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**Bioconcentration :
Flow-through fish test**

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Foreword

This translation has been made based on the original Japanese Industrial Standard established by the Minister of International Trade and Industry through deliberations at the Japanese Industrial Standards Committee in accordance with the Industrial Standardization Law.

To conform to the Overseas Standard, this Standard has been prepared based on OECD GUIDELINES FOR TESTING OF CHEMICALS PROPOSAL FOR UPDATING GUIDELINE 305, *Bioconcentration : Flow-through Fish Test* adopted on June 14, 1996.

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Bioconcentration : Flow-through fish test

Introduction This Japanese Industrial Standard has been prepared based on OECD Guidelines for testing of chemicals No. 305, *Bioconcentration : Flow-through fish test* adopted on June 14, 1996 without modifying the technical contents.

Information : OECD Guideline 305 adopted on June 14, 1996 consolidates and replaces the previous Guidelines 305A to E (1). The modified version of Guideline 305, as a result of an EEC "ring-test" (2), forms the basis of the consolidated Guideline.

1 Scope This Standard describes a procedure for characterizing the bioconcentration potential of substance, especially in the body of fish. The method gives sufficient details for performing the test while allowing adequate freedom for adapting the experimental design to the conditions in particular laboratories and for varying characteristics of test substances.

Although flow-through test regimes are much to be preferred, semi-static regimes are permissible, provided that the validity criteria are satisfied.

In addition, the portions underlined with dots are the matters not including the original OECD Guideline.

Information : The experiment design may be changed according to the conditions in particular laboratories, characteristics of the test substance, and the purposes of performing the test. The following items, for example, may be related to the change in the experiment design. Annex 2 (informative) shows the details at each item below.

- 1) Confirmation of a plateau or steady-state bioconcentration [3. 4])
- 2) Applicable compounds (4.2)
- 3) Radiolabelled test substance (4.2, 9.4.4)
- 4) Depuration test (4.2, 9.2.2 and 9.4.1)
- 5) Limit of bioconcentration in the quality of dilution water (8.2)
- 6) Test fish (8.4)
- 7) Analysis number of fish samples (9.2.3, 9.4.4)
- 8) Feeding (9.2.5)
- 9) Test concentration (5, 9.2.7)
- 10) Fish and water sampling schedule (9.4.1)
- 11) Lipid content (4.2, 9.4.4)

2 Normative references The following standards contain provisions which, through reference in this Standard, constitute provisions of this Standard. The most recent editions of the standards indicated below shall apply.

OECD GUIDELINES FOR TESTING OF CHEMICALS, No. 104 "Vapour Pressure" (Updated Guideline, Adopted by the Council on July 27, 1995).

OECD GUIDELINES FOR TESTING OF CHEMICALS, No. 105 "Water Solubility" (Updated Guideline, Adopted by the Council on 27 July, 1995).

OECD GUIDELINES FOR TESTING OF CHEMICALS, No. 107 "Partition Coefficient (n-Octanol/Water)" : Shake Flask Method (Updated Guideline, Adopted by the Council on 27 July, 1995).

OECD GUIDELINES FOR TESTING OF CHEMICALS, No. 111 "Hydrolysis as a Function of pH" (Original Guideline, Adopted by the Council on 12 May, 1981).

OECD GUIDELINES FOR TESTING OF CHEMICALS, No. 115 "Surface Tension of Aqueous Solutions" (Updated Guideline, Adopted by the Council on 27 July, 1995).

OECD GUIDELINES FOR TESTING OF CHEMICALS, No. 117 "Partition Coefficient (n-Octanol/Water), HPLC Method" (Original Guideline, Adopted by the Council on 30 March, 1989).

OECD GUIDELINES FOR TESTING OF CHEMICALS, No. 301A-301F "Ready Biodegradability" (Updated Guideline, Adopted by the Council on 17 July, 1992).

3 Definitions and symbols For the purpose of this Standard, the following definitions and symbols shall apply, and others shall be in accordance with the general terms used in Japanese Industrial Standards.

- 1) **Bioconcentration/bioaccumulation** is the increase in concentration of the test substance in or on an organism (specified tissues thereof) relative to the concentration of test substance in the surrounding medium.
- 2) The **bioconcentration factor** (BCF, or K_B) at any time during the uptake phase of this accumulation test is the concentration of test substance in/on the fish or specified tissues thereof [C_f as mg/g (ppm)] divided by the concentration of the chemical in the surrounding medium [C_w as mg/ml (ppm)].
- 3) The **steady state** is the condition which does not change significantly over a prolonged period of time, the concentration of the test substance in the surrounding medium being constant during this period of time. The factor of the concentration under the condition denotes the **steady state bioconcentration factor** (BCF_{ss} or K_B).
- 4) A **plateau** or **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 $\pm 20\%$ of each other, and there are no significant differences among the three sampling periods. 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.

The above-mentioned is important, especially in the determination of BCF_{ss} .

- 5) **Kinetic concentration factor, BCF_k** calculated directly from kinetic rate constants (k_1/k_2 —see below) are termed kinetic concentration factor, BCF_k .
- 6) The **octanol-water partition coefficient** (P_{ow}) is the ratio of a chemical's solubility in n-octanol and water at equilibrium (OECD Guidelines 107 and 117); also expressed as K_{ow} . The logarithm of P_{ow} is used as an indication of a chemical's potential for bioconcentration by aquatic organisms.

- 7) The **exposure** or **uptake phase** is the time during which the fish are exposed to the test chemical.
- 8) The **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 day⁻¹).
- 9) The **post-exposure** or **depuration (loss) phase** is the time, following the transfer of the test fish from a medium containing test substance to a medium free of that substance, during which the depuration (or the net loss) of the substance from the test fish (or specified tissue thereof) is studied.
- 10) The **depuration (loss) 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 in day⁻¹).

4 Principle of the test

4.1 Preparation of the test Before carrying out a test for bioconcentration, the following information about the test substance should be known:

- a) solubility in water [OECD Guideline 105];
- b) octanol-water partition coefficient, P_{ow} [OECD Guidelines 107, 117];
- c) hydrolysis [OECD Guideline 111];
- d) phototransformation in water determined under solar or simulated solar irradiation and under the irradiation conditions of the test for bioconcentration [OECD Guidance Document on Direct Phototransformation of Chemicals in Water] (3);
- e) surface tension (i.e. for substances where the log P_{ow} cannot be determined) [OECD Guideline 115];
- f) vapour pressure [OECD Guideline 104];
- g) ready biodegradability (where appropriate) [OECD Guidelines 301A to F].

4.2 Summary It is most validly applied to stable organic chemicals with log P_{ow} values between 1.5 to 6.0 (4), but may still be applied to superlipophilic substances (having log $P_{ow} > 6.0$). The pre-estimate of the bioconcentration factor (BCF), sometimes denoted as K_B , for such superlipophilic substances will presumably be higher than the steady-state bioconcentration factor (BCF_{ss}) value expected to be obtained from laboratory experiments. Preestimates of the bioconcentration factor for organic chemicals with log P_{ow} values up to about 9.0 can be obtained by using the equation of Bintein et al. (5). The parameters which characterise the bioconcentration potential include the uptake rate constant (k_1), the depuration rate constant (k_2) and the BCF_{ss} or BCF_k.

Radiolabelled test substances can facilitate the analysis of water and fish samples, and may be used to determine whether degradate identification and quantification should be made. If total radioactive residues are measured (e.g. by combustion or tissue solubilisation), the BCF is based on the parent compound, any retained metabolites and also assimilated carbon. BCFs based on total radioactive residues may

not, therefore, be directly comparable to a BCF derived by specific chemical analysis of the parent compound only. Clean-up procedures may be employed in radiolabelled studies in order to determine BCF based on the parent compound, and the major metabolites may be characterised if deemed necessary. BCF determination for parent compound should be based upon the concentration of the parent compound in fish and not upon total radiolabelled residues. BCFs based on total radiolabelled residues can serve as one of the criteria for determining if degradates identification and quantification is necessary. It is also possible to combine a fish metabolism study with a bioconcentration study by analysis and identification of the residues in tissues.

The test consists of two phases: the exposure (uptake) and post-exposure (depuration) phases. During the uptake phase, separate groups of fish of one species are exposed to at least two concentrations of the test substance. They are then transferred to a medium free of the test substance for the depuration phase. A depuration phase is always necessary unless uptake of the substance during the uptake phase has been insignificant (e.g. the BCF is less than 10). However, it is not limited on a certain purpose of the test [e.g. in the case that the determination of BCF_{ss} is carried out only, or it meets the criteria concerning a plateau bioconcentration shown in 4) of 3]. The concentration of the test substance in/on the fish (or specified tissue thereof) is followed through both phases of the test. In addition to the two test concentrations, a control group of fish is held under identical conditions except for the absence of the test substance, to relate possible adverse effects observed in the bioconcentration test to a matching control group and to obtain background concentrations of test substance.

The uptake phase is run for 28 days unless it is demonstrated that equilibrium has been reached earlier [see 4) of 3]. A prediction of the length of the uptake phase and the time to steady-state can be made from equations in 4.3. The depuration period necessary for determining BCF_k is then begun by transferring the fish to the same medium but without the test substance in another clean vessel. Where possible the bioconcentration factor is calculated preferably both as the ratio (BCF_{ss}) of concentration in the fish (C_f) and in the water (C_w) at apparent steady-state and as a kinetic bioconcentration factor, BCF_k as the ratio of the rate constants of uptake (k_1) and depuration (k_2) assuming first-order kinetics.

If a steady-state is not achieved within 28 days, especially in the case of determining BCF_{ss} only, the uptake phase should be extended until steady-state is reached, or 60 days, whichever comes first; the depuration phase is then begun.

In the case of determining BCF_k , the uptake rate constant, the depuration (loss) rate constant (or constants, where more complex models are involved), the bioconcentration factor, and where possible, the confidence limits of each of these parameters are calculated from the model that best describes the measured concentrations of test substance in fish and water.

The BCF is expressed as a function of the total wet weight of the fish. However, for special purposes, specified tissues or organs (e.g. muscle, liver), may be used if the fish are sufficiently large or the fish may be divided into edible (fillet) and non-edible (viscera) fractions. Since, for many organic substances, there is a clear relationship between the potential for bioconcentration and lipophilicity, there is also a corresponding relationship between the lipid content of the test fish and the observed bioconcentration of such substances. Thus, to reduce this source of variability in

test results for those substances with high lipophilicity (i.e. with $\log P_{ow} > 3$), bioconcentration should be expressed in relation to lipid content in addition to whole body weight. The lipid content should be determined on the same biological material as is used to determine the concentration of the test substance, when feasible.

4.3 Prediction of the duration of the uptake and depuration phases

4.3.1 Prediction of the duration of the uptake phase Before performing the test, an estimate of k_2 and hence some percentage of the time needed to reach steady-state may be obtained from empirical relationships between k_2 and the n-octanol/water partition coefficient (P_{ow}) or k_2 and the aqueous solubility (S).

An estimate of k_2 (day^{-1}) may be obtained, for example from the following empirical relationship (6):

$$\log_{10} k_2 = -0.414 \log_{10} (P_{ow}) + 1.47 \quad (r^2 = 0.95) \quad [\text{equation 1}]$$

For other relationships see (7).

If the partition coefficient (P_{ow}) is not known, an estimate can be made (8) from a knowledge of the aqueous solubility (S) of the substance using:

$$\log_{10} (P_{ow}) = -0.862 \log_{10} (S) + 0.710 \quad (r^2 = 0.994) \quad [\text{equation 2}]$$

where S = solubility (moles/l) ($n = 36$)

These relationships apply only to chemicals with $\log P_{ow}$ values between 2 and 6.5 (9).

The time to reach some percentage of "steady-state" may be obtained, by applying the k_2 -estimate, from the general kinetic equation describing uptake and depuration (first-order kinetics):

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

or, if C_w is constant:

$$C_f = \frac{k_1}{k_2} \cdot C_w (1 - e^{-k_2 t}) \quad [\text{equation 3}]$$

When "steady-state" is approached ($t \rightarrow \infty$), equation 3 may be reduced [see (10) (11)] to:

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

or

$$C_f/C_w = k_1/k_2 = \text{BCF}$$

Then $k_1/k_2 \cdot C_w$ is an approach to the concentration in the fish at "steady-state" ($C_{f,s}$).

Equation 3 may be transcribed to:

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

or

$$\frac{C_f}{C_{f,s}} = 1 - e^{-k_2 t} \quad [\text{equation 4}]$$

Applying equation 4, the time to reach some percentage of “steady-state” may be predicted when k_2 is pre-estimated using equation 1 or 2.

As a guideline, the statistically optimal duration of the uptake phase for the production of statistically acceptable data (BCF_k) is that period which is required for the curve of the logarithm of the concentration of the test substance in fish plotted against linear time to reach its mid-point, or $1.6/k_2$, or 80 % of steady-state but not more than $3.0/k_2$ or 95 % of steady-state (12).

Information : Consequently, the exposure time is regarded as the optimal duration for BCF_k , and it is preferred that the measurement of BCF should be carried out three or more times at not less than two days intervals in order to meet the BCF_{ss} criteria as fully as possible. BCF in the duration should be within ± 20 % of a specified range (provided that there is some significant difference). When the interval between $1.6/k_2$ and $3.0/k_2$ is short (e.g. $\log P_{ow} = 4$), obtain only BCF_k rounding up to $3.0/k_2$, or obtain both BCF_k and BCF_{ss} by carrying out exposure during the duration exceeding $3.0/k_2$ in order to obtain BCFs for three or more points of time [in this case, BCF_k is determined from a graphical method (10.2.2 and 10.2.3) or from computer method (10.2.4) which may have some lower accuracy].

The time to reach 80 percent of “steady-state” is (equation 4):

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

or

$$t_{80} = \frac{1.6}{k_2} \quad \text{[equation 5]}$$

Similarly 95 percent of “steady-state” is:

$$t_{95} = \frac{3.0}{k_2} \quad \text{[equation 6]}$$

For example, the duration of the uptake phase (up) for a test substance with $\log P_{ow} = 4.0$ would be (using equations 1, 5 and 6):

$$\log_{10} k_2 = -0.414 \cdot (4) + 1.47$$

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

$$\text{up (80 pct)} = 1.6/0.652, \text{ i.e. 2.45 days (59 hours)}$$

$$\text{or up (95 pct)} = 3.0/0.652, \text{ i.e. 4.60 days (110 hours)}$$

Similarly, for a test substance with $S = 10^{-5} \text{ mol/l}$ ($\log S = -5.0$), the duration of up would be (using equations 1, 2 and 5, 6):

$$\log_{10}(P_{ow}) = -0.862 \cdot (-5.0) + 0.710 = 5.02$$

$$\log_{10} k_2 = -0.414 \cdot (5.02) + 1.47$$

$$k_2 = 0.246 \text{ days}^{-1}$$

$$\text{up (80 pct)} = 1.6/0.246, \text{ i.e. 6.5 days (156 hours)}$$

$$\text{or up (95 pct)} = 3.0/0.246, \text{ i.e. 12.2 days (293 hours)}$$

Alternatively, the expression:

$$t_{eq} = 6.54 \times 10^{-3} P_{ow} + 55.31 \text{ (hours)}$$

may be used to calculate the time for effective steady-state to be reached (9).

4.3.2 Prediction of the duration of the depuration phase A prediction of the time needed to reduce the body burden to some percentage of the initial concentration may also be obtained from the general equation describing uptake and depuration (first order kinetics) (6) (13).

For the depuration phase, C_w is assumed to be zero. The equation may then be reduced to:

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

or

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

where $C_{f,0}$ is the concentration at the start of the depuration period.

50 percent depuration will then be reached at the time (t_{50}):

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

or

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

Similarly 95 percent depuration will be reached at:

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

If 80 % uptake is used for the first period ($1.6/k_2$) and 95 % loss in the depuration phase ($3.0/k_2$), then depuration phase is approximately twice the duration of the uptake phase.

It is important to note, however, that the estimations are based on the assumption that uptake and depuration patterns will follow first order kinetics. If first order kinetics are obviously not obeyed, more complex models should be employed [e.g. ref (6)].

Information : There are other prediction calculation, for example, when carrying out the preliminary test, exposure period satisfying the BCF_{ss} criteria or to reach near 80 % of the steady-state, and depuration period for 95 % of the initial concentration, can be estimated (9.1).

5 Information on the test substance In addition to the properties of the test substance given in clause 4 other information required is the toxicity to the fish species to be used in the test, preferably the asymptotic LC₅₀ (i.e. time-independent). An appropriate analytical method, of known accuracy, precision, and sensitivity, for the quantification of the substance in the test solutions and in biological material must be available, together with details of sample preparation and storage. Analytical detection limit of test substance in both water and fish tissues should also be known. When ¹⁴C labelled test substance is used, the percentage of radioactivity associated with impurities should be known.