<Bioconcentration test of substances in fish shellfish>

I: Bioconcentration test using fish (Aqueous exposure method)

I-I Scope of application

This section provides the method that should be the standard of test for evaluating the concentration of chemical substances in the body of fish, especially through water (via the gills). In principle, this test method is used for bioconcentration test in fish.

I-II Terms

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

I-III Test method

1 Overview of the test

The test method is a method for evaluating the uptake and accumulation of chemical substances through the water (via the gills) in fish bodies. In this test, test fish is exposed to test water in which chemical substances are dissolved, and the concentration of chemical substances in test water and test fish is measured, and then the steady-state bioconcentration factor (BCF_{SS}) is calculated. Further, if necessary, in addition to the above mentioned uptake phase, after the uptake phase is over, move the test fish to test water containing no chemical substance to provide a depuration phase. In this case, the kinetic bioconcentration factor (BCF_K) can be calculated throughout both the uptake and depuration phases.

2 Equipment and materials used in test

2-1 Equipment and instruments

All equipment and materials used should not have any harmful effect on the test fish due to dissolution, adsorption or leaching. The test water tank should be made of a chemically inert material and it should take the square or cylindrical shape with an appropriate capacity according to the flow rate. Use Teflon®, stainless steel, or glass tubing, and minimize the use of soft plastic tubing and use them only in unavoidable places. Test substance with high absorptivity like synthetic pyrethroids may require silanized glass.

2-2 Test water

(1) The test water is water for test and it does not contain the test substance and any solubilizing agent (solvent and dispersant). Use natural water obtained from an uncontaminated water source, dechlorinated tap water or artificially prepared water (dechlorinated tap water with known amounts of specific nutrients), and the water quality should be such that the selected fish species can survive without showing any abnormal appearance or behavior during the acclimation and test periods. For test water, measure at least pH, hardness, total particulate matter concentration, total organic carbon (TOC(1)) concentration. It is desirable to measure ammonium, nitrous acid and alkalinity as well.

(2) During the test periods, keep the water quality of test water constant. pH at the start of the test should be in the range of 6.0 to 8.5, and fluctuation range during the test periods should be within ±0.5 unit. Make sure that the test water does not affect the test results (for example, the effect due to test substance complex formation). Collect the test water regularly (at least at the start and end of the test) to ensure that it does not have any adversely effect on the fish activity, and measure the concentration of heavy metals, major anions and cations, agricultural chemicals, TOC, all particulate matters etc. (see test method description). If it can be confirmed that the quality of the test water is constant, the measurement frequency may be set every three months. In addition, if it can be demonstrated that the quality of the test water is constant over one year, the measurement frequency may be set every six months. In addition to TOC in test water, reduce the content of natural particles as much as possible. If necessary, filter the test water before use. Also, reduce the amount of organic carbon from test fish feces and residual food as much as possible.

2-3 Test fish

2-3-1 Selection of fish species

Cyprinus carpio (Carp) or Rice fish (*Oryzias latipes*; Japanese medaka) is recommended, but other fish species shown in the test method description may be used.

2-3-2 Farming and acclimatization

(1) Acclimatize the farmed fish at test water temperature for at least 2 weeks, and provide adequate food during that time. Water and food used during acclimatization should be of the same type as used for testing. Following the 48-hour observation period, record the mortality during the acclimatization period and use it for testing according to the following criteria.

- In the case of mortality rate exceeding 10% in 7 days: Do not use for test.
- In the case of mortality rate between 5% to 10% in 7 days: Acclimatize by extending the period by another 7 days. In the case of mortality rate of more than 5% in the next 7 days, do not use for the test.
- In the case of mortality rate of less than 5% in7 days, it can be used for the test.

(1) Total organic carbon (TOC) includes particulate organic carbon (POC) and dissolved organic carbon (DOC) (TOC = POC + DOC)

(2) Make sure that the fish used in the test is not sick or abnormal in appearance. Do not use the fish having any diseases in the test. Do not treat the fish for diseases etc. during 2 weeks before the start of the test or during the test period.

2-3-3 Feeding

(1) During acclimatization and test periods, provide appropriate amount of diet with known lipid and total protein content to keep the test fish healthy and maintain constant body weight. Set the feeding amount in consideration of fish species, test conditions and caloric value of the food, and provide the food every day during acclimatization and test periods (For example, in the case of *Cyprinus carpio* , about 1-2% of fish weight (wet weight). Set the feeding amount so that there is no rapid growth and increase of lipid content, and recalculate as appropriate from the weight of the test fish collected most recently from each test tank (once in a week etc.).

(2) Within 30 minutes to 1 hour after feeding, suck up the remaining food and feces from the test tank. Because the presence of organic carbon can limit the biological availability of the test substance, clean the test water tank throughout the test period and keep the organic carbon concentration as low as possible.

3 Conducting the test

3-1 Test water

(1) Test water is the water prepared by adding the test substance and a solubilizing agent to water to be used in the test. It is desirable that the test stock solution is prepared by simply mixing or stirring the test substance with test water. Minimize the use of solubilizing agent. Also, their critical micelle concentration must not be exceeded. Solvents that can be used include acetone, ethanol, methanol, *N, N*-dimethylformamide, and triethylene glycol. Dispersants that can be used are Tween®80, Methylcellulose 0.01%, NIKKOL®HCO-40 etc. The concentration of the solubilizing agent in the test water should be the same in all treatments, so that the solubilizing agent does not have any toxic effect on the test fish. The maximum concentration of solubilizing agent should be 100 mg/L (or 0.1 mL/L). Track the ratio of solubilizing and test substance with respect to the total amount of organic carbon in test water. Throughout the test period, the TOC concentration in test water should be 10 mg/L (±20%) or less (Except organic carbon concentration derived from the test substance and the solubilizing agent). The test substance concentration in test water should not be higher than the solubility in water, irrespective of whether any solubilizing agent is used or not. When using biodegradable solubilizing agent, care must be taken because it causes bacterial growth.

(2) In order to maintain the test substance concentration in the test water tank, a flow-through water system that continuously supplies and dilutes the test stock solution into the test water tank is effective. It is preferable to pass 5 times the test water volume of the test water tank at least one day. Although it is recommended to conduct the test with flowing-through mode, if this is not possible, a semi-static type test may be performed, provided that the validity criteria are satisfied. Check the flow rates of the test stock solution and test water 48 hours before the test and daily during the test period. The variation in flow rate of each test tank and the difference in flow rates between test water tanks should be within 20%.

(3) For test substance concentration in test water, if the change in concentration is observed before and after changing the test stock solution in the flowing water test, or if the change in concentration is observed before and after changing the water in the semi-static test, the test substance concentration (Cw) in test water may be calculated from the Time Weighted Average (TWA) as per the procedure given in Appendix 6 of the OECD Test Guideline 211.

3-2 Frequency of water quality measurement

During the test period, for all test water tanks, measure the dissolved oxygen concentration, TOC concentration, test water temperature and pH. Measure the total hardness for water tanks in a test vessel (1 water tank at the highest concentration) and the control. As for the dissolved oxygen concentration, measure at least three times during the uptake phase (at the start of the uptake phase, in the middle of the uptake phase, and at the end of the uptake phase), and once in a week during the depuration phase. With regard to TOC concentration, measure 24 hours and 48 hours prior to the start of the uptake phase, and once in a week during the uptake and depuration phase. Measure and record the test temperature once every day, pH at the start and end of the uptake phase and the depuration phase, and total hardness once during the uptake phase and the depuration phase. It is desirable to continuously monitor the test temperature in at least one test water tank.

3-3 Flow rate

Adjust the flow rate of test water according to the number of test fish in order to minimize the decrease in test substance concentration in test water due to the introduction of test fish at the start of the uptake phase and to avoid the decrease in dissolved oxygen concentration. Adjust the flow rate according to the fish species used. Normally, the recommended flow rate is 1-10 L/day per 1.0 g of fish weight (wet weight).

3-4 Condition of test fish

In each test tanks, the minimum fish weight at the start of the test is at least 2/3 of the maximum value. Use fish of the same age and source. Age and weight of the fish may significantly affect BCF. Therefore, record these details. In order to estimate the average fish weight at the start of the test, it is recommended to measure the weight of the acclimatized fish just before the start of the test.

3-5 Test water concentration

3-5-1 Conducting acute toxicity test (LC_{50} measurement)

Conduct the acute toxicity test set forth in the Notice as per the methods provided for in 71. of JIS K0102-2016 or OECD test guideline 203. However, these tests may be omitted when the data of maximum no-effect concentration (NOEC) is available for the test substance.

3-5-2 Setting the test concentration

(1) Conduct the test in at least 2 concentration levels. Set the test concentration in the high concentration level below 1% of acute toxicity value (LC_{50} value) of the test substance or below NOEC and keep it as low as technically possible. The appropriate guide is at least about 10 times higher than the quantification lower limit concentration of the test substance in the analysis of test water. For low concentration level, it should be 10 times lower concentration than high concentration level. However, if this is not possible due to toxicity and analytical sensitivity, a test substance (high purity, e.g. >98%) labeled with radioactive isotope may be used, or the test may be conducted at concentration ratio less than 10 times. Take precautions so that none of the test concentrations exceeds the water solubility of the test substance.

(2) In a test condition that is expected to have no BCF concentration dependency, test in only one concentration level may suffice. When applying the aqueous exposure method in one concentration level, set the test concentration to 1/10 or less of the solubility of the test substance in the test water. However, with regard to inorganic compounds, organometallic compounds, substances having surface activity, substances having a trifluoromethyl group or a tetrafluoroethylene group, or complex reaction products with unknown structure or mixtures with indefinite components, even if it is the above set concentration, the possibility of showing concentration dependency cannot be denied. Therefore, the aqueous exposure method in one concentration level is not suitable. In addition, even for substances having a high possibility of binding to a protein (substances that cannot be recovered from fish by solvent extraction), since the possibility of showing concentration dependency cannot be denied, the aqueous exposure method in one concentration level is not suitable.

In addition to a series of tests, provide for a comparison group containing only test water, or a comparison group containing only solubilizing agent when a solubilizing agent is used in the test stock solution.

3-6 Lighting and test temperature

Lighting time should be normally 12 to 16 hours. Track the type and characteristics of lighting. Note that the test substance may be photodegraded under the lighting conditions in the test. Use appropriate lighting to avoid exposure of artificial photoreaction products to test fish. Depending on the situation, use a suitable filter that blocks UV radiation below 290 nm. The test temperature is the recommended temperature for test fish, and its variation should be less than ±2℃.

3-7 Test period

3-7-1 Uptake phase

Uptake phase should be 28 days unless it is confirmed that the test substance concentration in the test fish reaches a steady state (see test method description) at an early stage of the uptake phase. For samples collected at intervals of at least 2 days, it is determined that the steady state has reached if the analysis results of three consecutive test substance concentrations are within ± 20%. However, when analyzing multiple test fishes together, the steady state is determined by at least four consecutive test fish analyses. When the steady state doesn't reach in 28 days, extend the uptake phase until the steady state is reached or 60 days, whichever is shorter, and calculate BCF (See BCF_{SS}, test method description) in the steady state. When BCF is less than 100 L/kg, even if the variation of test substance concentration in test fish exceeds 20%, after 28 days, it can be regarded that the steady state has reached. When depuration test is performed, calculate BCF (BCF_K, See test method description) based on kinetics. If uptake of the test substance is clearly not confirmed after 28 days, the test can be terminated. If the BCF_{SS} is 1000 L/kg or more (if BCF_{SS} is not obtained, when the average value of BCF in the last three consecutive measurements during the uptake phase is 1000 L/kg or more when conducting analysis for individual test fish, and when the average value of BCF in the last four consecutive measurements during the uptake phase is 1000 L/kg or more when analyzing multiple test fishes together), conduct analysis for each part separately. Conduct the analysis separately for four parts of head, internal organs, outer skin (including gills and digestive tract), and edible parts (other parts excluding the head, internal organs, and outer skin), and report the test substance concentration and BCF in the respective part.

3-7-2 Depuration phase

If the BCF_{SS} is 1000 L/kg or more (if BCF_{SS} is not obtained, when the average value of BCF in the last three consecutive measurements during the uptake phase is 1000 L/kg or more when conducting analysis for individual test fish, and when the average value of BCF in the last four consecutive measurements during the uptake phase is 1000 L/kg or more when analyzing multiple test fishes together), or when calculating BCF_K, provide for depuration phase. The depuration phase should preferably be the period until the test substance concentration in the test fish is sufficiently reduced (for example, 95% of the steady state concentration disappears) (See test method description). If the period until 95% test substance concentration in the test fish disappears is more than twice the normal uptake phase, the period may be shortened (for example, it can be the period until the test substance concentration in the test fish reduces to less than 10% of the steady state). However, for chemical substances whose uptake and depuration show a more complicated pattern than the one-compartment model based on the first-order rate equation, a longer depuration phase is required to determine the depuration rate constant. When extending the depuration phase, consider the possibility that the growth of the test fish may affect the test results.

3-8 Collection and analysis

3-8-1 Analysis method

(1) As for the analysis method, experimentally check whether the accuracy, precision, and reproducibility of the chemical analysis, as well as recovery of the test substance from the test water and test fish are sufficient or not. Also check that the test substance is not detected in test water. If necessary, correct the test substance concentration values in test water and test fish obtained by test with the recovery rate and the background value of the control. When collecting test water and test fish, minimize contamination and loss of the test substance (e.g. adsorption to the collection apparatus).

(2) Analyze test fish and test water immediately after collection to prevent degradation of the test substance. If analysis cannot be conducted promptly, preserve the sample in an appropriate manner. For the test substance, obtain information about the appropriate preservation method, preservation period and pretreatment before starting the test.

3-8-2 Analysis of test water

(1) Analyze the test water before and during the uptake phase to determine the test substance concentration. When depuration phase is provided for, analyze the test water during the depuration phase as well. Analyze test water at the same time as test fish before feeding. However, in the test water analysis at the start of the depuration phase, if it can be confirmed that no test substance is detected, the analysis of the test water in the test tanks and the control in the subsequent depuration phase may be omitted.

(2) For example, suck the test water with an inert tube from the center of the test tank and analyze it. At this time, a dirt of the test water is usually not removed by filtration or centrifugation. When removing it, report the justification or validity of the separation technique used. Particularly for highly hydrophobic chemicals (that is, chemicals with log *P*OW> 5), such treatment should not be performed because of adsorption to the filter material or the centrifuge vessel. Instead, take measures to keep the test tank as clean as possible. In addition, measure the TOC concentration during the uptake phase and the depuration phase.

3-8-3 Analysis of test fish

(1) As for the analysis of each test fish, analyze at least 4 fish per test group. However, if it is difficult to analyze each individual fish, analyze multiple test fish at the time of each analysis. In that case, it is desirable to have two or more groups.

(2) Analyze the test fish at least 5 times during the uptake phase. When depuration phase is provided for, analyze the test fish at least 4 times during the depuration phase. Transfer the test fish to a clean test tank before starting the depuration phase. In particular, if it is expected that uptake and depuration will not follow a simple first-order kinetic equation, more frequent analysis is recommended during both periods because it is difficult to calculate an accurate BCF (see the test method description). Perform euthanasia on the test fish that are collected in the most suitable way from the viewpoint of animal welfare, and measure its body weight and total length. Use an identifier code to the body weight and total length of each individual body, and link to the test substance concentration (lipid content too if applicable).

(3) Lipid content must be measured at least at the beginning and end of the uptake phase, and at the end of the depuration phase. Lipid content is measured by using the same test fish as the test substance concentration measurement. However, if measurement by using the same test fish is difficult, measure at least three additional fish at above mentioned three measurements. When it is clear that the test substance is not significantly detected in the test fish in the control group, measure only lipid content in the test fish in the control group, and measurement of the test substance concentration may be omitted.

(4) If BCF_{SS} is 1000 L/kg or higher, except when it can be considered that the test substance does not accumulate mainly in lipids, report BCF_{SS} (BCF_{SSL}) standardized with 5% lipid content (based on wet weight) too.

(5) When using chemical substances labeled with radioactive isotope in the test, either measure as total labeled products (i.e. parent compounds and metabolites), or clean up the sample and measure only parent compounds. When determining BCF on the basis of the parent compound, check and confirm the main metabolites at least at the end of the uptake phase.

3-8-4 Growth and measurement of test fish

Sample 5 to 10 fish at the start of uptake phase from the test fish before transferring them to the test water tank, and then individually measure body weight and total length. These test fish can be used to measure the test substance concentration and lipid content prior to the start of the uptake phase. Record the body weight and total length of the test fish collected during the test period before measuring the test substance concentration or lipid content. From these measurement values, estimate the fish weight and total length for the exposed group and the control group. A significant difference in the average growth rate of fish in the exposed group and the control group suggests that there is a toxicity effect of the chemical substance.

4 Calculating test results

4-1 Calculation of bioconcentration factor

Plot the test substance concentration (*Cf*) in test fish (or a specific tissue) during the uptake phase against time to obtain an uptake curve. When this curve has reached equilibrium, calculate BCF(BCF_{SS}) in the steady state from the following equation.

$$BCF_{SS}=\frac{\text{Average test substance concentration in test fish at steady state}}{\text{Average test substance concentration in test water at steady state}}$$

In addition, calculate kinetic bioconcentration factor (BCF_K) by using the following equation. The method of calculating k_1 and k_2 is shown in the test method description.

$$BCF_K=\frac{\text{Uptake rate constant } (k_1)}{\text{Depuration rate constant } (k_2)}$$

4-2 Growth dilution correction and lipid content standardization

(1) The growth of test fish during the depuration phase apparently reduces the test substance concentration in the test fish, and it has a huge impact on depuration rate constant (k_2). Therefore, when determining BCF_K, along with BCF_K, report BCF_K (BCF_{Kg}) that has been corrected for growth dilution. Depuration rate constant corrected for growth dilution (k_{2g}) is usually calculated by subtracting the growth rate constant (k_g) from the depuration rate constant (k_2). Furthermore, calculate BCF_{Kg} by dividing the uptake rate constant (k_1) by the depuration rate constant (k_{2g}) corrected for growth dilution. The growth dilution correction method, including methods other than the above, is shown in the test method description.

(2) When BCF_{SS} is 1000 L/kg or more, report BCF_K (BCF_{KL}) or BCF_{SS} (BCF_{SSL}) standardized with 5% lipid content along with BCF_K or BCF_{SS} (see test method description). In addition, when reporting BCF_K, report BCF_K corrected for growth dilution and standardized with 5% lipid content (that is, BCF_{KgL}). When test substance concentration and lipid content are measured by using the same fish at all sampling points, normalize the test substance concentration in each test fish by using the lipid content of that fish. If the growth of the test fish in the exposed group and the control group is similar, normalization may be conducted by using the lipid content of the test fish in the control group.

5 Validity of the test

For test to be valid following conditions apply:

- Temperature variation should be less than ±2°C (large variation in test water temperature will cause stress to test organisms and affect the biological parameters relevant to uptake and depuration).
- The dissolved oxygen concentration should not be below 60% of the saturated oxygen concentration.
- Variations in test substance concentration in test water should be kept within ±20% of the mean of the measured values during the uptake phase.
  (If the bioconcentration is extremely high, the variation of the test substance concentration during the uptake phase may become large. In this case, the variation of the test substance concentration in the steady state should be kept within ±20% of the average of the measured values.)
- The mortality or abnormalities such as disease should be less than 10% at the end of the test in both control and exposed fish. If the test is extended for several weeks or months, the mortality or abnormalities should be less than 5% per month and not more than 30% over the entire period in all groups.

6 Summarize the results

Summarize the test results in form 2-1, and attach the final report.

II: Bioconcentration test using fish (Minimized aqueous exposure method)

II-I Scope of application

Here, we provide the method that should become the standard of minimized bioconcentration test for the chemical substances present in the body of fish, especially through aqueous exposure (via the gills). This method should only be applied to substances that are not expected to be concentration dependent and for uptake and depuration rate constant estimation following the first order rate equation.

II-II Terms

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

II-III Test method

1 Overview of the test

This test method is a method for evaluating the uptake and accumulation of chemical substances through the water (gills) in the fish body. While the test conforms to I. Bioconcentration test using fish (Aqueous exposure method) conducted by using fish, the measurement of concentration of chemical substances in the test fish is reduced to 4 times (2 times in the uptake phase and 2 times in the depuration phase), and the bioconcentration factor (BCF_{Km}) by kinetics and the bioconcentration factor (minimized BCF_{SS}) in the steady state are calculated.

2 Equipment and materials used in test

Same as I: Bioconcentration test using fish (Aqueous exposure method).

3 Conducting the test

Same as I: Bioconcentration test using fish (Aqueous exposure method). However, the sampling schedule and the calculation method are as follows.

3-1 Analysis of test water

For determining the test substance concentration, analyze the test water at least once before the start of the uptake phase and at least 5 times during the uptake phase (two of which are at the same time as the analysis of the test fish). In addition, it should be once a week during the depuration phase. In the test water analysis at the start of the depuration phase, if it can be confirmed that no test substance is detected, the analysis of the test water in the exposed group and the control group in the subsequent depuration phase may be omitted.

3-2 Analysis of test fish

Analyze the test fish as follows and determine the test substance concentration in the test fish.

- As for the analysis of each test fish, analyze at least 4 fish per test group. However, if it is difficult to analyze each individual fish, analyze multiple test fish at the time of each analysis. In that case, it is desirable to have two or more groups.
- The analysis of the uptake phase is at the middle and end of the uptake phase (the end corresponds to the start of the depuration phase) (For example, 14 and 28 days after the uptake phase).
- The analysis of the depuration phase should be in the middle and at the end of the depuration phase (the test substance concentration should be preferably less than 10% of the maximum concentration, but at least until the depuration half-life of the test substance can be calculated) (for example, 7 and 14 days after the depuration phase). If depuration is expected to be early, the test substance concentration in the test fish should not fall below the lower limit of quantification.

4 Calculating test results

Using the test substance concentration (C_{f1}) in the test fish at the end of uptake (t_1) and the test substance concentration (C_{f2}) in the test fish at the end of depuration (t_2), calculate the depuration rate constant (k_2) in accordance with Equation 1.

$$k_2 = \frac{\ln(C_{f1}) - \ln C_{f2})}{t_2 - t_1} \quad \text{[Equation 1]}$$

By using the depuration rate constant (k_2) determined above, average test substance concentration (C_w) in the test water during the uptake phase, and test substance concentration (C_{f1}) in the test fish at the end of the uptake phase (t_1), calculate the uptake rate constant (k_1) in accordance with Equation 2.

$$k_1 = \frac{C_f \cdot k_2}{C_w(1 - e^{-k_2 t})} \quad \text{[Equation 2]}$$

Furthermore, by using the ratio of the uptake rate constant (k_1) and the depuration rate constant (k_2), calculate the bioconcentration factor (BCF_{Km}) based on the kinetics of the simplified aqueous exposure method according to Equation 3.

$$BCF_{Km} = \frac{k_1}{k_2} \quad \text{[Equation 3]}$$

Assuming that steady state reached during the uptake phase, by using the test substance concentration in the test water ($C_{w\text{-}minSS}$, mg/L) and the test substance concentration in the test fish at the end of the uptake phase ($C_{f\text{-}minSS}$, mg/kg wet weight), calculate the bioconcentration factor (minimized BCF_{SS}) in the steady state in the minimized aqueous exposure method according to Equation 4.

$$\text{minimised } BCF_{SS} = \frac{C_{f\text{-}minSS}}{C_{w\text{-}minSS}} \quad \text{[Equation4]}$$

Lipid content measurement and correction for growth dilution should be same as I: Bioconcentration test using fish (Aqueous exposure method).

5 Validity of the test

Same as I: Bioconcentration test using fish (Aqueous exposure method).

6 Summarizing the results

Summarize the test results in form 2-1, and attach the final report.

III: Bioconcentration test by using fish (Dietary exposure method)

III-I Scope of application

Here, we provide the method that should become the standard of test for evaluating the concentration of chemical substances in the body of fish, especially through feed. This test can be applied to test substances with water solubility of less than 0.01 mg/L and log Pow of more than 5. The water solubility is actually measured value, but log P_{ow} may be estimated by (Q)SAR etc. In addition, for substances whose concentration is difficult to maintain in the test water in the aqueous exposure method, or substances whose bioconcentration factor (BCF) that can be calculated from the lower limit of quantification of the test substance in the test fish exceeds about 1000 L/kg, since it is difficult to use the aqueous exposure method, the dietary exposure method may be used. However, consult the authorities before starting the test.

In principle, this test is not applied to complex reaction products with unknown structure or mixtures with indefinite components.

III-II Terms

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

III-III Test method

1 Overview of the test

This test method is a method for evaluating the uptake and depuration of chemical substances through the test substance-spiked feed in the fish body. The test consists of two periods of uptake and depuration. During the uptake phase, the test fish is given the feed with the chemical substance mixed in it, and after that, the test fish is given the feed containing no chemical substance during the depuration phase. During both periods of the test, calculate the chemical concentration in the test feed and test fish is measured and the dietary biomagnification factor (BMF, see the test method description). As BMF, calculate either or both of dietary biomagnification factor (BMF_K) by kinetics and dietary biomagnification factor (BMF) at the end of the uptake phase.

2 Equipment and materials used in test

2-1 Equipment and instruments

All equipment and instruments shall be the same as those described in I: Bioconcentration test using fish (aqueous exposure method). Use the flow-through or semi-static water system that supplies a sufficient amount of test water to the test tank and record the flow rate.

2-2 Test water

Water for test shall be the same as that described in I: Bioconcentration test using fish (Aqueous exposure method).

2-3 Test fish

2-3-1 Selection of fish species

Fish species provided for in I: Bioconcentration test using fish (Aqueous exposure method) can be used. The test fish should be of the recommended size (see test method description) and should be of the size that allows individual analysis.

2-3-2 Farming and acclimatization

Acclimatization condition before test, mortality rate during acclimatization, and disease tolerance should be the same as I: Bioconcentration test using fish (Aqueous exposure method).

2-3-3 Diet

Diet should not contain the test substance and a medium (organic solvent or oil). Commercial fish feed (powdered or slowly settled pellet shape or crumbled feed) with at least a known protein and lipid content is recommended. In order to increase the feeding efficiency, the feed should be of uniform size and adjusted to an appropriate size according to the test fish at the start of the test. The size of the feed may be adjusted to the growth of the test fish at the start of the depuration phase. An example of a suitable commercial feed composition is given in the test method description. Measure the lipid content in the feed of the exposed group and the control group before the start of the uptake phase and at the end of the uptake phase. In the test report, mention information such as nutrients, moisture, fiber, ash, etc. in the feed.

3 Conducting the test

3-1 Test food

(1) The test food is obtained by spiking the test substance and a medium to the feed. Based on the physical and chemical properties and solubility of the test substance, add to the feed by using the following method as a reference (see test method description). When adding the test substance to the feed, ensure its uniformity in the test feed. In the test report, mention the method and procedure of spiking to the feed.

- If the test substance is soluble and stable in triglycerides, dissolve the test substance in a small amount of oil (fish oil or edible vegetable oil) before mixing with the feed. In this case, minimize the amount of oil in consideration of the original lipid content of the feed.
- After dissolving in a suitable organic solvent (volatile solvent such as hexane, acetone and tetrahydrofuran) and mixing with the feed, distill off the solvent that was added for dispersing and ensuring uniformity of the test substance in the test feed (Crystallization of test substance occurs due to evaporation of the organic solvent, which may reduce the biological availability of the test substance). Moreover, since the components (for example, lipids or proteins) in the feed are extracted by adding the organic solvent, which may affect the uniformity of the components in the feed, minimize the amount of the organic solvent added to the feed.
- Directly spike the non-sticky liquid test substance to the feed and mix it well to promote uniformity and good assimilation.

(2) During the uptake phase and the depuration phase, give a nutritionally equivalent feed or the test feed to the exposed group and the control group. When oil or an organic solvent is used as

the test substance addition medium, for the test food of the control group, add the same amount of medium (excluding the test substance) as the exposed group. For the test food to which the test substance is spiked, store it under a condition where the test substance in the test food is stably maintained, and report this method.

3-2 Feeding

(1) During the acclimatization period and depuration phase, give the food, and during the uptake phase, given the test food (however, for the control group, give test feed with no test substance spiked) in a certain amount (for example, about 1-2% of fish weight (wet weight) in the case of carp). When conducting the test under the flow-through conditions, temporarily stop the flowing water while the fish is feeding. Set the dieting amount such that rapid growth of test fish and a significant increase in lipid content are avoided. Record the actual food amount set during the test. Set dieting at the start of the test based on the measured body weight of the acclimatized fish before the start of the test. Adjust the feeding amount on the basis of the test fish body weight (wet weight) at each sampling considering growth during the test. Estimate the body weight and length of fish in the test and comparison groups from the fish collected at the time of each collection (do not measure the body weight and length of the fish remaining in the test and comparison group water tanks). It is important to maintain a constant amount feeding throughout the test period.

(2) Make sure that the test fish is eating all the unspiked food and the spiked food. Set the feeding amount so that the test fish consumes all the unspiked food and the spiked food once a day. If there is all the unspiked food and the spiked food that is consistently remaining as it is, the amount given to the fish may be divided. For example, if dividing once a day into twice a day, the second feeding should be done at regular intervals to allow as much time as possible until sampling the test fish.

(3) In order to avoid the test substance from dispersing in the water from the spiked food and the test fish from being exposed to the test substance in the water, remove all remaining food and feces from the water tanks of the exposed group and the control group within 1 hour or preferably within 30 minutes of feeding. To adsorb all dissolved substances, a system that continuously cleans the water with an activated carbon filter can be used. The flow-through system is useful for quickly removing feed particles and dissolved material(2).

3-3 Frequency of water quality measurement

Same as I: Bioconcentration test using fish (Aqueous exposure method). However, the TOC concentration may be measured only before the start of the test as part of the characterization of the test water.

(2) As a result of depuration from the test fish or elution from the feed, the presence of the test substance in the test water may not be completely avoided. Therefore, measuring the test substance concentration in water at the end of the uptake phase is one measure, and particularly when using the semi-stop water method, it is useful for checking whether aqueous exposure has occurred or not.

3-4 Flow rate

Adjust the flow rate of test water according to the number of test fish in order to maintain appropriate dissolved oxygen concentration and reduce stress on test animals. Normally, the recommended flow rate is 1-10 L/day per 1.0g fish weight (wet weight).

3-5 Condition of test fish

The minimum fish weight at the start of the test should be at least 2/3 of the maximum value. Use fish of the same age and source.

Decide the number of test fish is determined in consideration of the number of collections and the number of fish at that time. An example of a sampling schedule including the number of test fish is shown in the test method description.

3-6 Concentration of spiked diet

In principle, conduct the test in 1 concentration level. At the same time, set a control group giving the food with no test substance spiked to it. Set the test concentration according to the sensitivity of analysis (the concentration of the test substance in the fish during the depuration phase can be measured up to less than 10% of the test substance concentration in the fish body at the end of the uptake phase), the toxicity of the test substance (if known, the maximum no-effect concentration) (NOEC)), and repelling behavior. From previous knowledge, test substance concentration in the range of 1-1000 μg/g is a practical range for chemical substances that do not exhibit a specific toxicity mechanism. For confirm that the test fish is properly taking the spiked food and that the test results are valid, for the time being, it is recommended to add the reference substance(3) with known BCF and BMF to the spiked food of the exposed group and the control group, and calculate the BMF in the same way as the test substance.

3-7 Lighting and test temperature

Same as I: Bioconcentration test using fish (Aqueous exposure method).

3-8 Test period

3-8-1 Uptake phase

Uptake phase should be normally 7-14 days. The experiment starts when the spiked food is first given. As for counting the experiment day, from the time of feeding until just before the next feeding (for example, one hour before) becomes one day. The uptake phase is until immediately before the first feeding of the no-spiked (only medium spiked it) food (for example, one hour before). Ensure that the test substance concentration in the fish is sufficiently high considering the analytical sensitivity so that a decline in the test substance concentration in the fish body of up to at least 10% can be measured during the depuration phase. In addition, to confirm the accumulation behavior of the test substance in the test fish, you may extend the uptake phase (up to 28 days) and conduct additional analysis.

(3) Examples of reference materials are shown in the test method explanation. In addition to these examples, once knowledge is accumulated and appropriate substances come to light in the future, these substances may also be used as reference substances.

3-8-2 Depuration phase

(1) In principle, the depuration phase should be 28 days. If it is necessary to further confirm the extent of test substance depuration from the test fish, extend the period. The depuration phase starts when the test fish is given with food that does not contain the test substance and a medium. If the test substance concentration in the test fish is below the lower limit of quantification at the beginning of depuration (for example, after 7 days or 14 days), the subsequent analysis may be stopped and the test may be terminated. Even if the half-life cannot be obtained at the end of the depuration phase, calculate the dietary biomagnification factor (BMF) from the results of the depuration tests performed.

(2) In a test with the uptake phase of 10 days or more, the test can be terminated if the BMF at the end of the uptake phase is less than 0.007, and the following ① and ② are satisfied.

① The test validity condition is satisfied.

②The lack of uptake is not due to test design issues (for example, reduced biological availability due to test feed preparation failure, lack of analytical sensitivity, fish not eating the test feed, etc.).

3-9 Analysis

In the analysis, comply with the matters described in I: Bioconcentration test using fish (aqueous exposure method).

3-9-1 Analysis of diet sample

For the spiked and unspiked diet of the exposed group and the control group, measure at least 3 samples for the test substance concentration and the lipid content at least before and at the end of the uptake phase.

When testing the materials labeled with radioactive isotope, analyze the test feed in conformance with I: I: Bioconcentration test using fish (Aqueous exposure method).

3-9-2 Analysis of test fish

(1) For the analysis of the test fish, sample 5 to 10 fish from the exposed group and the control group, and conduct analysis on the individual test fish. However, if it is difficult to analyze each individual fish, analyze multiple test fish at the time of each analysis. In that case, it is desirable to have two or more groups. Sample the test fish at the same time (feeding time is the rough guide for this). If there is test diet remaining in the digestive tract of the test fish, it will affect the measurement of the test substance concentration in the test fish. Therefore, sample just before the next round of feeding (for example, 1 hour prior to the feeding). If there is a possibility of residual diet, it is desirable to remove the digestive tract and analyze separately. The presence or absence of residual diet in the digestive tract may be confirmed in the preliminary study.

(2) For the exposed group and the control group, upon completion of the uptake phase and during the depuration phase, analyze the test fish 4 times to 6 times (for example, on 1st, 3rd, 7th, 14th, and 28th day). In addition, when it is necessary to confirm the concentration of each detailed tissue, conduct the test for each site. Conduct the test separately for five parts of head, internal organs, outer skin (including gills), digestive tract and edible parts (other parts excluding the head, internal organs, outer skin, and digestive tract), and report the test substance concentration and BMF in the respective part. For the test fish in the control group, if the test substance concentration is not detected at the start of the depuration phase, it is sufficient to analyze 2-3 fish at the end of the depuration phase. In all analyses, after euthanizing the test fish collected the most suitable way from the viewpoint of animal welfare, measure their body weight and the total length for each fish. (ensure that the same number of test fish are collected from the exposed group and the control group). Assign an identification code to the body weight and total length of each individual body, and match with the test substance concentration (lipid content too if applicable).

(3) When testing the materials labeled with radioactive isotope, replace the test water analysis in I: Bioconcentration test using fish (aqueous exposure method) with test feed analysis, and comply with the matters described in 3-5-2 Test concentration setting (1).

(4) It is preferable that the lipid content in the test fish in the exposed group and the control group is measured at the time of each collection. However, at least measure at the start and end of the uptake phase and at the end of the depuration phase. For the lipid content at the start of the uptake phase, the test fish collected in 3-9-3 Measurement of growth of test fish may also be used. Measure the lipid content for the same fish as the test fish used for measuring the test substance concentration. If measurement is not possible, measure for the test fish collected separately. When it is clear that the test substance is not significantly detected in the test fish in the control group, measure only lipid content in the test fish in the control group, and measurement of the test substance concentration may be omitted. Describe the method of quantifying the lipid content in the test report.

3-9-3 Measurement of weight of test fish

For calculating the growth rate constant (k_g), measure the body weight (wet weight) of the test fish collected in 3-9-2 Analysis of test fish. As the fish weight at the start of the experiment, immediately before feeding the test diet for the first time, collect at least as many test fish (5-10 fish) as the test fish analysis during the test period, and measure the fish weight.

4 Calculating test results

4-1 Calculation of assimilation efficiency and biomagnification factor

Calculate the relationship between the natural logarithm of the test substance concentration (C_f) in the test fish during the depuration phase and the depuration phase by the method of least squares. The slope of the straight line is the depuration rate constant (k_2), and the intercept is the test substance concentration in the fish at the end of the uptake phase (mg/kg, extrapolated value $C_{0,d}$). Use these values, The feeding rate constant (I), uptake duration (t), and the mean measured concentration of the test substance in the food (C_{food}), and calculate the chemical assimilation efficiency (α: the absorption of test substance across the gut) as per Equation 5.

$$\alpha = \frac{C_{0,d} \cdot k_2}{I \cdot C_{food}} \cdot \frac{1}{1\text{-}e^{-k_2 t}} \qquad \text{[Equation5]}$$

Furthermore, calculate BMFK according to Equation 6 using the feeding rate constant (I), assimilation efficiency (α), and depuration rate constant ($k2$).

$$BMF_k = \frac{I \cdot \alpha}{k_2} \qquad \text{[Equation6]}$$

In addition, use the following equation to calculate BMF at the end of the uptake phase. When it seems to have reached the steady state, calculate BMFss from the following equation.

$$BMF = \frac{\text{Average test substance concentration in test fish at the end of the uptake phase}}{\text{Average test substance concentration in the test feed}}$$

4-2 Growth dilution and lipid content correction

(1) The growth of test fish during the depuration phase apparently reduces the test substance concentration in the test fish, and it has a huge impact on depuration rate constant (k_2). Therefore, when calculating BMF_K, also report BMF_K (BMF_{Kg}) corrected for growth dilution. The depuration rate constant (k_{2g}) corrected for growth dilution is usually calculated by subtracting

the growth rate constant (k_g) from the depuration rate constant (k_2). Furthermore, by using the depuration rate constant (k_{2g}) corrected for growth dilution in Equation 6, calculate BMFK (BMF_{Kg}) corrected for growth dilution. The method of growth dilution correction is shown in Test Method Explanation 6.6 including methods other than the above method.

(2) Correct BMF by using the lipid content of the test fish and test food unless it is clear that the test substance is hardly accumulated in lipid[4]. If lipid content measurement is not done for all collected test fish, calculate the average lipid content (w/w). Divide the average lipid content of the test fish by the average lipid content of the test feed to calculate the lipid content correction factor (Lc). Divide BMF, BMF_K, and BMF_{Kg} at the end of the uptake phase by the lipid content correction factor to calculate BMF(BMF_L), BMFK (BMF_{KL}), and BMF_{Kg} (BMF_{KgL}) corrected for the lipid content at the end of the uptake phase.

(3) When the test substance and lipid content are measured in the same test fish at the time of each collection, plot the test substance concentration data corrected for lipid content against the time axis to obtain $C_{0,\,d}$ and k_2 corrected for lipid content. Calculate the chemical assimilation efficiency (α) by using the amount of feed corrected for lipid content (I_{lipid}) and the test substance concentration in the test feed corrected for lipid content ($C_{food\text{-}lipid}$) (see test method description). Use these values to calculate BMF_{KgL} (when calculating BMF corrected for lipid content and growth dilution, correct by using the growth rate constant per lipid content and, not the wet weight of the test fish).

5 Validity of the test

For test to be valid following conditions apply:

- Temperature variation is less than ±2°C.
- The dissolved oxygen concentration should not be less than 60% of the saturated oxygen concentration.
- Regarding the test substance concentration in the test feed of the exposed group (measure at least 3 nos. each), the variation between the average value before the start of the uptake phase and the average value at the end of the uptake phase is within ± 20%.
- Regarding the test substance concentration in the test feed of the exposed group (measure at least 3 nos. each), the variation between samples is within ± 15% of the average value.
- The test substance concentration in the test feed or the test fish in the control group is not detected or it is less than the lower limit of quantification as compared to the exposed group.
- The mortality or abnormalities such as disease should be less than 10% at the end of the test in both control and exposed treated fish. If the test is extended for several weeks or months, the mortality death or abnormalities should be less than 5% per month and not more than 30% over the entire period in all groups.

6 Summarizing the results

Summarize the test results in form 2-2, and attach the final report.

(4) This method follows a different procedure than the aqueous exposure method and it is limited to the feeding method. Therefore, "correction" is used instead of "standardization" to prevent misunderstanding.

Test method description

1. Definitions and units

Uptake phase is the period during which the fish is exposed to the chemical substance.

Depuration phase is a period for examining the process (half-life) where the chemical substance taken into the fish body decreases due to depuration or metabolism.

Uptake rate constant (k_1) is the numerical value defining the rate of increase in the concentration of test substance in/on test fish (or specified tissues thereof) when the fish are exposed to that chemical (k_1 is expressed in L/kg/day).

Depuration rate constant (k_2 is the numerical value defining the rate of reduction in the concentration of the test substance in the test fish (or specified tissues thereof) following the transfer of the test fish from a medium containing the test substance to a medium free of that substance (k_2 is expressed with day^{-1}).

Steady-state is reached in the plot of test substance in fish (C_f) against time when the curve becomes parallel to the time axis and three successive analyses of C_f made on samples taken at intervals of at least two days are within ± 20% of each other, and there is no significant increase of C_f in time between the first and last successive analysis. When pooled samples are analysed at least four successive analyses are required. For test substances which are taken up slowly the intervals would more appropriately be seven days.

Bioconcentration factor (BCF) refers to the concentration of test substance in/on the fish or specified tissues thereof (C_f as mg/kg) divided by the concentration of the chemical in the surrounding medium (C_w as mg/L) (BCF is expressed in L/kg).

The steady-state bioconcentration factor (BCF_{SS}) is the value obtained by dividing concentration of the test substance in the fish (C_f, mg/kg wet weight) at steady-state with the concentration of test substance in the test water (C_w, mg/L) at steady state.

The lipid-normalized steady-state bioconcentration factor (BCF_{SSL}) is normalized BCF_{SS} with 5% lipid content.

The kinetic bioconcentration factor (BCF_K) is the ratio of the uptake rate constant, k_1, to the depuration rate constant, k_2 (i.e. k_1/k_2). Essentially, this value is theoretically equal to BCF_{SS} when the uptake of chemicals into the test fish and their depuration follows the first order rate equation. However, if the chemical concentration in the test fish does not reach a steady state, or if BCF_K is adjusted for growth dilution, it may deviate from BCF_{SS}.

The growth corrected kinetic bioconcentration factor (BCF_{Kg}) is the BCF_K corrected for growth dilution of the test fish during the test period.

The lipid normalized kinetic bioconcentration factor (BCF_{KL}) is BCF_K normalized with 5% lipid content.

The lipid normalized, growth corrected kinetic bioconcentration factor BCF (BCF_{KgL}) is BC_{FK} standardized at 5% lipid content and corrected for growth dilution of test fish during the test period.

The octanol-water partition coefficient (P_{OW}) is the ratio of a chemical's solubility in n-octanol and water at equilibrium (OECD Test Guideline 107, 117, 123). It is often written as K_{OW}.

Dissolved organic carbon (DOC) is carbon derived from organic substances dissolved in test water.

Particulate organic carbon (POC) is carbon derived from suspended organic sources in the test media.

Total organic carbon (TOC) is carbon originating from all organic sources in the test media, including particulate and dissolved sources.

UVCB substances (chemical substances of unknown or variable composition, complex reaction products and biological materials) are substances having components whose composition is unknown or indefinite, complex reaction products or biological substances.

The dietary biomagnification factor (BMF) is the ratio of the concentration of a chemical substance in the predator to the concentration of the chemical in the predator's feed (or food). The BMF obtained by this test method is the concentration of chemical substances through diet. In OECD Test Guideline 305, for distinguishing from the BMF (concentration of chemical substances through water and diet) obtained in the environment, it is defined as dietary BMF.

Dietary biomagnification factor (BMF) at the end of the uptake phase is defined as the chemical substance concentration (C_{fish}, mg/kg wet weight) in the test fish at the end of the uptake phase divided by the chemical substance concentration in the test diet (C_{food}, mg/kg). In OECD Test Guideline 305, BMF estimated to have reached the steady state during the uptake phase is defined as steady-state bioconcentration factor (BMF_{SS}; indicative steady-state BMF).

The kinetic biomagnification factor (BMF_K) is the ratio of the product of assimilation efficiency (α) and food ingestion rate constant (I) to the depuration rate constant (k_2) ($I \times \alpha / k_2$).

Assimilation efficiency (α) is a measure of the relative amount of chemicals absorbed into the body from the digestive tract (α is dimensionless, but often expressed as a percentage rather than a ratio).

Food ingestion rate constant (I) is the amount of test feed (g food/g fish/day) ingested by each test fish per day with respect to the estimated average total test fish weight.

Growth dilution corrected kinetic dietary biomagnification factor (BMF_{Kg}) is the BMF_K corrected for growth dilution of the test fish during the test period.

Lipid corrected kinetic dietary biomagnification factor (BMF_{KL}) is the BMF_K divided by the lipid content correction factor (L_c).

Lipid correction factor (L_c) is the average lipid content of the test fish divided by the average lipid content of the test diet.

Lipid-corrected growth-corrected kinetic BMF (BMF_{KgL}) is BMF_{Kg} divided by lipid correction factor (L_c).

2. Water solubility of test substance

Obtain the water solubility of the test substance by referring to standard test methods such as OECD Test Guideline 105. In the report of bioconcentration test, describe the measurement results, measurement method and measurement temperature. The upper limit concentration of water solubility of the test substance to be obtained is 100 mg/L.

3. Water quality parameters test water that should be measured (Test method "2-2 Test water")

Refer to the OECD Test Guideline etc. for the upper limit concentration of each measurement item in the test water. If the concentration is difficult to achieve, confirm beforehand that the test fish can be raised with the test water to be used.

Substance
pH
Hardness
Total particulate matter
Total organic carbon
Ammonium
Nitrous acid
Alkalinity
Nonionic ammonia
Residual chlorine
Total organophosphorus insecticide
Total organic chlorine insecticide and polychlorinated biphenyl
Total organic chlorine
Aluminum
Arsenic
Chromium
Cobalt
Copper
Iron
Lead
Nickel
Zinc
Cadmium
Mercury
Silver
Calcium
Magnesium
Sodium
Potassium
Chloride ion
Sulfate ion

4. Test fish

4. 1 Fish species that can be used for test (Test method "2-3-1 Selection of fish species")

The fish species that can be used for the test, the recommended test temperature, and the total length [length from the tip of the head (rostral tip) to the tip of the tail (tail tip)] are as follows. Although carp or medaka is recommended, if other fish species are used, report the reasons of fish species selection.

Fish species	Recommended test temperature range (°C)	Recommended total length of test organism (cm)
Common carp *Cyprinus carpio* (Carp family)	20 – 25	8.0 ± 4.0
Medaka (Ricefish) *Oryzias latipes* (Medaka family)	20 – 25	4.0 ± 1.0
Zebra-fish *Danio rerio* (Carp family)	20 – 25	3.0 ± 0.5
Fat head minnow Fathead minnow *Pimephales promelas* (Carp family)	20 – 25	5.0 ± 2.0
Guppy *Poecilia reticulata* (Poeciliidae family)	20 – 25	3.0 ± 1.0
Bluegill *Lepomis macrochirus* (Sunfish family)	20 – 25	5.0 ± 2.0
Rainbow trout *Oncorhynchus mykiss* (Salmonidae)	13 – 17	8.0 ± 4.0
Three-spined stickleback *Gasterosteus aculeatus* (Gasterosteidae)	18 – 20	3.0 ± 1.0

4. 2 Test fish farming and acclimatization (Test method "2-3-2 Farming and acclimatization")

If there is a difference between the test temperature and the temperature of the farming pond during farming and acclimatization, for example, with the methods given in (1) or (2) below, it can be acclimated in the acclimatization water tank. During acclimatization, test fish with damaged gills or damaged skin or weak or diseased test fish are removed. In addition, adjust the amount of food to be given so that the lipid content does not change drastically during the test period. It is preferable that the farm pond and the acclimatization tank having flowing water.

(1) If the test temperature is higher than the temperature of the pond, keep it higher than the temperature within 5°C of the pond for 1 day, and then gradually increase the temperature within 3°C a day, and finally at the same temperature as the test temperature, breed it for 5-7 days.

(2) If the test temperature is lower than the temperature of the pond, keep it lower than the temperature within 3 of the pond for 1 day, and then gradually decreases the temperature within 2°C a day, and finally at the same temperature as the test temperature, breed it for 7-10 days.

5. Precautions to be taken when using the aqueous exposure method

5. 1 Solubilizing agents (Test method "3-1 Test water")

Minimize the use of solubilizing agents when using them to prepare stock solutions of the appropriate concentrations.

48 hours LC_{50} value (mg/L, w/v) of the solubilizing agent used in concentration tests

Solvent		Dispersant	
Methanol	16,200	HCO-10	5,300
Ethanol	12,000	HCO-20	>50,000
Acetone	11,200	HCO-40	>100,000
N,*N*-Dimethylformamide	9,800	HCO-50	>100,000
Dimethyl sulfoxide	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: Medaka Water temperature: 25°C
HCO: Polyoxyethylene hydrogenated castor oil

5. 2 Flow rate (Test method "3-3 Flow rate")

Normally, the recommended flow rate is 1-10 L/day per 1.0g of fish weight (wet weight). However, if the test substance concentration can be maintained within ±20% and the dissolved oxygen concentration exceeds 60% of the saturated oxygen concentration, it may be lowered than the recommended flow rate.

5. 3 Test concentration (Test method "3-5-2 Setting test concentration")

Uptake of the test substance into the fish body may be limited depending on the set test concentration (Concentration dependence of BCF). In such a case, it is necessary to carry out the test in at least 2 concentration levels, and in some cases, 3 or more concentration levels in order to confirm that uptake is not restricted. Set the test concentration in the 1st concentration level below 1% of acute toxicity value (LC_{50} value) of the test substance or below NOEC and keep it as low as technically possible. The concentration should be at least 10 times higher than the lower limit concentration of quantification in the test water by the analytical method. 2nd concentration level should have concentration that is 10 times lower than the 1st concentration level. However, if this is not possible due to toxicity and analytical sensitivity, a test substance (high purity, e.g. >98%) labeled with radioactive isotope may be used, or the test may be conducted at concentration ratio less than 10 times. In addition, even for test substances where the test must be performed at test concentrations in the vicinity of water solubility, it is recommended to conduct the test in at least two concentration levels so as to ensure the reliability of the concentration set.

For multi-component substances such as UVCB substances, the test concentration may be set so that the components subject to evaluation are below water solubility.

5. 4 Example of a theoretical sampling schedule for test substance where log P_{OW} = 4 (See test method "3-8 Sampling and analysis")

Sampling	Sampling schedule		Number of water samples	Number of fish samples (1)
	Minimum required frequency (days)(2)	Additional sampling (days)(2)		
Before uptake phase				
1	-1 0		1-2(3)	4(4) (3(6))
Uptake phase				
2	0.3	0.4	1-2	4
3	0.6	0.9	1-2	4
4	1.2	1.7	1-2	4
5	2.4	3.3	1-2	4
6	4.7		1-2	4-8(5) (3(6))
When providing a depuration phase				Move fish to water not containing any test substance
7	5.0	5.3	1-2	4
8	5.9	7.0	1-2(7)	4
9	9.3	11.2	1-2(7)	4
10	14.0	17.5	1-2(7)	4-8(5) (3(6))

(1) The value in parentheses is the number of samples for additional collection.

(2) When log P_{OW} is 4.0, the estimated value of k_2 is 0.652 day$^{-1.}$ Total period of the test is $3 \times t_{SS}$ = 3 × 4.6 days. That is, it is set to 14 days. For the estimation of t_{SS} (Time required for reaching the steady state), see "6. Calculation method of BCF".

(3) Sample the water after supplying the water that is at least 3 times of water tank capacity.

(4) Sampling these fish from farming groups.

(5) If high-precision curve fitting or metabolite knowledge is required, it is necessary to sample more test fish. However, at the end of the uptake phase and depuration phase, it is especially better to collect more test fish.

(6) At the start of the test, at the end of the uptake phase and at the end of the depuration phase, if the same fish as the one sampling for the test substance concentration measurement cannot be used for the lipid content measurement, sample at least 3 test fish additionally for the lipid content measurement. In that case, three fish from the control group instead of the exposed group may be used for lipid content measurement.

(7) In the test water analysis at the start of the depuration phase, if it can be confirmed that no test substance is detected, it is not necessary to measure the test substance in the test water during the subsequent depuration phase.

5. 5 Comparison of growth (Test method "3-8-4 Measurement of growth of test fish")

Calculate the growth rate constant of the fish in the exposed group and the control group, and confirm the difference in growth between the exposed group and the control group by a statistical method, for example, *t* test, or when there are multiple test concentrations, *F* test. If there is a

significant difference in growth between the exposed group and the control group, it is desirable to consider the impact on the reliability and completeness of the test.

6. Test results processing

6. 1 Prediction of the length of the uptake phase for test design (Test method "Uptake phase 3-7-1)"

By using the empirical relationship between depuration rate constant (k_2) and *n*1-octanol/water partition coefficient (P_{OW}), uptake rate constant (k_1) and BCF, it is possible to estimate k_2 before conducting the test and *t*x, the time required for the test substance concentration in the test fish to reach X% of the steady state. However, these equations can be applied only when the update and the depuration follow the 1st order rate equation. When they clearly do not follow the 1st order rate equation, these predictions are not valid. Relation between X and t_x is expressed with the following equation.

$$\frac{X}{100}=1-e^{-k_2 t_x} \quad \text{[Equation A6.1]}$$

The estimated value of $k_2(day^{-1})$ can be obtained with several methods. For example, the following empirical equation can be used:

$$\log k_2=1.47-0.414\log P_{ow} \quad (r^2=0.95) \quad \text{[Equation A6.2}^{(\text{Note 1})}\text{]}$$

or

$$k_2=\frac{k_1}{BCF} \quad \text{[Equation A6.3]}$$

In the case of a chemical substance where log P_{OW} exceeds 3, in the above equation,

$$k=520\cdot W^{-0.32} \quad (r^2=0.85) \quad \text{[Equation A6.4]}$$

and

$$BCF=10^{(0.910\cdot\log P_{ow}-1.975\cdot\log(6.8\cdot10^{-7}P_{ow}+1)-0.786} \quad (r^2=0.90) \quad \text{[Equation A6.5}^{(\text{Note 3})}\text{]}$$

W=Average fish weight at the end of uptake / start of depuration (g wet weight)

In addition, there is another method "Note 4" for estimating k_2. For example, if metabolism is considered fast, it may be better to estimate k_2 using a complex model [(Note 5, 6)]. However, the more complex is the model, the more attention must be paid to interpreting the prediction results. Therefore, as for the handling of the estimation results, carefully judge by comparing with the test substance structure and other relevant information (for example, preliminary test results).

The time required to reach a certain percentage of the steady state can be calculated by substituting the estimated value of k_2 into a general rate equation describing the uptake and depuration (first order rate equation). However, if growth during the test period is significant, it is more appropriate to use the growth dilution corrected depuration rate constant (k_{2g}) described in 6.6 "Growth dilution correction for BCF by kinetics".

$$\frac{dC_f}{dt}=k_1 C_w-k_2 C_f \quad \text{[Equation A6.6]}$$

When C_w is constant

$$C_f=\frac{k_1}{k_2}\cdot C_w(1-e^{-k_2 t}) \quad \text{[Equation A6.7]}$$

As the steady state approaches ($t \rightarrow \infty$), Equation A6.7 may be omitted as follows (Note 7、8).

$$C_f=\frac{k_1}{k_2}\cdot C_w \quad \text{[Equation A6.8]}$$

or

$$\frac{C_f}{C_w}=\frac{k_1}{k_2}=\text{BCF} \quad \text{[Equation A6.9]}$$

In other words, BCF·C_w is the approximated value of the test substance concentration in the test fish ($C_{f\text{-}SS}$) at steady state.

Equation A6.7 can be rewritten as follows.

$$C_f=C_{f\text{-}ss}\cdot\left(1-e^{-k_2 t}\right) \quad \text{[Equation A6.10]}$$

or

$$\frac{C_f}{C_{f\text{-}ss}}=1-e^{-k_2 t} \quad \text{[Equation A6.11]}$$

By using Equation A6.2 or Equation A6.3, we can estimate k_2 beforehand and insert it in Equation A6.11 for predicting t_X.

When the accumulation exceeds 95% of the steady state, BCF_{SS} can be calculated. As for the statistically optimal uptake phase for calculating BCF_K, the least required period is the period where the test substance concentration in the test fish reaches 50% ($0.69/k_2$) of the steady state(Note 9).

Time (t_{80}) where it is reaches 80% of the steady state is expressed as follows.

$$0.80=1-e^{-k_2 t_{80}} \quad \text{[Equation A6.12]}$$

or

$$t_{80}=\frac{-\ln(0.20)}{k_2}=\frac{1.6}{k_2} \quad \text{[Equation A6.13]}$$

Similarly, time (t_{95}) where it is reaches 95% of the steady state is expressed as follows.

$$t_{95}=\frac{-\ln(0.05)}{k_2}=\frac{3.0}{k_2} \quad \text{[Equation A6.14]}$$

For example, for the test substance having $\log P_{OW}=4$, t_{80} and t_{95} (by using Equation A6.2, Equation A6.13, and Equation A6.14) can be expressed as follows:

$\log k_2=1.47-0.414\cdot 4$

$k_2=0.652\text{day}^{-1}$

$t_{80}=\frac{1.6}{0.652}=2.45$ days (59 hours)

$t_{80}=\frac{3.0}{0.652}=4.60$ days (110 hours)

Instead, by using the following equation, we can calculate the time (t_{eSS}) required for reaching the steady state(Note 10).

$t_{eSS}=6.54\cdot10^{-3}\cdot P_{ow}+55.31$ (hours) [Equation A6.15]

Results for the test substance having log P_{OW} = 4 can be expressed as follows:

$t_{eSS}=6.54\cdot10^{-3}\cdot10^{4}+55.31=121$ (hours)

6. 2 Predicting the length of depuration phase for test design (Test method "Depuration phase 3-7-2")

By using a general formula describing uptake and depuration, it is possible to predict the time required for the test substance concentration in test fish to decrease to a certain percentage of the initial concentration during the depuration phase. However, these equations can be applied only when the update and the depuration follow the 1st order rate equation. When they clearly do not follow the 1st order rate equation, these predictions are not valid. (See Equation A6.6) (Note 11).

In the depuration phase, assuming that C_w is zero, it can be expressed with the following equations:

$$\frac{dC_f}{dt}=-k_2C_f$$ [Equation A6.16]

or

$$C_f=C_{f,0}\cdot e^{-k_2t}$$ [Equation A6.17]

In the above equations, $C_{f,0}$ is the test substance concentration in the test fish at the start of the depuration phase.

By using Equation A6.2 or Equation A6.3, we can estimate k_2 beforehand and insert it in Equation A6.17 for predicting time t_Y required for Y% depuration. Time (t_{50}) required for 50% depuration is expressed with the following equation:

$$\frac{C_f}{C_{f,0}}=\frac{1}{2}=e^{-k_2t_{50}}$$

or

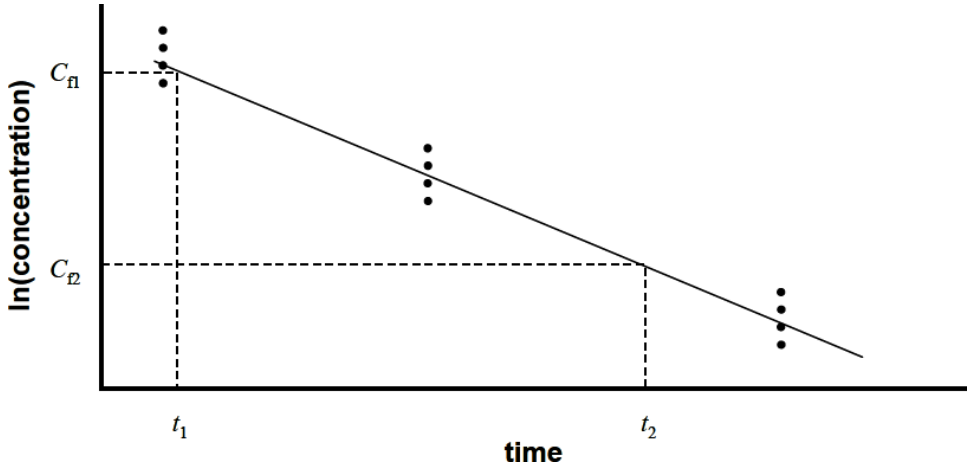
$$t_{50}=\frac{-\ln(0.50)}{k_2}=\frac{6.93}{k_2}$$

Similarly, time (t_{95}) required for 95% depuration is expressed with the following equation:

$$t_{95}=\frac{-\ln(0.05)}{k_2}=\frac{3.0}{k_2}$$

6. 3 Sequential method: Determination of depuration rate constant k_2 (Test method "Calculation of bioconcentration factor 4-1")

When the test substance concentration (natural logarithm) in the test fish during the depuration phase is plotted on a straight line with respect to the time axis (depuration follows the first order rate equation), k_2 can be explained with simple two compartment/two parameter models.



When k_2 is not plotted on a straight line, it shows the possibility that depuration has a more complex pattern than the first order rate constant. The pattern of depuration when deviating from the first order rate equation may be explained by a graphical solution.

For calculating k_2 from multiple sampling points, take ln(concentration) on the vertical axis and time on the horizontal axis and perform regression analysis. The slope of the resulting regression line is the depuration rate constant k_2. From the intercept, it is possible to calculate the average value of the test substance concentration in the test fish at the beginning of the depuration phase ($C_{0,d}$; equal to the average value of the test substance concentration in the test fish at the end of the uptake phase, but including the error range).

$$C_{0,d}=e^{\text{intercept}} \qquad \text{[Equation A6.18]}$$

When there are only two sampling points for calculating k_2, (at the beginning of the depuration phase in the simplified aqueous exposure method (i.e., at the end of the uptake phase) and at the end of the depuration phase)

$$k_2=\frac{\ln(C_{f1})-\ln(C_{f2})}{t_2-t_1} \qquad \text{[Equation A6.19]}$$

In the above equation, $\ln(C_{f1})$ and $\ln(C_{f2})$ are the natural logarithms of test substance concentration in the test fish at time t_1 and t_2 respectively, and t_2 and t_1 are times from the start of depuration of two sampling points. However, when this method is used, standard error or confidence interval of k_2 cannot be obtained.

6. 4 Sequential fitting method (Sequential method): Determination of intake rate constant k_1 (Test method ("4-1 Calculation of bioconcentration factor")

k_1 can be calculated from a series of consecutive times-concentration data in the uptake phase. In that case, use a computer program to fit the data to the following model.

$$C_f(t)=C_w(t)\cdot\frac{k_1}{k_2}\cdot(1-e^{-k_2 t}) \qquad \text{[Equation A6.20]}$$

In the above equation, k_2 is the value calculated in 6. 3 "Sequential method", and $C_f(t)$ and $C_w(t)$ are test substance concentration in the test fish and test water at time t respectively.

When there are only two sampling points for calculating k_2, (at the beginning of the depuration phase in the simplified aqueous exposure method (i.e., at the end of the uptake phase) and at the end of the depuration phase), use the following equation.

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

[Equation A6.21]

In the above equation, k_2 is the value calculated in 6.3 "Sequential method", C_f is the test substance concentration in the test fish at the start of depuration phase, and C_w is the average test

substance concentration in the test water during the uptake phase. However, when this method is used, standard error or confidence interval of k_1 cannot be obtained.

By plotting data of measured sampling point, the validity of k_1 and k_2 can be visually assessed.
If it is determined that the value of k_1 obtained by the sequential fitting method is not appropriate, calculate k_1 and k_2 by using the simultaneous method (See 6. 5 "Simultaneous method").

6. 5 Determining uptake rate constant and depuration rate constant with the Simultaneous fitting method (Simultaneous method) (Test method "4-1 Calculation of bioconcentration factor)

By using a computer program, k_1 and k_2 can be calculated from a series of continuous time-concentration data and the following model equation.

$$C_f=C_w\cdot\frac{k_1}{k_2}\cdot(1-e^{-k_2t}) \qquad 0<t<t_c$$ [Equation A6.22]

$$C_f=C_w\cdot\frac{k_1}{k_2}\cdot(e^{-k_2(t-t_c)}-e^{-k_2t}) \qquad t>t_c$$ [Equation A6.23]

In the above equation, t_c = Time at the end of the uptake phase

This method can directly calculate the standard error of k_1 and k_2. In Equation A6.22 and Equation A6.23, by replacing k_1/k_2 with BCF, it is possible to estimate the standard error of BCF and 95% confidence interval. This is particularly useful when comparing differences in BCF calculation results depending on whether logarithmic conversion of the test substance concentration in test fish is done or not.

When k_1 and k_2 are calculated by the simultaneous fitting method, there is a strong correlation between k_1 and k_2. In most cases, k_2 can be calculated with relatively high accuracy from the depuration curve. Therefore, it is recommended to calculate k_1 and k_2 by using the sequential fitting method first. When calculating k_1 and k_2 by the sequential fitting method, it is recommended to handle the same data for both (converting / not converting the test substance concentration in the test fish to natural logarithm). By plotting the data of the measured sampling points, visually assess the validity of the curve that is plotted. If it is determined that k_1 obtained by the sequential fitting method is not valid, calculate k_1 and k_2 by using the simultaneous fitting method. Assess the validity of the curve once again, and compare k_1, k_2 and BCF with the results obtained by the sequential fitting method.

If neither method is deemed valid, it is possible that they do not follow the first order rate equation and other more complex models should be used. One of the most common factors that makes it difficult to determine validity is the growth of the test fish during the test period.

6.6 Growth dilution correction for BCF by kinetics (Test method "4-2 (1) Growth dilution correction and lipid content standardization")

Apply when the update and depuration of the chemical substance follows the first order rate equation. If the first order rate equation is not followed, it is recommended to use the mass based approach.

The method for correcting dilution associated with growth may be inaccurate or it may not correct appropriately. For example, if a fast-growing fish is tested for a chemical substance that is excreted very slowly, the error in the two rate constants (k_2 and k_g) required for the depuration rate constant corrected for growth (k_{2g}) also increases, and k_{2g} may become very small. In such a case, a substitute method based on the amount of the test substance may be used. Although BCF_{SS} is also affected by growth, there is currently no suitable method for correcting BCF_{SS} for growth dilution.

<u>Growth dilution correction by growth rate constant subtraction method</u>

As a standard method, convert all individual body weight data to natural logarithm, divide into exposed group and control group, and plot ln (fish weight) or ln (1/fish weight) against time (days). This process is performed separately for the data of the uptake and depuration phases. The growth

rate constant (k_g) used for growth dilution correction is generally desirable to use body weight data for the entire study period. However, if there is a statistically significant difference between the two growth rate constants during the uptake and depuration phases, use the rate constant during the depuration phase and report.

Calculate the depuration rate constant corrected for growth (k_{2g}) by subtracting the growth rate constant (k_g) from the depuration rate constant (k_2).

$$k_{2g}=k_2-k_g \qquad \text{[Equation A6.24]}$$

Divide the uptake rate constant by the depuration rate constant corrected for growth to calculate the BCF (BCF_{Kg}) based on the kinetics corrected for growth.

$$BCF_{Kg}=\frac{k_1}{k_{2g}} \qquad \text{[Equation A6.25]}$$

Growth dilution correction based on the amount of test substance

As a substitute method of the “growth rate constant subtraction method”, the following method can be used.

- Convert the test substance concentration in the test fish during the depuration phase (that is, the amount of test substance per unit mass of the fish) into the amount of test substance in the test fish.
- Plot ln (amount of test substance) with respect to time (depuration phase), and calculate the depuration rate constant from the slope.
- However, when calculating k_1, use the method described in 6.3 "Sequential method" and 6.5 "Simultaneous fitting method", note to use the usual k_2 calculated from the test substance concentration in the test fish.

6.7 Standardization with 5% lipid content (Test method "4-2 (2) Growth dilution correction and lipid content standardization")

When BCF_{SS} is 1000 L/kg or higher, the BCF (BCF_K or BCF_{SS}) for 5% lipid content (based on wet weight) should be reported unless it is clear that the test substance is hardly accumulated in lipid. Fish concentration data or BCF should be standardized to values per 5% lipid content (based on wet weight).

Two methods are recommended for lipid content measurement: the chloroform / methanol extraction method and the Smedes method. When using other methods, check in advance that the same extent of extraction efficiency and accuracy as the recommended two types of methods can be obtained. It is preferable to measure the lipid content for the same fish as the test fish used for measuring the test substance concentration.

$$C_{f,L}=\frac{0.05}{L}\cdot C_f \qquad \text{[Equation A6.26]}$$

$C_{f,L}$ = Test substance concentration in test fish standardized with 5% lipid content (mg/kg wet weight)

L=Lipid content (based on wet weight)

C_f =Test substance concentration in test fish (mg/kg wet weight)

For all sampling points, use with regard to BCF_{SS}, use the average value at the end of the uptake phase for the exposed group unless the test substance analysis and lipid content measurement are conducted by using the same fish. Standardize BCF_K by using the mean value of lipid content. However, if the lipid content changes significantly during the uptake phase or the depuration phase, report the BCF standardized with the lipid content by using appropriate values along with this.

$$BCF_{SSL}=\frac{0.05}{L_n}\cdot BCF_{SS} \qquad \text{[Equation A6.27]}$$

[Equation A6.28]

$$BCF_{KL}=\frac{0.05}{L_n}\cdot BCF_K$$

BCF_{SSL}=BCF_{SS} standardized with 5% lipid content

BCF_{KL}=BCF_K standardized with 5% lipid content

L_n=Average lipid content (based on wet weight)

BCF_{SS}=BCF in steady state

BCF_K=BCF based on kinetics

When test substance analysis and lipid content measurement are conducted by using the same fish at all sampling points, standardize the test substance concentration in each test fish by using the lipid content of that fish.

7. II: Bioconcentration test using fish (Minimized aqueous exposure method)

As for the principle of this method, it is based on the fact that while the bioconcentration factor in the aqueous exposure method can be calculated as BCF_{SS} from the ratio of the test substance concentration in the test fish to the test substance concentration in the test water, but it can also be calculated from the ratio of the uptake rate constant k_1 and the depuration rate constant k_2 as BCF_K .

Measure the test substance concentration (C_{f1}) in the test fish at the end of the uptake phase (t_1), and then measure the test substance concentration (C_{f2}) in the test fish again at a certain time (t_2) during the depuration phase. This will make it possible to calculate the depuration rate constant (k_2) by using Equation A6.19 given in see "6. BCF calculation method".

The uptake rate constant k_1 can be calculated by using Equation A6.20 given in "6. BCF calculation method". (In this equation, C_f becomes C_{f1} and t becomes t_1) (Note 12, 13). Therefore, the bioconcentration factor (BCF_{Km}) by kinetics in minimized aqueous exposure method becomes as follows.

$$BCF_{Km}=\frac{k_1}{k_2} \qquad \text{[Equation A7.1]}$$

If possible, test substance concentration in test fish and BCF_{Km} should be corrected for growth dilution as described in "6. BCF calculation method".

The minimized BCF_{SS} required to evaluate the validity of the result of BCF_{Km} is the BCF calculated on the assumption that the steady state was reached at the end of the uptake phase, and it is different from BCF_{SS} provided for in I: Bioconcentration test using fish (aqueous exposure method).

$$\text{minimised } BCF_{ss}=\frac{C_{f\text{-}minSS}}{C_{w\text{-}minSS}} \qquad \text{[Equation A7.2]}$$

$C_{f\text{-}minSS}$=Test substance concentration in the test fish assuming that steady state was reached at the end of the uptake phase (mg/kg wet weight)

$C_{w\text{-}minSS}$=Test substance concentration in the test water assuming that steady state was reached at the end of the uptake phase (mg/L)

8. III: Bioconcentration test by using fish (Dietary exposure method)

8. 1 Examples of appropriate ingredient levels in the diet (Test method "2-3-3 Diet")

Main ingredients	Diet
Crude protein	≦55.0%
Crude fat	About 10-15%
Crude fiber	≧2.0%
Moisture	≧12%
Ash	≧8%

8. 2 Examples of additive techniques to diet (Test method "3-1 Test diet")

General points

- As for the test diet of the control, it should be prepared in exactly the same way as the test diet with the test substance added to it except that it does not contain the test substance.
- In order to check the test substance concentration in the test diet, the test substance should be extracted from the sample in an appropriate manner in triplicate, and the test substance concentration or radioactivity in the extract should be measured. It is desirable to consider the analytical sensitivity of the test substance, and analyze the minimum amount of diet. Variation between samples should be small and analysis recovery rate should be high (> 85%) (The concentration of the test substance in triplicate samples collected before the start of the uptake phase must not vary by more than ±15% from the mean value).
- At least triplicate samples should be taken and analyzed at day 0 and at the end of the uptake phase to determine the test substance concentration in the test diet during the dietary exposure method.

Preparation of liquid sample diet (example)

The test substance concentration to be added to the diet should be, for example, 500 μg test substance/g feed. Add an appropriate amount of test substance and a known amount of diet to a glass bottle or egg-plant shaped flask. The amount of diet should be sufficient during the uptake phase (consider increasing the amount of die due to the growth of the test fish). As for the mixture of diet/test substance, it should be mixed overnight (for example, by using a rotor mixer, or by rotating if using an eggplant-shaped flask). The food supplemented with the test substance should be stored under conditions (for example, refrigerated) that can maintain the stability of the test substance in the diet until the end of the test.

Preparation of diet with corn oil or fish oil (example)

Pulverize the solid test substance with a mortar etc. as necessary to make a fine powder. The liquid test substance can be added directly to corn oil or fish oil. Dissolve the test substance in a known amount (For example, 5-15 mL) of corn oil or fish oil. Transfer the oil to which the test substance added to it into an appropriately sized eggplant-shaped flask. Wash the container used to prepare this oil twice with a small amount of oil and add it to the eggplant-shaped flask to ensure that the dissolved test substance is transferred. For ensuring complete dissolution/ dispersion in the oil (or when multiple test substances are used in the test), add a micro stirrer bar, cap the flask, and stir the mixture overnight at high speed. Add the appropriate amount of feed to the test and mix the contents of the eggplant-shaped flask for at least 30 minutes, preferably overnight by continuous rotation. The feed to which the test substance has been added should be stored under conditions (for example, refrigeration) that allow stable storage of the test substance in the feed until use.

Preparation of diet with organic solvent (example)

Dissolve an appropriate amount of the test substance for administration in an appropriate amount of an organic solvent (for example, cyclohexane or acetone, but higher amount if necessary depending on the amount of diet added). Mix a fixed amount or all of this solution with food suitable for the dose concentration required for the test. The diet/ test substance mixture can be mixed in a stainless steel mixing container. For the diet added in the container, leave it in the draft of the test laboratory, sufficiently evaporate the solvent, or mix it by continuous rotation in the eggplant-shaped flask. Excess solvent can be removed by air or nitrogen purge as required. Care must be taken that the test substance does not crystallize when the solvent is removed. The diet to which the test substance has been added should be stored under conditions (for example, refrigeration) that allow stable storage of the test substance in the feed until use.

8.3 Example of a theoretical sampling schedule following a 10-day uptake phase and a 42-day depuration phase (Test method "3-9 Analysis")

Sampling	Sampling schedule		Number of diet samples	Number of fish samples	
	Day of period	Additional fish samples?		Exposed group	Control group
Uptake phase					
1	0	Possible(1)(2)	3 – Exposed group 3 – Control group(1)	0	5 – 10 (8 – 13)(2)
1A(3)	1-3			5 – 10	5 – 10
2	10	Yes(4)	3 – Exposed group 3 – Control group(1)	10 – 15(4) (13 – 18)(5)	5 – 10 (8 – 13)(5)
Depuration phase					
3	1	Yes(4)		10 – 15(4)	5 – 10
4	2			5 – 10	5 – 10
5	4			5 – 10	5 – 10
6	7	Yes(4)		10 – 15(4)	5 – 10
7	14			5 – 10	5 – 10
8	28			5 – 10	5 – 10
9	42	Yes(4)		10 – 15(4) (13 – 18)(5)	5 – 10 (8 – 13)(5)
Total				59 – 120 (63 – 126)(4, 5)	50 – 110 (56 – 116)(4, 5)

(1) Analyze three samples from the control group and the exposed group for test substance concentration and lipid content.
(2) Sample from the acclimatization tank as soon as possible at the start of uptake; and sample at least 3 fish for lipid content measurement at the start of uptake.
(3) Collecting at the beginning of the uptake phase will allow the calculation of the in vivo absorption rate, which can be compared with the in vivo absorption rate of the test substance calculated from the data of the depuration phase (optional).
(4) Five additional fish can be collected for tissue analysis.
(5) At the start of the uptake phase, at the end of the uptake phase and at the end of the depuration phase, if the same fish as the one sampled for the test substance concentration measurement cannot be used, sample at least 3 test fish additionally for the lipid content measurement. In many cases, three fish from the control area can only be used to measure the lipid content.

Points to keep in mind during the period and sampling: The uptake phase begins when at the time of first feeding of the test diet containing the test substance. Each experiment day is from one feeding until just before the next feeding 24 hours later. The first sampling (1 in the table above) should be done immediately before the first feeding (e.g. 1 hour before). Sampling during the test should ideally be performed immediately before feeding on the next day (for example, sampling of the test fish after 10 days of uptake is after about 23 hours of feeding after 10 days of uptake).

The uptake phase ends immediately before given the food without any test substance added to it, and the depuration phase starts from that point (for fish in the exposed group, until 24 hours after giving the last food with the test substance added to it, it is highly likely that the test fish has digested the test diet). For this reason, sampling at the end of uptake should be carried out immediately before

giving the first diet without the test substance added to it, and the first sampling of the depuration phase should be done about 23 hours after giving the first without the test substance added to it.

8. 4 Calculation of assimilation efficiency and dietary biomagnification factor (Test method "4-1 Calculation of dietary biomagnification factor")

In order to calculate the in vivo absorption rate, first estimate the depuration rate constant by using the average test substance concentration in the test fish during the depuration phase according to Test Method Description 6.3 (estimate by using the "Sequential method", that is, standard linear regression). Amount of feed I and uptake phase t are the known parameters of the test. The average concentration of the test substance in the test food C_{food} is the measurement variable in the test. The extrapolated value $C_{0,d}$ of the test substance concentration in the test fish at the start of the depuration phase is usually calculated from the intercept of the plot of ln (concentration) and the number of days of depuration.

The in vivo absorption rate of the test substance (α, absorption of the test substance from the digestive tract) is calculated as follows.

$$\alpha=\frac{C_{0,d}\cdot k_2}{I\cdot C_{food}}\cdot\frac{1}{1-e^{-k_2 t}} \qquad \text{[Equation A8.1]}$$

$C_{0,d}$ = The extrapolated value of test substance concentration in test fish at the start of depuration phase (mg/kg)

k_2 = Depuration rate constant (not corrected for growth dilution) calculated according to the equation given in the Test Method Description 6.3 (/day)

I = Total feeding amount (g Feed/g Fish/day)

C_{food} = Test substance concentration in test feed (mg/kg feed)

t = Uptake phase (days)

However, the amount of feed I used for accurately determining the assimilation efficiency α may need to be corrected for the growth of the test fish. In tests where the test fish grows significantly during the uptake phase, the appropriate amount of feed during the uptake phase will be lower than the amount of feed set, and as a result, a value higher than the "actual" assimilation efficiency is obtained (Note: I is effectively set off between Equation A8.1 and Equation A8.4. Therefore, this is not important for the calculation of the BMF itself). The average amount of feed I_g corrected for dilution with growth can be calculated in several ways. However, a direct and rigorous method is to use the growth rate constant (k_g) and estimate the weight of the test fish at each sampling during the uptake phase. In other words;

$$W_{f(t)}=W_{f,0}\times e^{k_g t} \qquad \text{[Equation A8.2]}$$

$W_{f(t)}$ = Average test fish weight on the uptake date t

W_{f0} = Average test fish weight at the start of the test

In this way, the average body weight ($W_{f,end\text{-}of\text{-}uptake}$) of the test fish on the last day of the uptake phase can be estimated. Since the amount of feed is set based on W_{f0}, the appropriate amount of feed on each experiment day in the uptake phase can be calculated by using these two body weights. If rapid growth is observed during the uptake phase, instead of I, calculate the amount of feed I_g (g feed/g fish/day) corrected for growth dilution as follows.

$$I_g = \frac{I \times W_{f0}}{W_{f\,end\text{-}of\text{-}uptake}} \qquad \text{[Equation A8.3]}$$

Once the assimilation efficiency is obtained, BMF_K can be calculated by multiplying it with the amount of feed I (or when used to calculate I_g, α) and dividing by the total depuration rate constant k_2.

$$BMF_K = \frac{I \times \alpha}{k_2} \qquad \text{[Equation A8.4]}$$

Growth dilution corrected dietary biomagnification factor should be calculated in the same way by using growth dilution corrected depuration rate constant (calculated in accordance with Test Method Description 6.6). *If* I_g is used to calculate α, here also I_g must be used instead of I.

$$BMF_{Kg} = \frac{I \times \alpha}{k_{2g}} \qquad \text{[Equation A8.5]}$$

α = assimilation efficiency (absorption of test substance from the digestive tract)

k_2 = Depuration rate constant (not corrected for growth dilution) calculated according to the equation given in the Test Method Description 6.3 (/day)

k_{2g} = Depuration rate constant corrected for growth dilution(/day)

I = Total feeding amount (g Feed/g Fish/day)

Depuration half-life ($t_{1/2}$) corrected for growth dilution is calculated as follows.

$$t_{1/2} = \frac{0.693}{k_{2g}} \qquad \text{[Equation A8.6]}$$

When the test substance concentration in the tissue is measured during the uptake phase in which the test substance is taken up linearly, the assimilation efficiency (α) the test substance can be estimated as follows.

$$\alpha = \frac{C_{fish}(t)}{I \times C_{food} \times t} \qquad \text{[Equation A8.7]}$$

$C_{fish}(t)$ =Test substance concentration in test fish at time t (mg/kg wet weight)

8.5 Lipid content correction (Test Method "4-2 Growth dilution and lipid content correction")

If lipid content has been measured for the same test fish that was analyzed for the test substance in all samplings, the individual test substance concentration should be corrected with the lipid content, and plotting ln (lipid content corrected concentration) with respect to the depuration phase (days) would give $C_{0,d}$ and k_2. By using C_{food} per lipid content, the in vivo absorption rate per lipid content (Equation A8.1) can be calculated (That is, C_{food} multiplied by the average lipid content of the feed). By calculating using Equation A8.4 and Equation A8.5, the BMF corrected for lipid content (growth dilution correction) can be calculated directly.

Alternatively, calculate the average lipid content (w/w) of the exposed group and the control group in the test fish and the test feed (With regard to the test feed and the test fish in the control group, usually measure at the beginning and end of uptake. For the test fish in the exposed group,

usually calculate only from the measurement data at the end of uptake). Depending on the test, the lipid content of the test fish may increase significantly. In such cases, it is more appropriate to use the average value of the lipid content of the test fish calculated from the measured lipid content at the end of uptake and at the end of depuration. In general, the lipid content of each test should be calculated by using only data of the exposed group.

Lipid content correction factor (L_c) is calculated as follows.

$$L_c = \frac{L_{fish}}{L_{food}} \qquad \text{[Equation A8.8]}$$

L_{fish} and L_{food} are the average lipid content in the test fish and the test food respectively.

The lipid content correction factor is used for calculating the oral biomagnification factor (BMF_L) corrected for lipid content.

$$BMF_L = \frac{BMF}{L_c} \qquad \text{[Equation A8.9]}$$

8.6 Evaluation of the difference between the measured value ($C_{0,m}$) of the test substance concentration in the test fish at the end of the uptake phase and the extrapolated value ($C_{0,d}$) of the test substance concentration in the test fish at the start of the depuration phase

Compare the measured value ($C_{0,m}$) of the test substance concentration in the test fish at the end of the uptake phase and the extrapolated value ($C_{0,d}$) of the test substance concentration in the test fish at the start of the depuration phase If they are very close, it corroborates the first order rate model used to calculate the depuration parameters.

Depending on the test, there may be a significant difference between the extrapolated value ($C_{0\,d}$) and the actually measured value ($C_{0,m}$). If C0 d is significantly lower than $C_{0,m}$ ($C_{0,d} << C_{0,m}$), this difference may suggest that there is undigested test food in the digestive tract. This can be confirmed experimentally by dissecting the digestive tract and conducting site-specific tests if additional (total test fish) samples are preserved at the end of the uptake phase. Or, when applied to linear regression of depuration duration, if the first sampling of depuration is shown to be inappropriate by a statistically valid rejection test, when performing linear regression to calculate k_2, it is appropriate to exclude the first sampling of depuration. In such a case, if the uncertainty in the linear regression is greatly reduced and if it is clear that the first order rate was observed during the depuration phase, it is appropriate to use values of $C_{0,d}$ and k_2 to calculate the in vivo absorption efficiency. In this case, the written report must fully explain the reasons. During the depuration phase, the test substance depuration may not follow the first order rate.

If $C_{0,d}$ is much higher than the measured value ($C_{0,d} >> C_{0,m}$), it means that the test substance was depurated very rapidly (i.e. in the very early sampling of the depuration phase, it got closer to the lower limit of quantification of the analysis method, See Test method explanation 8.7), the depuration phase deviated from first order rate, linear regression to calculate k_2 and $C_{0,d}$ is inadequate, or in some samplings, there were problems with the measured test substance concentration. In such cases, check if the linear regression plot has any evidence that the measured sample is at or near the lower limit of quantification, or that there is a rejection and clear curvature (which suggests that it doesn't follow the first order rate), and explain it in a test report. In addition, describe and explain about reevaluation of linear regression for improving the estimated values.

8.7 Guidance about the test substance depurated very rapidly

Some test substances cannot be measured at the very early stage of the depuration phase (that is, after sampling on the second day of depuration), and it may not be possible to calculate a reliable concentration $C_{0,d}$ at time 0 and k_2. This situation was also found in the ring tests performed by using benzo[*a*] pyrene and it is documented in a validation report. In such a case, even if linear

regression is conducted, the reliability will be low, $C_{0,d}$ becomes an unrealistically high predicted value, and the in vivo absorption efficiency becomes much higher than 1. In such a case, it is possible to calculate a higher estimate of k_2 and a higher estimate of BMF ("upper bound" BMF).

By using data points where the test substance concentration for the depuration phase, including the first "undetected" concentration (concentration set as the lower limit of quantification), could be measured, and by preparing a linear regression (using natural logarithmically converted concentration over time), the estimated value of k_2 can be obtained. In some cases, only two data points (for example, depuration sampling date, day 1 and day 2) can be calculated. In this case, k_2 can be estimated by using equation A6.19 given in test method explanation 6.3. If it is clearly estimated that $C_{0,d}$ is much larger than the value obtained by the test, this estimated value of k_2 follows Equation A8.1, and the value of $C_{0,d}$ in the equation is replaced with concentration at time 0 with the actual measurement ($C_{0,m}$), which can be used for estimation of in vivo absorption efficiency. If the measurement of $C_{0,m}$ is not possible, the lower detection limit in test fish tissue should be used. If the value of α obtained in this manner exceeds 1, as the worst case, assume that in vivo absorption efficiency is 1.

BMF estimated higher can be estimated by using Equation A8.4, and it should be described as "(<<) value". For example, if the depuration half-life is less than 3 days when the amount of food is 3% of test fish weight, assuming that α=1 as the "worst case", the BMF_K will be less than about 0.13. Given that the purpose of the estimation and the fact that this value is conservative in the first place, there is no need to compensate for dilution with growth or lipid content of the test fish or food.

8.8 Examples of reference substances (Test Method "3-6 Spiked diet concentration")

Examples of reference substances used in the dietary exposure method include the following substances: (Note 14, 15, 16).

Test substance	Water solubility*1	log K_{ow}*2	BCF	BMF at the end of uptake phase	BMF_{Kg}	BMF_{KgL}
Decahydro naphthalene (CAS: 91-17-8)	0.889 mg/L (literature value, 25℃)	4.20 (estimated value)	1800 (*cis*) 1900 (*trans*) (average value, 2nd concentration level)	0.0893 (*cis*) 0.109 (*trans*) (after 10 days)	0.112 (*cis*) 0.137 (*trans*)	0.301 (*cis*) 0.369 (*trans*)
Ethylcyclohexane (CAS: 1678- 91-7)	6.3 mg/L (literature value, 20℃)	4.56 (measured value)	2100 (average value after 56 days, 2nd concentration level)	0.0162 (after 10 days)	0.0172	0.0512
2,4-Dichloropheny-4′-nitrophenyl ether (CAS: 1836-75-5)	1 mg/L (literature value, 22℃)	4.64 (literature value)	3400 (average value after 70 days, 2nd concentration level)	0.0517 (after 10 days)	0.0622	0.179
o-Terphenyl (CAS: 84-15-1)	1.24 mg/L (literature value, 25℃)	5.52 (estimated value)	1400 (BCFss, 2nd concentration level)	0.0343 (after 13 days)	0.0370	0.0912
Methoxychlor (CAS: 72-43-5)	0.1 mg/L (literature value, 25℃)	5.08 (literature value)	620 (BCFss, 2nd concentration level)	0.0128 (after 13 days)	0.0138	0.0340
N,N ′-Di-2-naphthyl-*p*-phenylenediamine (CAS: 93-46-9)	0.001446 mg/L (estimated value)	6.39 (estimated value)	1100 (BCFss, 2nd concentration level)	0.0206 (after 10 days)	0.0252	0.0802

*1 Calculated from Wskowwin v. 1.42 (US Environmental Protection Agency, USA). When there is a literature value available, the same is indicated, and when there is no literature value, an estimated value is indicated.

*2 Calculated from Kowwin v. 1.68 (US Environmental Protection Agency, USA). When there is a literature value available, the same is indicated, and when there is no literature value, an estimated value is indicated

(Note 1) Spacie A. and Hamelink J.L. (1982). Alternative models for describing the bioconcentration of organics in fish. Environ. Toxicol. Chem. 1: 309-320.

(Note 2) Sijm D.T.H.M., Verberne M.E., de Jonge W.J., Pärt P. and Opperhuizen A. (1995). Allometry in the uptake of hydrophobic chemicals determined in vivo and in isolated perfused gills. Toxicol. Appl. Pharmacol. 131: 130-135.

(Note 3) Bintein S., Devillers J. and Karcher W. (1993). Nonlinear dependence of fish bioconcentration on n-octanol/water partition coefficient. SAR QSAR Environ. Res. 1: 29-39.

(Note 4) Kristensen P. (1991). Bioconcentration in fish: comparison of BCF's derived from OECD and ASTM testing methods; influence of particulate matter to the bioavailability of chemicals. Danish Water Quality Institute, Hørsholm, Denmark.

(Note 5) Arnot J.A., Meylan W., Tunkel J., Howard P.H., Mackay D., Bonnell M. and Boethling R.S. (2009). A quantitative structure-activity relationship for predicting metabolic biotransformation rates for organic chemicals in fish. Environ. Toxicol. Chem. 28: 1168-1177.

(Note 6) OECD (2011). QSAR Toolbox 2.1. February 2011. Available from: http://www.oecd.org/document/54/0,3746,en_2649_34379_42923638_1_1_1_1,00.html.

(Note 7) Branson D.R., Blau G.E., Alexander H.C. and Neely W.B. (1975). Bioconcentration of 2,2',4,4' tetrachlorobiphenyl in rainbow trout as measured by an accelerated test. T. Am. Fish. Soc. 104: 785-792.

(Note 8) Ernst W. (1985). Accumulation in aquatic organisms, in Appraisal of tests to predict the environmental behaviour of chemicals, Sheeman, P., et al., Editors. John Wiley & Sons Ltd, New York, NY, USA: 243-255.

(Note 9) Reilly P.M., Bajramovic R., Blau G.E., Branson D.R. and Sauerhoff M.W. (1977). Guidelines for the optimal design of experiments to estimate parameters in first order kinetic models. Can. J. Chem. Eng. 55: 614-622.

(Note 10) Hawker D.W. and Connell D.W. (1988). Influence of partition coefficient of lipophilic compounds on bioconcentration kinetics with fish. Wat. Res. 22: 701-707.

(Note 11) Konemann H. and van Leeuwen K. (1980). Toxicokinetics in fish: Accumulation and elimination of six chlorobenzenes by guppies. Chemosphere. 9: 3-19.

(Note 12) Springer T.A., Guiney P.D., Krueger H.O. and Jaber M.J. (2008). Assessment of an approach to estimating aquatic bioconcentration factors using reduced sampling. Environ. Toxicol. Chem. 27: 2271-2280.

(Note 13) Hashizume N., Inoue Y., Murakami H., Ozaki H., Tanabe A., Suzuki Y., Yoshida T., Kikushima E. and Tsuji T. (2013). Resampling the bioconcentration factors data from Japan's chemical substances control law database to simulate and evaluate the bioconcentration factors derived from minimized aqueous exposure tests. Environ. Toxicol. Chem. 32: 406-409.

(Note 14) Inoue Y., Hashizume N., Kikushima E., Otsuka M. (2011). Comparison of nitrofen uptake via water and food and its distribution in tissue of common carp, *Cyprinus carpio* L. Bull. Environ. Contam. Toxicol. 87 (3): 287.

(Note 15) Inoue Y., Hashizume N., Yoshida T., Murakami H., Suzuki Y., Koga Y., Takeshige R., Kikushima E., Yakata N., Otsuka M. (2012). Comparison of the Bioconcentration and Biomagnification Factors for Poorly-Water-Soluble Chemicals using Common Carp (*Cyprinus carpio* L.). Arch. Environ. Contam. Toxicol. 63 (2):241-248.

(Note 16) Hashizume N., Tanabe A., Inoue Y., Sawada T., Murakami H., Suzuki Y., Sumi S., Tsubokura Y., Yoshida T., Ajimi S., Tsuji T., Furukawa K. (2014). Prediction of the bioconcentration factor in common carp (*Cyprinus carpio* L.) using data from the dietary exposure bioaccumulation fish test. Environ. Toxicol. Chem. 33 (6): 1406-1414.

[Form 2-1]

Bioconcentration test report (Aqueous exposure method, Minimized aqueous exposure method)

1. General matters

Name of the new chemical substances (In accordance with IUPAC nomenclature)			
Synonym			
CAS Number			
Structural formula or rational formula (When both are not known, outline of the production method)			
Molecular weight			
Purity of the new chemical substance used in the test (%)			
Lot number of the new chemical substance used in the test			
Name and percentage content of purity			
Vapor pressure			
Solubility in water or Solubility in test water	(Measurement method:)		
1-octanol/water partition coefficient			
Melting point			
Boiling point			
Properties at normal temperature			
Stability			
Solubility in solvent, etc.	Solvent	Solubility	Stability in solvent

[Remarks]

1. Write down the physical and chemical properties to the extent possible.
2. In the "Vapor pressure" column, write down the vapor pressure of the test substance.
3. In the "Stability" column, write down stability with respect to temperature, light, etc.
4. In the "Solubility etc. in solvent" column, write down the solubility of the test substance in the solvent and its stability in the solvent.
5. In the "Solubility in water or solubility in test water" column, write down the measurement method. In addition, when applying the aqueous exposure method in one concentration level, attach the test water solubility written report, and when applying the minimized aqueous exposure method, attach the water solubility written report.

2. Acute toxicity test

Test fish (academic name)		
LC_{50} or NOEC	LC_{50} (　　hr)　/　NOEC	
Use of auxiliary substance	Yes　/　No	
Name and its concentration if any auxiliary agent is used	Name	Concentration (mg/L)

[Remarks]
1. Circle either LC_{50} or NOEC, and write down its value.

3. Test method

Test method			
Test fish (academic name)			
Lipid content (%)	At the start of uptake phase: At the end of uptake phase: At the end of depuration phase (if conducted):		
Fish body weight (g)	At the start of uptake phase: At the end of uptake phase: At the end of depuration phase (if conducted):		
Set concentration of test substance (mg/L)	1st concentration level		
	2nd concentration level		
Use of auxiliary agent		Yes　/　No	
Name and its concentration if any auxiliary agent is used		Name	Concentration (μg/L)
			1st concentration level:
			2nd concentration level:
			1st concentration level:
			2nd concentration level:

[Remarks]
1. In the "Test method" column, fill in the type of test used (aqueous exposure method, minimized aqueous exposure method).
2. When applying the aqueous exposure method and minimized aqueous exposure method in one concentration level, draw a diagonal line in the 2nd concentration level.

4. Test results

(1) Bioconcentration test results table

	Uptake phase	(day)	(day)	(day)	(day)	(day)
1st concentration level	Test substance concentration in water (unit)					
	Test substance concentration in fish (unit)					
	BCF					
2nd concentration level	Test substance concentration in water (unit)					
	Test substance concentration in fish (unit)					
	BCF					

[Remarks]

When applying the aqueous exposure method and minimized aqueous exposure method in one concentration level, draw a diagonal line in the column that does not apply.

(2) BCF in steady state or kinetics

		Bioconcentration factor	
		1st concentration level	2nd concentration level
Aqueous exposure method	BCF_{SS}・BCF		
	BCF_{SSL}		
	BCF_{K}		
	BCF_{Kg}		
	BCF_{KL}		
	BCF_{KgL}		
Minimized aqueous exposure method	minimised BCF_{SS}		
	BCF_{Km}		
Checking the steady state		Individual analysis or collective analysis	Individual analysis or collective analysis

[Remarks]

1. As the method of checking the steady state, circle either individual analysis or collective analysis that is used for fish analysis.
2. In addition to BCF_{SS}・BCF, fill in the values of BCF_{SSL}, BCF_{K}, BCF_{Kg}, BCF_{KL}, BCF_{KgL} when available.
3. Based on the applied test method, draw a diagonal line in the column that does not apply.

(3) BCF and half-life by each tissue

Tissue	1st concentration level	2nd concentration level
Head		
Internal organs		
Outerskin		
Edible part		

	1st concentration level	2nd concentration level
Half-life in depuration test (days)		

[Remarks]

If the BCF is 1,000 times or more and less than 5,000 times, in addition to (2) Steady state or kinetic BCF, measure sure to fill in the BCF by tissue and the half-life in the depuration test.

5. Test water and fish body analysis method

(1) Test water and fish body analysis flow (briefly describe the procedure and steps.)

(2) Type of the analyzer used and its condition

6. Recovery rate (average values)

Recovery rate from water	(%)	
Recovery rate from fish body	(%)	

7. Considerations

* To the extent possible, write down the considerations of the test results (accumulative property of the test substance).

8. Others

Facility where the test was administered	Name	
	Location	TEL FAX
Study director	Title and name	
	Years of experience	
Test number		
Test period	From YYYY/MM/DD to YYYY/MM/DD	

[Remarks]

1. Fill in this form by copying from the final written report.
2. Fill in the same test number as the final written report.
3. The person responsible for preparing this form shall write down his department and name outside the column of this form.

[Form 2-2]

Bioconcentration test results written report (Dietary exposure method)

1. General matters

Name of the new chemical substances (In accordance with IUPAC nomenclature)			
Synonym			
CAS Number			
Structural formula or rational formula (When both are not known, outline of the production method)			
Molecular weight			
Purity of the new chemical substance used in the test (%)			
Lot number of the new chemical substance used in the test			
Name and percentage content of purity			
Vapor pressure			
Solubility in water	(Measurement method:)		
1-octanol/water partition coefficient			
Melting point			
Boiling point			
Properties at normal temperature			
Stability			
Solubility in solvent, etc.	Solvent	Solubility	Stability in solvent

[Remarks]

1. Write down the physical and chemical properties to the extent possible.
2. In the "Vapor pressure" column, write down the vapor pressure of the test substance.
3. In the "Stability" column, write down stability with respect to temperature, light, etc.
4. In the "Solubility etc. in solvent" column, write down the solubility of the test substance in the solvent and its stability in the solvent.
5. Note down the measurement method in the “Water solubility” column.

2. Test method

Test Method		
Test fish (academic name)		
Lipid content of test fish (%)	At the start of uptake phase: At the end of uptake phase: At the end of depuration phase:	
Feed		
Set concentration of test substance		
Lipid content of feed (%)	At the start of uptake phase: At the end of uptake phase:	
Name of the organic solvent or oil used and its concentration	Name	Concentration (unit)
Feed amount (g Feed/g Fish/day)		
Reference substance		

3. Test results

(1) Bioconcentration test measurement results

		At the start of uptake phase	At the end of uptake phase	Depuration phase					
		(day)	(day)	(day)	(day)	(day)	(day)	(day)	(day)
Test substance	Concentration in test feed (mg/kg)								
	Concentration in fish (mg/kg)								
	Fish weight (g)								
Reference substance (Test group)	Concentration in test feed (mg/kg)								
	Concentration in fish (mg/kg)								
	Fish weight (g)								
Reference substance (Comparison group)	Concentration in test feed (mg/kg)								
	Concentration in fish (mg/kg)								
	Fish weight (g)								

[Remarks]
1. Draw diagonal lines in the columns that do not apply.

(2) Dietary biomagnification factor

	Test substance	Reference substance (test group)	Reference substance (comparison group)
BMF			
BMF_L			
BMF_K			
BMF_{Kg}			
BMF_{KL}			
BMF_{KgL}			

[Remarks]
1. Draw diagonal lines in the columns that do not apply.

(3) BMF of test by each tissue

Tissue	Test substance	Reference substance
Head		
Internal organs		
Outer skin		
Digestive tract		
Edible parts		

[Remarks]
1. Draw diagonal lines in the columns that do not apply.

4. Feed and fish body analysis method
(1) Feed and fish body analysis flow (briefly describe the procedure and steps.)

(2) Type of the analyzer used and its condition

5. Recovery rate (average values)

Recovery rate from feed	(%)	
Recovery rate from fish body	(%)	

6. Considerations

* To the extent possible, write down the considerations of the test results (accumulative property of the test

substance).

7. Others

Facility where the test was administered	Name	
	Location	TEL FAX
Study director	Title and name	
	Years of experience	
Test number		
Test period	From YYYY/MM/DD to YYYY/MM/DD	

[Remarks]

1. Fill in this form by copying from the final written report.
2. Fill in the same test number as the final written report.
3. The person responsible for preparing this form shall write down his department and name outside the column of this form.