"Bioaccumulation: Sequential Static Fish Test"

1. INTRODUCTORY INFORMATION

- Prerequisites
  - Water solubility (high precision value not needed)
  - Stability and reactivity in water
  - Acute toxicity to test fish species
  - Analytical method specific to the test chemical

- Guidance information
  - Information on the possible disappearance of the test chemical from the test medium due to volatilisation, sorption and/or degradation in the test system is necessary. It is useful to estimate possible analytical difficulties and, with consideration of the prerequisite information, to fix workable concentration ranges in which the chemical can be tested.

  If extreme losses are to be expected by volatilisation, e.g. when the water/air partition coefficient \( < 10^3 \), or by sorption to parts of the test apparatus, or to the feed of the test species, e.g. \( P_{ow} > 10^5 \), then either renew the test medium regularly (to maintain a sufficiently high test concentration) or use a flow-through test system. Relevant properties:

  - \( P_{ow} \) (Partition coefficient, n-octanol water)
  - Sorptivity, e.g. \( K_{oc} \)
  - Volatility, water/air partition coefficient
  - Purity/composition of test chemical
  - Solubility in solvents other than water

- Qualifying statements
  - This test procedure is applicable as long as the test chemical can be reliably analysed in the test organisms and the test medium, and as long as it can be demonstrated that sorption isotherms have been measured (i.e. the bioconcentration factor (BCF) given is based on steady-state measurements). If these prerequisites can be observed (generally with chemicals having a \( P_{ow} \leq 10^5 \)), the Test Guideline can be used at both the screening and the confirmatory testing level.

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Users of this Test Guideline should consult the Preface, in particular paragraphs 3, 4, 7 and 8.
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- The rate (i.e., order of kinetics) and extent of depuration are essential data for the final evaluation of the bioaccumulation behaviour of a test chemical. Incomplete depuration may indicate reaction of the test chemical or part of it with cell constituents of the test organism.

- After testing a series of chemicals (preferably including those with known bioaccumulation behaviour), it is recommended that a regression equation which correlates the BCF's of the test chemicals with their $P_{ow}$'s in a given test species be established (for example in the form of $\log \text{BCF} = a \cdot \log P_{ow} + b$). This can be used to compare a measured BCF with the corresponding calculated BCF. Good agreement between the two values is a reliable proof that the test procedure was suitable for the test chemical.

- In case the test chemical is not stable and forms metabolites, an accumulation test with such metabolites must be considered. In justifying the validity of the BCF found, the magnitude of the BCF must be carefully considered.

- This Test Guideline has been submitted to the OECD Laboratory Intercomparison Test Programme (1978-1980).

**Recommendations**

- It is recommended that results of static test procedures such as that given in the present Test Guideline be carefully compared with flow-through test procedures (see 305 E) in order to detect whether different results sometimes found with the same chemical in the two approaches are based upon differences intrinsic to these approaches and/or upon differences in experimentation.

- Based on results with 1,2,4-trichlorobenzene (TCB) from the OECD Bioaccumulation Ring Tests (April, 1980), it was found that the variability of BCF's in several fish species was significantly reduced by relating the BCF's to the lipid content of the fish. Therefore, it is recommended that BCF's be normalised on this basis.

- It is recommended that measured BCF's of test chemicals be related to the BCF of a reference chemical of known bioaccumulation behaviour (e.g., BCF of test chemical as a percentage of BCF of reference chemical). Variation of results caused, for example, by different lipid contents, species differences, etc., should thus be minimised considerably.
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- **Standard documents**

  This Test Guideline is based upon work undertaken to study the influence of the physical-chemical properties of pesticides on their bioaccumulation potential. Some of the work to date has been published (1,2).

  With regard to definitions of terms and experimental techniques, this Guideline relies to a great extent on a recent ASTM draft proposal (3).

2. **METHOD**

A. **INTRODUCTION, PURPOSE, SCOPE, RELEVANCE, APPLICATION AND LIMITS OF TEST**

  The potential of a chemical to bioaccumulate in aquatic organisms can be determined by measuring the bioconcentration factor, which is the ratio of the concentration of the compound in the test organism to that in water under steady-state conditions (apparent plateau).

  This Test Guideline describes a stepwise procedure which measures, under static test conditions, the dependence of the bioaccumulation of a test compound in fish on its concentration in water (uptake or sorption isotherm) and on its rate of depuration after termination of uptake when the fish are transferred to non-contaminated water.

  Since it was found that physical-chemical properties govern the bioaccumulation of test pesticides in organisms of the aquatic food chain (algae, daphnia, fish), it has been concluded that this procedure is generally applicable to organic chemicals, provided they have a certain lipophilicity and stability as described in Specificity, below.

- **Definitions and units**

  **Bioconcentration** is the increase in concentration of a test compound in or on a test organism (or specified tissues thereof) relative to the concentration of test compound in the ambient water.

  **Bioconcentration factor (BCF)** is the ratio of the test substance concentration in the test fish \((C_f)\) to the concentration in the test water \((C_w)\) at steady-state.
Median Tolerance Limit (TLₘ) is the concentration of the test compound expressed in mg/l, at which 50 per cent of the test fish will die in a specified time.

Uptake is the process of sorbing a test compound into and/or onto aquatic organisms.

Uptake (sorption) isotherm is a curve showing the dependence of the concentration of test compound in the test organism upon its concentration in water at steady-state which can be described by the Freundlich sorption equation

\[ x/m = K \cdot C_e^{1/n} \]

where \( x/m \) is \( \mu g \) test compound sorbed per g fish
K and \( 1/n \) are constants
\( C_e \) is the steady-state concentration in water

Plateau (steady-state) is a condition in which the amounts of test compound being taken up and depurated are equal at a given water concentration.

Depuration (or elimination or clearance) is the process of losing a sorbed test compound from aquatic organisms after they have been placed in water free of the test compound. Generally, this process cannot be described with sufficient accuracy by first-order kinetics.

Depuration rate constant is the slope \( k_d \) of the curve describing the depuration of the concentration of the test compound taken up by the test organism with time according to the equation

\[ 1/R_t = k_d \cdot t + c \text{ [g.µg}^{-1}] \]

where \( R_t \) is the residue of test compound at depuration time \( t \) and \( c \) is \( 1/R_{t0} \), the reciprocal of the starting residue concentration at \( t = 0 \).

**Reference substances**

In some cases when investigating a new substance, reference substances may be useful; however, specific reference substances cannot yet be recommended.
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* Principle of the test method *

In the *first step* of the procedure the time interval needed to reach the steady-state concentration (apparent plateau) in the fish is determined by exposing one group of fish to one concentration of the test compound in water (true solution, solvents only to the extent specified in reference 3, Chapter 9.1; preferably ≤ 1/10 of TLM at 96 hours) without renewal of the test solution. Preferably, a low concentration which can be analysed with sufficient accuracy is used in order to reach plateau as quickly as possible. For example, if the test concentration is ≤ 0.5 ppm and the fish/water ratio about 1 g/litre, the plateau generally is reached within 4 days with compounds specified in (d) of Specificity, below.

In the *second step* of the procedure the dependence of bioaccumulation upon the concentration of the test compound in water is determined (sorption isotherm).

For this purpose, groups of fish are exposed to at least three concentrations (the highest concentration preferably ≤ 1/10 of TLM at 96 hours) for the time interval found necessary to reach the plateau in the first step, and the concentrations of test compound in the fish and water are analysed.

In the *third step* of the procedure the rate constant of depuration is determined. For this purpose, one group of fish exposed to the highest test compound concentration in the second step is transferred to non-contaminated water from which depurated test compound is removed continuously. Concentration of test compound in the fish is periodically determined until about 80 per cent depletion is reached or for a period of up to 10 days, whichever comes first.

* Quality criteria *

Reproducibility

In studies with a limited number of $^{14}$C-labelled pesticides, a standard deviation about the mean in the order of ± 25 per cent was observed. More repetitions of individual studies (compounds) are needed to define reproducibility more precisely, especially when non-labelled compounds are used as standards, and the usefulness of different fish species is to be determined.
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Sensitivity

Sensitivity of the procedure depends essentially on the sensitivity of the analytical method available for the compound to be studied.

Specificity

The procedure described is for use with non-ionised, organic chemicals with the following properties:

(a) not readily degradable is a microbial degradation test (e.g. Modified OECD Screening Test, Test Guideline 301 E)

(b) relatively stable in the aquatic environment (e.g. half-life > 4 days for structural transformation of test compound into more polar derivatives)

(c) soluble in water at < 2000 ppm

(d) partition coefficient in n-octanol/water, $P_{ow} > 10^3$

(e) volatility: water/air partition coefficient $> 10^2$

(f) chemicals ionising under physiological pH's are also suitable for this test if it can be shown that complex or ion pair formation does not cause an increase of $P_{ow}$ over $10^3$.

In cases of compounds with solubilities $> 2000$ ppm, the decision not to perform a fish bioaccumulation test can be taken irrespective of the result of a prior biodegradation screening test. The result "not readily degradable" for a highly water soluble compound has consequences with regard to ecotoxicity and further degradability testing but not with regard to fish bioaccumulation testing. Among the compounds with solubilities $< 2000$ ppm, those with $P_{ow} < 10^3$ will also be excluded from bioaccumulation testing in fish.

Those test compounds with $P_{ow} > 10^3$ which decompose according to (b) into derivatives with high water solubility (e.g. $P_{ow} < 10^3$) generally need not be tested for bioaccumulation.

Test compounds of very low water solubility with a volatility greater than that defined in (c) might require a modified test procedure. In those cases the test solution of step 1 and 2 experiments should be regularly renewed to maintain an analysable concentration of the test compound (see Test solution, below) or a flow-through test should be used.
Ability to standardise

The procedure was established with $^{14}\text{C}$-labelled pesticides such as 2,4-D, Atrazine, DDT and others (see reference 1). Radio-labelled compounds with properties defined in Specificity, above, can be used for standardisation, provided that suitable equipment is available. The use of non-labelled compounds as standards requires sensitive (limits of quantitation in the ppb region) and compound-specific analytical methods.

B. DESCRIPTION OF THE TEST PROCEDURE

- Preparations

Test compound

The following properties must be known to properly design the study:

- solubility in water
- partition coefficient n-octanol/water
- stability in water (hydrolysis, photolysis if appropriate)
- degradability by aquatic micro-organisms (e.g. sludge)
- volatility from water
- TLm at 96 hours for the fish species to be used in this bioaccumulation study
- analytical grade material

Test solution

- Composition of the water:
  - Deionised water supplemented with

\[
\begin{align*}
\text{CaSO}_4\cdot2\text{H}_2\text{O} & \quad 37.9 \text{ mg/l} \\
\text{MgSO}_4\cdot7\text{H}_2\text{O} & \quad 61.6 \text{ mg/l} \\
\text{NaHCO}_3 & \quad 37.8 \text{ mg/l} \\
\text{KCl} & \quad 3.0 \text{ mg/l} \\
\text{pH} & \quad 7.0 - 7.2
\end{align*}
\]

Before the fish are introduced this test water is circulated for 24 hours through a charcoal filter.
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- Concentrations of test compound: The test compound should be in true solution; no detergent should be used; if necessary solvents such as ethanol or acetone can be used to help in the dissolution of the test compound, but their final concentration should not exceed 0.5 ml/litre.

- Concentrations of test compound > 0.5 ppm should be avoided (see Principle of the test method, above).

- During depuration the concentration of released test compound in water is kept essentially at zero by continuously circulating the depuration water through a charcoal filter.

- Compounds of very low solubility in water with significant volatility [see Specificity, section (e)] require regular renewal of the test solution in the step 1 and 2 experiments to maintain a concentration which can be analysed with sufficient accuracy.

**Test conditions**

*Fish*

- Species: this Test Guideline has been established with catfish (*Ictalurus melas*). However, other species such as zebra fish or carp should be equally suitable. The only consequence might be a certain variation in the constant K of the sorption isotherm and the depuration rate constant when comparing catfish with other species under identical experimental conditions.

- Size: fish of a weight range of 0.5 - 2 g should be used.

- Concentration of biomass: about 1.0 g fish/litre water, but identical for all 3 steps.

- Number per sample to be analysed: 4-5 catfish or, with other species, a number of fish equivalent to the weight of the catfish.

- Adaptation to test conditions: at least 24 hours.

*Experimental conditions*

- Aeration: continuously with filtered air (ca 7-8 mg O₂/l).
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- Temperature: 20 ± 2°C for catfish; to be adapted to other species.

- Feeding schedule: The fish are not fed during step 1 and 2 experiments. During step 3 fish obtain feed once a day (corresponding to 10 per cent of their fresh weight) after samples have been taken for analysis.

- Constant concentration of biomass: The starting ratio of g fish/litre water is maintained during step 1 of the study by simultaneously removing a corresponding volume of the test solution when the fish are removed for analysis.

- Sampling schedule:

<table>
<thead>
<tr>
<th>Step 1</th>
<th>Interval (hrs)</th>
<th>Water</th>
<th>Fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1 concentration)</td>
<td>Fortified sample</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Blank</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>+</td>
<td>+ (e.g. Plateau)</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 2</th>
<th>Interval (hrs)</th>
<th>Water</th>
<th>Fish</th>
</tr>
</thead>
<tbody>
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<td>(≥3 concentration)</td>
<td>Fortified sample</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Blank</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>e.g. 48</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 3</th>
<th>Interval (hrs)</th>
<th>Water</th>
<th>Fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1 concentration)</td>
<td>Blank</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>e.g. - 48</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0 (i.e. before depuration)</td>
<td>+</td>
<td>+ (= plateau)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>114</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(etc., if needed, until 10 days)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total number of samples: 11 water, 19 fish

Generally, the recommendations regarding apparatus, dilution water, test material and test organisms given in chapters 7 through 11 of reference 3 are also valid for this proposed method, except those which relate specifically to flow-through experimentation.
3. DATA AND REPORTING

- **Treatment of results**

  Bioaccumulation processes in an aquatic environment can be characterised by measuring two parameters, the sorption isotherm under steady-state conditions and the depuration rate. From the sorption isotherm, the bioaccumulation factor is derived; from the depuration curve the rate constant and the depuration half-lives (see Definitions, above).

  The results of the second step (see Principle of the method, above) are presented graphically by plotting, on a double-logarithmic scale, the fish and water concentrations of the test compound at steady-state (at least three pairs). The slope and the constant of the isotherm are determined either graphically or by regression analysis. Provided the slope is approximately 1, then the constant of the isotherm is identical with the bioaccumulation factor BCF. This also proves that the test had been performed under steady-state conditions.

  The results of the third step (see Principle of the method, above) are presented graphically by plotting, on a linear scale, the reciprocal of the fish residue (test compound concentration) against depuration time. The result should be a straight line following the equation

  \[ \frac{1}{R_f} = k_\text{d} \cdot t + c \ [g \cdot \mu g^{-1}] \]

  The slope \( k_d \) and the constant \( c \) (the latter corresponding to \( 1/R_f \) at \( t = 0 \)) are determined either graphically or by regression analysis. The depuration half-life, \( \tau \), for a given initial residue \( R_0 \) in fish is then given by the equation

  \[ \frac{1}{k_d \cdot R_0} = \tau \]

  i.e. starting depuration from different \( R_0 \) values will yield different depuration half-life times.

- **Evaluation of results**

  **Step 1:** When catfish are used, the fish steady-state concentration of the test compound should correspond to that which can be calculated from its partition coefficient according to reference 2. For other species, the corresponding equation relating the steady-state concentration
with the partition coefficient has still to be determined. Some variation in the constants of these equations might be expected.

Exceptions where the expected plateau is not reached are (a) significant degradation of the test compound into polar compounds by the fish, (b) significant degradation into compounds less polar (more lipophilic) than the test compound, or reaction of the test compound with body constituents and (c) diseases of the test fish during the study.

Cases (a) and (b) require the metabolic behaviour of the test compound in fish to be checked; case (c) requires repetition of the test with healthy fish.

**Step 2:** Plotting the steady-state concentrations fish/water on a double-logarithmic scale should give a straight line with a slope of about 1. Regression analysis of the curve should show a \( r^2 \geq 0.9 \).

**Step 3:** Plotting the reciprocal of the fish residue against depuration time on a linear scale should result in a straight line. Regression analysis of the curve should show a \( r^2 \geq 0.9 \). In cases in which depuration must be started with very low fish residues (e.g. for reasons of toxicity), depuration half-life might be longer than 10 days. The half-life must then be calculated from the values obtained within 10 days using the equations given under Treatment of results, above. Compounds showing a non-linear depuration behaviour (variation of constant \( k_{hl} \) with time) should be looked at with regard to their metabolic and toxicological behaviour in fish.

- **Test report**

The relevant data with regard to properties of the test compound and the test fish, as well as the test conditions and the sampling schedule as described above, should be given in detail.

All data measured during experimental steps 1, 2 and 3 should be summarised in individual tables which also should show the limits of quantitation of the analytical method used.

The result of step 2 should be presented in form of the equation

\[
\frac{x}{m} = k \cdot C_e^{1/n} \quad \text{or} \quad \log \frac{x}{m} = \log k + \frac{1}{n} \log C_e \quad [\mu g \cdot g^{-1}]
\]

including the correlation coefficient \( (r^2) \).
The result of step 3 should be presented in form of the equation

\[ 1/R_t = k_{II} \cdot t + c \left[ g \cdot \mu g^{-1} \right], \]

including the correlation coefficient (r²). Depuration half-life time of the test compound should be given in a normalised form by calculating \( \tau \) for \( R_0 = 1 \) ppm, i.e.

\[ \tau = \frac{1}{k_{II}}. \]

- **Interpretation of results**

Interpretation and evaluation of the results obtained for an individual test compound, especially with respect to environmental relevance, can only be made when agreement on a suitable reference compound has been reached. The characteristic data finally obtained for a compound tested, i.e. \( k \) at \( C_e = 1 \) ppm and \( \tau \) at \( R_0 = 1 \) ppm, can then further be normalised by relating them to the respective data of the reference compound, which each are considered as 100 per cent.

4. **Literature**

