviating from the linear kinetic theory.

12-2. Method for determining the uptake rate constant (k_1) by plotting
Calculate k_1 from the following formula using the obtained k_2:

$$k_1 = \frac{C_f / K_2}{C_w \times (1 - e^{-k_2 t})}$$

[Formula 1]

Determine C_f by reading the value at the central point of the uptake curve obtained by plotting the logarithmic concentration against time.

12-3. Method for determining the uptake and elimination rate constants using a computer
A better way to determine the bioconcentration coefficient and the rate constants k_1 and k_2 is to perform nonlinear parameter estimation programs by computer.

These programs calculate k_1 and k_2 in the following model equation from a pair of consecutive time-concentration data:

$$C_f = C_w \cdot \frac{k_1}{k_2} \times (1 - e^{-k_2 t})$$

[Formula 2]

$$C_f = C_w \cdot \frac{k_1}{k_2} \times (e^{-k_2(t - t_c)} - e^{-k_2 t})$$

[Formula 3]

Here, t_c = the completion time of the uptake period

Through this approach, the standard deviations of k_1 and k_2 can also be calculated.
In most cases, k_2 can be obtained from the elimination curve with a relatively high precision. Due to the strong correlation between k_1 and k_2, when calculating these two parameters simultaneously, it is recommended to first calculate k_2 from the elimination data and then k_1 from the uptake data using a nonlinear regression equation.

13. Interpretation of the results

Interpret the results carefully when the measured concentration in the test solution is close to the detection limit of the analytical method.

Bioconcentration data obtained with sufficiently high precision should result in clear uptake and elimination curves. The variations in the uptake/elimination constants between the two concentrations must not exceed 20%. Report any apparent difference in the uptake/elimination rates between the two concentrations and possible explanations for it. An appropriately designed test usually results in a confidence limit of BCF_{SS} within ± 20%. If the concentration rate is high, it is recommended to individually determine the concentration rate, etc., for different parts of the body.