"Bioaccumulation: Semi-Static Fish Test"

1. INTRODUCTORY INFORMATION

- **Prerequisites**
  - Water solubility of the test substance (high precision not needed)
  - Stability and reactivity in water
  - Acute toxicity data related to the test organism
  - Precise procedure for quantitative chemical analysis of test substance

- **Guidance information**
  - If the experimentally determined bioconcentration factor (BCF) is lower than the BCF predicted from the n-octanol/water partition coefficient – BCF regression line, any data on metabolism of related compounds may be helpful.
  - Composition of test substance; existence of various components.
  - Partition coefficient, octanol-water (P<sub>ow</sub>), vapour pressure.
  - Purity/composition of test substance, solubility in other solvents.

- **Qualifying statements**

  The semi-static procedure is not appropriate for testing of chemicals of very low water solubility or high volatility. With such chemicals it is too difficult to keep concentration in the water sufficiently constant, even if attempts are made to saturate the walls of the test tank with the chemical prior to the test. For these types of chemicals, a continuous flow test procedure should be selected. In justifying validity of the BCF found, the magnitude must be considered. If the test chemical is converted to metabolites, repetition of the test should be considered.

This Test Guideline has been tested in the OECD Laboratory Intercomparison Test Programme (1978-1980).
"Bioaccumulation: Semi-Static Fish Test"

- **Recommendations**

  This Test Guideline is appropriate for testing of chemicals with a water solubility of approximately 1 - 500 mg/l. As the greatest need to test chemicals for bioaccumulation with fish may be with those chemicals of intermediate water solubility, the semi-static test would be an appropriate and rapid procedure for handling this important group of chemicals.

  It is recommended that BCF values be related to the lipid content of fish; this procedure reduces variability of BCF determined in several fish species.

  Whenever possible the uptake phase should always be followed by a depuration phase in order to estimate the rate of depuration of the chemical from the fish.

- **Standard documents**

  This Test Guideline is based primarily on the "Second proposal for a standard method for screening chemicals and products for acute toxicity to freshwater fish using a semistatic procedure" to ISO developed by a Swedish working group for standardisation of toxicity tests (SIS/SK33/TK7/AG1) and the "Proposed standard practice for measuring bioconcentration of chemicals with fishes", ASTM-draft N° 8, August 29, 1978. This final draft has been worked out by an ad hoc working group consisting of representatives from four Swedish laboratories*.

2. METHODS

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

  This Test Guideline describes a procedure for characterising the potential of a test chemical to bioconcentrate in aquatic biota. The bioconcentration potential is estimated by measuring the bioconcentration factor – i.e. the ratio of the concentration of the test material in the test fish (Cₐ) to that in the test water (Cₑ) at steady-state.

* Swedish Water and Air Pollution Research Institute, laboratories of the Swedish Environment Protection Board for brackish water toxicology, special analysis and general investigations.
"Bioaccumulation: Semi-Static Fish Test"

The present procedure is based on semi-static conditions (renewal of test solutions every 48 hours). Therefore, test chemicals with very low water solubility can also be characterised as to their bioconcentration potential without adding (or with addition of only very small amounts) solvents or other auxiliary substances which may affect the results. Also, volatile test compounds may be characterised by the present semi-static procedure; in this case, the test container should be equipped with a tightly fixed cover.

• **Definitions**

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

  **Uptake** is the process of sorbing the test substance into and/or onto the test organism.

  **Depuration** or **elimination** or **clearance** is the process of losing a sorbed test substance from the test organisms.

  **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.

  **Steady-state** is a condition in which the concentration of the substance in the fish is constant in time.

  **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.

  **Induced metabolism** is a change in the rate of bioconcentration or depuration during a bioconcentration test due to latent induction of enzymes. Induced metabolism usually results in a faster rate of depuration causing a corresponding reduction in the amount of test substance bioconcentration.

• **Reference substances**

In some cases when investigating a new substance, reference substances may be useful; however, specific reference substances cannot yet be recommended.
"Bioaccumulation: Semi-Static Fish Test"

- **Principle of the test method**

  At least two groups of test fish (with at least fifty organisms per group) of one species is exposed for a period of time (up to four weeks) to water containing added test substance, at two or more concentrations. The test solution is renewed at regular intervals. Fish are fed throughout the test; no test substance is added to the food. Fish and water are periodically removed from the test chamber and analysed for test substances. Bioconcentration factors are calculated from the measured concentrations in test organisms and test solution at steady-state. Therefore, one important part of the data analysis entails checking whether or not steady-state is attained (See Data and Reporting).

- **Quality criteria**

  **Sensitivity**

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

  **Specificity**

  The procedure proposed is applicable to organic chemicals having the following properties:

  a) not readily degradable in a microbial degradation test (e.g. modified OECD screening test, see Test Guideline 301E)

  b) relatively stable in the aquatic environment

  c) soluble in water at < 2000 mg/l.

B. **DESCRIPTION OF THE TEST PROCEDURE**

- **Preparations**

  **Chemical analysis**

  Before any biological experiments are carried out, the analytical method for the particular substance must be tested. It must be shown experimentally that the recovery as well as the reproducibility are satisfactory both on water and organisms. Blank samples (of untreated water,
solvents, etc.) should be regularly analysed to ensure that no contamination occurs. The detection level should be determined, and no quantification should be based on signals which are less than 10 times the instrument noise. Organisms and water samples should be removed in a way that no contamination or losses by adsorption occur.

Apparatus

Construction materials: Construction materials and commercially purchased equipment that may come in contact with any water into which test organisms are placed should not contain any substances which leach or dissolve in water to the extent that they may influence the results of the test. In addition, materials and equipment that contact stock solutions or test solutions should be chosen to minimise sorption of toxicants from water. In principle, the adsorption and/or leaching to or from the material must be checked in an in vitro pre-test. Glass, stainless steel or polyethylene plastics may be used in order to minimise leaching, dissolution and sorption. Rubber, copper, brass, zinc and lead must not come into contact with dilution water, stock solutions or test solutions.

Test aquarium: The test container should be a conventional, rectangular, all-glass (or stainless steel) aquarium. The size will depend on the required volume of test solution, which in turn depends on the number and size of fish used in the test. The weight of all fish in a test jar must not exceed 0.8 gram per litre of test solution. For tests with fifty individuals of zebra fish (10 x 0.2 to 10 x 0.4 g) the volume of the test jar should be at least 25 litres, containing 20 litres of test solution. In order to minimise loss of test substance due to evaporation, the test container should be equipped with a tightly fixed cover.

Equipment for temperature regulation: Water baths, or preferably an experimental room, capable of maintaining a temperature of 23.5 ± 1°C for zebra fish, should be used.

Cleaning containers and equipment: Test containers must be cleaned before use. They must be washed with detergent and rinsed with water, then carefully rinsed with acetone, and finally the acetone is removed by rinsing the containers with the water to be used in the experiment.
Reagents

Dilution water: The standard dilution water may be prepared from glass distilled or deionised water according to the recipe given below. Dilution water may also be obtained from an uncontaminated well or spring or from tap water passed through an activated carbon filter, provided the ionic composition of the water is close to that recommended in the recipe.

The dilution water should be prepared at least one day before use. The water must be prepared and stored in thoroughly cleaned tanks which do not leach or dissolve in the water to the extent that they influence the results of the test (e.g. glass, stainless steel, polyvinylchloride (PVC), unplasticised polyethylene and polypropylene). Prior to use, the dilution water should be intensively aerated. Adequate aeration will bring the pH and the concentration of dissolved oxygen and carbon dioxide into equilibrium with the air. The concentration of dissolved oxygen in the dilution water should be between 90 and 100 per cent of air saturation value. The water, after aeration should have a pH value of 7.8 ± 0.2 and a hardness of 100 mg/l, expressed as calcium carbonate.

The standard dilution water has the following ionic composition in parts per million by weight:

<table>
<thead>
<tr>
<th>Ion</th>
<th>Parts per million</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>29.5</td>
</tr>
<tr>
<td>Magnesium</td>
<td>7.5</td>
</tr>
<tr>
<td>Sodium</td>
<td>56.5</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>100.0</td>
</tr>
<tr>
<td>Chloride</td>
<td>60.5</td>
</tr>
<tr>
<td>Sulphate</td>
<td>56.0</td>
</tr>
<tr>
<td>Nitrate</td>
<td>3.5</td>
</tr>
</tbody>
</table>

It is prepared by adding stock solutions of analytical reagents to deionised or distilled water whose conductivity does not exceed 5 conductivity units (10 uOhm/cm).

Stock solution 1: Dissolve 320 g CaCl₂·6H₂O, 29 g NaCl and 9 g NaNO₃ and make up to 1 litre.

Stock solution 2: Dissolve 151 g MgSO₄·7H₂O and 79 g Na₂SO₄ and make up to 1 litre.

Stock solution 3: Dissolve 27 g NaHCO₃ and make up to 1 litre.

For each 100 litres of standard water to be prepared, add 50 ml each of solutions 1 and 2 and 500 ml of solution 3.
"Bioaccumulation: Semi-Static Fish Test"

Test solutions: Stock solutions of the test substances may be prepared daily, unless it is known that the material is stable in solution, in which case sufficient stock solution for 14 days may be made up. In general, the stock solution should be prepared by dissolving the substance in 100 per cent acetone. It is recommended that the volume of stock solution added to the dilution water be at a maximum 25 ml acetone per litre of test solution.

It is necessary to report deviations from the described methods for preparation of stock solution and test solution.

If the test substance is marketed as a formula, both the designated active ingredient and the complete formula should be tested, and the report should state the specification of the material tested and whether the concentrations refer to the pure substance or to the formula.

Test organism

The recommended test species is Brachydanio rerio, commonly known as the zebra fish.

The stock population should be maintained for a minimum of 2 weeks in water of quality and temperature similar to the dilution water and fed on a normal diet. The fish should be free from observable diseases; if normal prophylactic treatment for the prevention of disease is followed (including the use of medicated food) the fish should be left for at least two weeks after treatment before use in the test. However, if more than 5 per cent of the stock fish become diseased, then the whole population should be discarded.

A representative sample of the fish population should be measured in order to ensure compliance with the requirements that individual total lengths should be between 25 and 35 mm, and total weights between 0.2 and 0.4 g.

The fish should be moved from one vessel to another every second day.

• Performance of the test

Test fish should be exposed to two (or more) concentrations of the substance in water.
"Bioaccumulation: Semi-Static Fish Test"

As a guideline, the highest concentration should be less than 1/50 of the TLm at 96 hours for the test species and at least 2.5 times higher than the detection limit in water and, if possible, each exposure concentration should differ from another by a factor of ten.

The duration of the bioconcentration test is based on the physical properties of the test substance. In general, highly fat soluble compounds require longer exposure duration times to approach steady-state compared to compounds with lower fat solubility. The test might be discontinued when steady-state is obtained – but in general the uptake period should be two to four weeks (14-28 days).

The fish must be fed (1-2 hours before transfer to new test solution), using a normal amount and type of food, during the test period. Fifty fish should be used in each experimental concentration of the substance assayed. The fish should be transferred from the acclimatising aquarium to the test jars only by aid of a small mesh dip net of soft material (e.g. nylon) and must not rest on any dry surface. They must not be held out of water longer than necessary. Any specimen accidently dropped or otherwise mishandled during the transfer must be discarded.

The test solutions should not be aerated. They are renewed every 48 hours for the purpose of maintaining a more or less uniform concentration and an adequately dissolved oxygen content. This renewal is accomplished by transferring the test fish quickly (using a dip net) to test jars with fresh test solutions, which must be prepared early enough to reach equilibrium between test substance and dilution water.

Chemical analysis of the test substance should be carried out on water samples from each of the test jars, initially and after 48 hours, in order to confirm that concentrations remain acceptably constant between the times of test water exchange. Simultaneously, measurements should be made of the pH, concentration of dissolved oxygen, and temperature of the test solutions in each jar. The dissolved oxygen content of the test solutions should be greater than 70 per cent of the air saturation value.

Such checking of the concentration of test substance in water should be repeated at least twice for each renewal.
"Bioaccumulation: Semi-Static Fish Test"

Sampling

In general, it is advisable to analyse both water and organism samples as soon as possible after they have been collected to prevent degradation or loss of test substance and to determine whether or not steady-state has been reached.

If water samples cannot be analysed immediately, the test substance may be extracted into a solvent rendering it inert or easier to store until it can be analysed. Fish samples may be stored in deep frozen condition.

Water: Water samples should be obtained by pipetting from the most central point of the test tank. The pipette should be rinsed with a small volume of acetone after collecting the sample and put, together with the sample, into the sample vessel.

Water samples are best collected directly into glass vessels of appropriate volume, from which the test substance can be extracted or analysed. These vessels might include separatory funnels in the case of organic compounds or scintillation vials for radioactive test substances.

Test fish: As a guideline, at least seven uptake samples should be taken according to the following schemes:

For a 4-week test: after 22, 23, 24, 25, 26, 27 and 28 days.

For a 2-week test: after 8, 9, 10, 11, 12, 13 and 14 days.

In addition, a zero sample is taken at zero time.

Each sample consists of 7 fish.

When removing test fish for analyses, they should be netted or trapped in a random manner with as little disturbance as possible. If two or more concentrations are tested, separate nets should be used for each concentration.

Fish should be rinsed with dilution water if accompanied by extraneous matter, blotted dry, and killed by pithing the brain with a dissecting needle or by severing the spinal cord above the opercular region with scissors.

The seven individuals in each sample are then combined to form a composite sample.

After weighing, the sample is usually ground up or homogenised to promote extraction of test substance or to enhance solution of the tissue. Procedures for grinding, extraction,
"Bioaccumulation: Semi-Static Fish Test"

separating impurities, determining lipid content, etc., are described in the publication cited in references (1) and (2), Section 4, Literature.

When determining bioconcentration of test substances which concentrate in lipids, it is often desirable to determine the percentage of lipids in the total tissue weight. Results between samples are frequently less variable when based on lipid weight rather than on total weight.

Organism samples can be wrapped in aluminium foil and frozen if they are not to be analysed immediately.

- **Analytical means**

Prior to analysing fish or water for the test substance, control samples should be spiked with several different concentrations of the test substance and then analysed. Final values of $C_w$ (concentration in the water) and $C_f$ (concentration in the fish) should be corrected for recoveries and background.

Analytical detection limits of test substance in both fish and water should be determined before the bioconcentration test begins and should be documented in the test report. As a guideline, the limit of detection may be defined as a signal 10 times higher than the background noise level.

If possible, results reported as "not detected at the limit of detection" should be minimised by pretest method development. These results are invalid input for rate constant calculations.

3. **DATA AND REPORTING**

- **Treatment of results**

A sample of seven fish is taken each day during the last seven days, i.e. 49 fish altogether.

If $X_i$ is the weight concentration in sample $i$, one calculates in the usual manner

\[
m = \frac{1}{7} \sum_{i=1}^{7} X_i \tag{1}
\]

\[
S^2 = \frac{1}{7} \sum_{i=1}^{7} (X_i - m)^2 \tag{2}
\]
The data are then plotted as shown below:

\[ m + \Delta \uparrow X_1 \]
\[ m \mid \quad \circ \quad \circ \quad \circ \quad \circ \]
\[ m - \Delta \]

where

\[ \Delta = 1.94 \, S \] (3)

Provided there is no time trend, at most one of the data points should fall outside the two limits.

In addition, the following quantities are calculated:

\[ m' = \frac{1}{4} \sum_{i=4}^{7} X_i \]  \[ (4) \]

\[ S'^2 = \frac{1}{4} \sum_{i=4}^{7} (X_i - m')^2 \]  \[ (5) \]

The results should be reported as follows:

a) Which points, if any, are outside the limits and whether they are above or below; e.g.

\[-1, -1, +7\]

b) \[ m' \pm \Delta' \]  \[ (6a) \]

where \[ \Delta' = 3.2 \, S' \]  \[ (6b) \]

c) The individual data points and, if possible (as a service), a chart according to the figure above, are approximately Student distributed with 6 and 3 degrees of freedom, respectively, when no time trend exists.

Note:

- It has been assumed here that the individual data are Gaussian distributed (each one of them is the sum of 7 independent stochastic variables) and, therefore, if \( \mu \) and \( \mu' \) are the corresponding true mean values,

\[ \frac{\mu - m}{S} \sqrt{6} \]

and

\[ \frac{\mu' - m'}{S'} \sqrt{3} \]
are approximately Student distributed with 6 and 3 degrees of freedom, respectively, when no time trend exists.

- Step (b) above is suggested in order to always get a numerical value for the accumulation in a standard format, even in those cases where a time trend is present.

  Expression (6) then gives a kind of 95 per cent confidence interval.

- This form of analysis is simple and gives the experimenter an immediate impression of how well his experiment has worked.

**Test report**

The test report should comprise at least the following sections:

- Properties of the test material - water solubility, basis for assuming that exposures will be below the toxic effect concentration for the test species and test conditions, and the analytical limits of detection for the parent compound(s) in fish and water

- Fish and water – species, size, number of fish per exposure, diet composition, aquaria size, fish-to-volume loading, frequency of water replacement, temperature and water chemistry values

- Exposure concentrations and decrease in concentration of test substance between renewals of test solution

- Uptake duration

- Fish sampling schedule

4. **Literature**
