

# OECD GUIDELINES FOR TESTING OF CHEMICALS

## PROPOSAL FOR UPDATING GUIDELINE 305

### Bioconcentration: Flow-through Fish Test

#### INTRODUCTION

1. In the Detailed Review Paper on Bioaccumulation prepared by Japan (February 1990), it was recommended that the existing five Guidelines for bioaccumulation, 305A to E, be consolidated into a single method. At the same time, work was proceeding on the updating of Guideline 305 E, Flow-through Fish Test (1), modified as a result of an EEC "ring-test" (2), and it was agreed that this modified version of 305 E should form the basis of the consolidated Guideline.

2. This present Guideline 305 consolidates and replaces the previous Guidelines 305 A to E. It describes a procedure for characterising the bioconcentration<sup>(1)</sup> potential of substances in fish under flow-through conditions. Although flow-through test regimes are much to be preferred, semi-static regimes are permissible, provided that the validity criteria (see paragraph 12) are satisfied.

3. Before carrying out a test for bioconcentration, the following information about the test substance should be known :

- (a) solubility in water [Guideline 105];
- (b) octanol-water partition coefficient,  $P_{ow}$ <sup>(2)</sup> [Guidelines 107, 117];
- (c) hydrolysis [Guideline 111];
- (d) phototransformation in water determined under solar or simulated solar irradiation and under the irradiation conditions of the test for bioconcentration [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) [Guideline 115];
- (f) vapour pressure [Guideline 104];
- (g) ready biodegradability (where appropriate) [Guidelines 301 A to F].

4. 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. It is most validly applied to stable organic chemicals with  $\log P_{ow}$  values between 1.5 and 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  $BCF_{ss}$ .

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(1) See Annex 1 for definitions and units.

(2) Sometimes denoted by  $K_{ow}$ ; determined by an HPLC method in Guideline 117.

5. 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.

### **PRINCIPLE OF THE TEST**

6. 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). 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.

7. The uptake phase is run for 28 days unless it is demonstrated that equilibrium has been reached earlier (see Annex 1.4). A prediction of the length of the uptake phase and the time to steady-state can be made from equations in Annex 4. The depuration period 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,  $BCF_K$  (Annex 1.5) as the ratio of the rate constants of uptake ( $k_1$ ) and depuration ( $k_2$ ) assuming first-order kinetics<sup>(3)</sup>.

8. If a steady-state is not achieved within 28 days, the uptake phase should be extended until steady-state is reached, or 60 days, whichever comes first; the depuration phase is then begun.

9. 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.

10. 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

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(3) If first-order kinetics are obviously not obeyed, more complex models should be employed (see Annex 6).

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.

### **INFORMATION ON THE TEST SUBSTANCE**

11. In addition to the properties of the test substance given in the Introduction (paragraph 3) other information required is the toxicity to the fish species to be used in the test, preferably the asymptotic  $LC_{50}$  (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  $^{14}C$  labelled test substance is used, the percentage of radioactivity associated with impurities should be known.

### **VALIDITY OF THE TEST**

12. For a test to be valid the following conditions apply:

- the temperature variation is less than  $\pm 2^{\circ}C$ ;
- the concentration of dissolved oxygen does not fall below 60% saturation;
- the concentration of the test substance in the chambers is maintained within  $\pm 20\%$  of the mean of the measured values during the uptake phase;
- the mortality or other adverse effects/disease in both control and treated fish is less than 10% at the end of the test; where the test is extended over several weeks or months, death or other adverse effects in both sets of fish should be less than 5% per month and not exceed 30% in all.

### **REFERENCE COMPOUNDS**

13. The use of reference compounds of known bioconcentration potential would be useful in checking the experimental procedure, when required. However, specific substances cannot yet be recommended.

### **DESCRIPTION OF THE METHOD**

#### **Apparatus**

14. Care should be taken to avoid the use of materials, for all parts of the equipment, that can dissolve, sorb or leach and have an adverse effect on the fish. Standard rectangular or cylindrical tanks, made of chemically inert material and of a suitable capacity in compliance with loading rate (see paragraph 31), can be used. The use of soft plastic tubing should be minimised. Use teflon (R), stainless steel and/or glass tubing. Experience has shown that for substances with high adsorption coefficient, such as the synthetic pyrethroids, silanized glass may be required. In these situations the equipment will have to be discarded after use.

#### **Water**

15. Natural water is generally used in the test and should be obtained from uncontaminated and uniform quality source. The dilution water must be of a quality that will allow the survival of the chosen fish species for the duration of the acclimation and test periods without them showing any abnormal appearance or behaviour. Ideally, it should be demonstrated that the test species can survive, grow and reproduce in the dilution water (e.g. in laboratory culture or a life-cycle toxicity test). The water should be characterised at least by pH, hardness, total solids, total organic carbon and, preferably also ammonium, nitrite and alkalinity and, for marine species, salinity. The parameters which are important for optimal fish well-being are not fully known, but Annex 2 gives recommended maximum concentrations of a number of parameters for fresh and marine test waters.

16. The water should be of constant quality during the period of a test. The pH value should be within the range 6.0 to 8.5, but during a given test it should be within a range of  $\pm 0.5$  pH units. In order to ensure that the dilution water will not unduly influence the test result (for example, by complexation of the test substance) or adversely affect the performance of the stock of fish, samples should be taken at intervals for analysis. Determination of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl,  $\text{SO}_4$ ), pesticides (e.g. total organophosphorous and total organochlorine pesticides), total organic carbon and suspended solids should be made, for example, every three months where a dilution water is known to be relatively constant in quality. If water quality has been demonstrated to be constant over at least one year, determinations can be less frequent and intervals extended (e.g. every six months).

17. The natural particle content as well as the total organic carbon (TOC) of the dilution water should be as low as possible to avoid adsorption of the test substance to organic matter which may reduce its bioavailability. The maximum acceptable value is 5 mg/l for particulate matter (dry matter, not passing a 0.45  $\mu\text{m}$  filter) and 2 mg/l for total organic carbon (see Annex 2). If necessary, the water should be filtered before use. The contribution to the organic carbon content in water from the test fish (excreta) and from the food residues should be as low as possible. Throughout the test, the concentration of organic carbon in the test vessels should not exceed the concentration of organic carbon originating from the test substance and, if used, the solubilising agent by more than 10 mg/l ( $\pm 20\%$ ).

### **Test Solutions**

18. Prepare a stock solution of the test substance at a suitable concentration. The stock solution should preferably be prepared by simply mixing or agitating the test substance in the dilution water. The use of solvents or dispersants (solubilising agents) is not recommended (see paragraphs 38 and 39); however this may occur in some cases in order to produce a suitably concentrated stock solution. Solvents which may be used are, ethanol, methanol, ethylene glycol monomethyl ether, ethylene glycol dimethyl ether, dimethylformamide and triethylene glycol. Dispersants which may be used are Cremophor RH40, Tween 80, methylcellulose 0.01% and HCO-40. Care should be taken when using readily biodegradable agents as these can cause problems with bacterial growth in flow-through tests. The test substance may be radiolabelled and should be of the highest purity (e.g. preferably  $>98\%$ ).

19. For flow-through tests, a system which continuously dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) is required to deliver the test concentrations to the test chambers. Preferably allow at least five volume replacements through each test chamber per day. The flow-through mode is to be preferred, but where this is not possible (e.g. when the test organisms are adversely affected) a semi-static technique may be used provided that the validity criteria are satisfied (see paragraph 12). The flow rates of stock solutions and dilution water should be checked both 48 hours before and then at least daily during the test. Include in this check the determination of the flow-rate through each test chamber and ensure that it does not vary by more than 20% either within or between chambers.

### **Selection of species**

20. Important criteria in the selection of species are that they are readily available, can be obtained in convenient sizes and can be satisfactorily maintained in the laboratory. Other criteria for selecting fish species include recreational, commercial, ecological importance as well as comparable sensitivity, past successful use etc. Recommended test species are given in Annex 3. Other species may be used but the test procedure may have to be adapted to provide suitable test conditions. The rationale for the selection of the species and the experimental method should be reported in this case.

### **Holding of fish**

21. Acclimate the stock population of fish for at least two weeks in water (see paragraph 16) at the test temperature and feed throughout on a sufficient diet (see paragraph 33) and of the same type to be used during the test.

22. Following a 48-hour settling-in period, mortalities are recorded and the following criteria applied:

- mortalities of greater than 10% of population in seven days: reject the entire batch;
- mortalities of between 5 and 10% of population in seven days: acclimate for seven additional days;
- mortalities of less than 5% of population in seven days: accept the batch - if more than 5% mortality during second seven days reject the entire batch.

23. Ensure that fish used in tests are free from observable diseases and abnormalities. Discard any diseased fish. Fish should not receive treatment for disease in the two weeks preceding the test, or during the test.

## **PERFORMANCE OF THE TEST**

### **Preliminary Test**

24. It may be useful to conduct a preliminary experiment in order to optimise the test conditions of the definitive test, e.g. selection of test substance concentration(s), duration of the uptake and depuration phases.

### **Conditions of Exposure**

#### **Duration of uptake phase**

25. A prediction of the duration of the uptake phase can be obtained from practical experience (e.g. from a previous study or an accumulation study on a structurally related chemical) or from certain empirical relationships utilising knowledge of either the aqueous solubility or the octanol/water partition coefficient of the test substance (see Annex 4).

26. The uptake phase should be run for 28 days unless it can be demonstrated that equilibrium has been reached earlier (see Annex 1.4). If the steady-state has not been reached by 28 days, the uptake

phase should be extended, taking further measurements, until steady-state is reached or 60 days, whichever is shorter.

### **Duration of the depuration phase**

27. A period of half the duration of the uptake phase is usually sufficient for an appropriate (e.g. 95%) reduction in the body burden of the substance to occur (see Annex 4 for explanation of the estimation). If the time required to reach 95% loss is impractically long, exceeding for example twice the normal duration of the uptake phase (i.e. more than 56 days) a shorter period may be used (e.g. until the concentration of test substance is less than 10% of steady-state concentration). However, for substances having more complex patterns of uptake and depuration than are represented by a one-compartment fish model, yielding first order kinetics, allow longer depuration phases for determination of loss rate constants. The period may, however, be governed by the period over which the concentration of test substance in the fish remains above the analytical detection limit.

### **Numbers of test fish**

28. Select the numbers of fish per test concentration such that a minimum of four fish per sample are available at each sampling. If greater statistical power is required, more fish per sample will be necessary.

29. If adult fish are used, report whether male or female, or both are used in the experiment. If both sexes are used, differences in lipid content between sexes should be documented to be non-significant before the start of the exposure; pooling all male and all female fish may be necessary.

30. In any one test, select fish of similar weight such that the smallest are no smaller than two-thirds of the weight of the largest. All should be of the same year-class and come from the same source. Since weight and age of a fish appear sometimes to have a significant effect on BCF values (4) record these details accurately. It is recommended that a sub-sample of the stock of fish is weighed before the test in order to estimate the mean weight (paragraph 46).

### **Loading**

31. Use high water-to-fish ratios in order to minimise the reduction in  $C_w$  caused by the addition of the fish at the start of the test and also to avoid decreases in dissolved oxygen concentration. It is important that the loading rate is appropriate for the test species used. In any case, a loading rate of 0.1-1.0 g of fish (wet weight) per litre of water per day is normally recommended. High loading rates can be used if it is shown that the required concentration of test substance can be maintained within  $\pm 20\%$  limits, and that the concentration of dissolved oxygen does not fall below 60% saturation.

32. In choosing appropriate loading regimes, take account of the normal habitat of the fish species. For example, bottom-living fish may demand a larger bottom area of the aquarium for the same volume of water than pelagic fish species.

### **Feeding**

33. During the acclimation and test periods, feed an appropriate diet of known lipid and total protein content to the fish in an amount sufficient to keep them in a healthy condition and to maintain body weight. Feed daily throughout the acclimation and test periods at a level of approximately 1 to 2% of body weight per day; this keeps the lipid concentration in most species of fish at a relatively constant level during the test. The amount of feed should be re-calculated, for example, once per week, in order to maintain consistent body weight and lipid content. For this calculation, the weight of the fish in each

test chamber can be estimated from the weight of the fish sampled most recently in that chamber. Do not weigh the fish remaining in the chamber.

34. Siphon uneaten food and faeces daily from the test chambers shortly after feeding (30 minutes to 1 hour). Keep the chambers as clean as possible throughout the test so that the concentration of organic matter is kept as low as possible (see paragraph 17), since the presence of organic carbon may limit the bioavailability of the test substance (4).

35. Since many feeds are derived from fishmeal, the feed should be analysed for the test substance. It is also desirable to analyse the feed for pesticides and heavy metals.

### **Light and temperature**

36. The photoperiod is usually 12 to 16 hours and the temperature ( $\pm 2^\circ\text{C}$ ) should be appropriate for the test species (see Annex 3). The type and characteristics of illumination should be known. Caution should be given to the possible phototransformation of the test substance under the irradiation conditions of the study. Appropriate illumination should be used avoiding exposure of fish to unnatural photoproducts. In some cases it may be appropriate to use a filter to screen out UV irradiation below 290 nm.

### **Test concentrations**

37. Expose fish under flow-through conditions to at least two concentrations of the test substance in water. Normally, select the higher (or highest) concentration of the test substance to be about 1% of its acute asymptotic  $\text{LC}_{50}$ , and to be at least ten-fold higher than its detection limit in water by the analytical method used. The highest test concentration can also be determined by dividing the acute 96h  $\text{LC}_{50}$  by an appropriate acute/chronic ratio (e.g. appropriate ratios for some chemicals are about 3, but a few are above 100). If possible, choose the other concentration(s) such that it differs from the one above by a factor of ten. If this is not possible because of the 1% of  $\text{LC}_{50}$  criterion and the analytical limit, a lower factor than ten can be used or the use of  $^{14}\text{C}$  labelled test substance should be considered. No concentration used should be above the solubility of the test substance.

38. Where a solubilising agent is used its concentration should not be greater than 0.1 ml/l and should be the same in all test vessels (paragraph 18). Its contribution (together with the test substance) to the overall content of organic carbon in the test water should be known. However, every effort should be made to avoid the use of such materials.

### **Controls**

39. One dilution water control or if relevant, one control containing the solubilising agent should be run in addition to the test series, provided that it has been established that the agent has no effects on the fish. If not, both controls should be set up.

### **Frequency of Water Quality Measurements**

40. During the test, dissolved oxygen, TOC, pH and temperature should be measured in all vessels. Total hardness and salinity (if relevant) should be measured in the control(s) and one vessel at the higher (or highest) concentration. As a minimum, dissolved oxygen and salinity (if relevant) should be measured three times - at the beginning, around the middle and end of the uptake period - and once a week in the depuration period. TOC should be measured at the beginning of the test (24h and 48h prior to test initiation of uptake phase) before addition of the fish and, at least once a week, during both uptake and depuration phases. Temperature should be measured daily, pH at the beginning and end of

each period and hardness once each test. Temperature should preferably be monitored continuously in at least one vessel.

### **Sampling and Analysis of Fish and Water**

#### **Fish and water sampling schedule**

41. Sample water from the test chambers for the determination of test substance concentration before addition of the fish and during both uptake and depuration phases. As a minimum, sample the water at the same time as the fish and before feeding. During the uptake phase, the concentrations of test substance are determined in order to check compliance with the validity criteria (paragraph 12).

42. Sample fish on at least five occasions during the uptake phase and at least on four occasions during the depuration phase. Since on some occasions it will be difficult to calculate a reasonably precise estimate of the BCF value based on this number of samples (especially when other than simple first-order depuration kinetics are indicated), it may be advisable to take samples at a higher frequency in both periods (see Annex 5). Store the extra samples as described in paragraph 48 and analyse them only if the results of the first round of analyses prove inadequate for the calculation of the BCF with the desired precision.

43. An example of an acceptable sampling schedule is given in Annex 5. Other schedules can readily be calculated using other assumed values of  $P_{ow}$  to calculate the exposure time for 95% uptake.

44. Continue sampling during the uptake phase until a steady-state has been established (see Annex 1.4) or for 28 days, whichever is the shorter. If the steady-state has not been reached within 28 days continue until a steady-state has been attained or 60 days, whichever is shorter (see paragraphs 25 and 26). Before beginning the depuration phase transfer the fish to clean tanks.

#### **Sampling and sample preparation**

45. Obtain water samples for analysis e.g. by siphoning through inert tubing from a central point in the test chamber. Since neither filtration nor centrifuging appears always to separate the non-bioavailable fraction of the test substance from that which is bioavailable (especially for super-lipophilic chemicals i.e. those chemicals with a  $\log P_{ow} > 5$ ) (4) (7), samples may not be subjected to those treatments. Instead, measures should be taken to keep the tanks as clean as possible (see paragraph 34) and the content of total organic carbon should be monitored during both the uptake and depuration phases (see paragraph 40).

46. Remove an appropriate number of fish (normally a minimum of four) from the test chambers at each sampling time. Rinse the sampled fish quickly with water (paragraph 16), blot "dry", kill instantly, using the most appropriate and humane method, and then weigh.

47. It is preferable to analyse fish and water immediately after sampling in order to prevent degradation or other losses and to calculate approximate uptake and depuration rates as the test proceeds. Immediate analysis also avoids delay in determining when a plateau has been reached.

48. Failing immediate analysis, store the samples by an appropriate method. Obtain, before the beginning of the study, information on the proper method of storage for the particular test substance - for example, deep-freezing, holding at 4°C, duration of storage, extraction, etc.

#### **Quality of analytical method**

49. Since the whole procedure is governed essentially by the accuracy, precision and sensitivity of the analytical method used for the test substance, check experimentally that the precision and reproducibility of the chemical analysis, as well as recovery of the test substance from both water and fish are satisfactory for the particular method. Also, check that the test substance is not detectable in the dilution water used. If necessary, correct the values of  $C_w$  and  $C_f$  obtained from the test for the recoveries and background values of controls. Handle the fish and water samples throughout in such a manner as to minimise contamination and loss (e.g. resulting from adsorption by the sampling device).

### **Analysis of fish samples**

50. If radiolabelled materials are used in the test, it is possible to analyse for total radio label (i.e. parent and metabolites) or, the samples may be cleaned up so that parent compound can be analysed separately. Also, the major metabolites may be characterised at steady-state or at the end of the uptake phase, whichever is the sooner (see paragraph 5). If the BCF in terms of total radiolabelled residues is  $\geq 1000$ , it may be advisable, and for certain categories of chemicals such as pesticides strongly recommended, to identify and quantify degradates representing  $\geq 10\%$  of total residues in fish tissues at steady state. If degradates representing  $\geq 10\%$  of total radiolabelled residues in the fish tissue are identified and quantified, then it is also recommended to identify and quantify degradates in the test water.

51. The concentration of the test substance should usually be determined for each weighed individual fish. If this is not possible, pooling of the samples on each sampling occasion may be done but pooling does restrict the statistical procedures which can be applied to the data. If a specific statistical procedure and power are important considerations, then an adequate number of fish to accommodate the desired pooling, procedure and power, should be included in the test. See references (8) and (9) for an introduction to relevant pooling procedures.

52. BCF should be expressed both as a function of total wet weight and, for high lipophilic substances, as a function of the lipid content (see paragraph 10). Determine the lipid content of the fish on each sampling occasion if possible. Suitable methods should be used for determination of lipid content (see reference 10 and Annex II : reference 2). Chloroform/methanol extraction technique may be recommended as standard method (11). The various methods do not give identical values (12), so it is important to give details of the method used. When possible, the analysis for lipid should be made on the same extract as that produced for analysis for the test substance, since the lipids often have to be removed from the extract before it can be analysed chromatographically. The lipid content of the fish (as mg/kg wet weight) at the end of the experiment should not differ from that at the start by more  $\pm 25\%$ . The tissue percent solids should also be reported to allow conversion of lipid concentration from a wet to a dry basis.

## **DATA AND REPORTING**

### **Treatment of results**

53. Obtain the uptake curve of the test substance by plotting its concentration in/on fish (or specified tissues) in the uptake phase against time on arithmetic scales. If the curve has reached a plateau, that is, become approximately asymptotic to the time axis, calculate the steady state  $BCF_{ss}$  from:

54. When no steady state is reached, it may be possible to calculate a  $BCF_{ss}$  of sufficient precision

$$\frac{C_f \text{ at steady - state (mean)}}{C_w \text{ at steady - state (mean)}}$$

for hazard assessment from a "steady-state" at 80% ( $1.6/k_2$ ) or 95% ( $3.0/k_2$ ) of equilibrium.

55. Also, determine the concentration factor ( $BCF_k$ ) as the ratio  $k_1/k_2$ , the two first-order kinetic constants. The depuration rate constant ( $k_2$ ) is usually determined from the depuration curve (i.e. a plot of the decrease in test substance concentration in the fish with time). The uptake rate constant ( $k_1$ ) is then calculated given  $k_2$  and a value of  $C_f$  which is derived from the uptake curve. See Annex 6 for a description of these methods. The preferred method for obtaining  $BCF_k$  and the rate constants,  $k_1$  and  $k_2$ , is to use non-linear parameter estimation methods on a computer (2). Otherwise, graphical methods may be used to calculate  $k_1$  and  $k_2$ . If the depuration curve is obviously not first-order, then more complex models should be employed (see references in Annex 4) and advice from a biostatistician sought.

### **Interpretation of results**

56. The results should be interpreted with caution where measured concentrations of test solutions occur at levels near the detection limit of the analytical method.

57. Clearly defined uptake and loss curves are an indication of good quality bioconcentration data. The variation in uptake/depuration constants between the two test concentrations should be less than 20%. Observed significant differences in uptake/depuration rates between the two applied test concentrations should be recorded and possible explanations given. Generally the confidence limit of BCFs from well-designed studies approach  $\pm 20\%$ .

### **Test Report**

58. The test report must include the following information:

Test substance:

- physical nature and, where relevant, physicochemical properties;
- chemical identification data (including the organic carbon content, if appropriate);
- if radiolabelled, the precise position of the labelled atom(s) and the percentage of radioactivity associated with impurities.

Test species:

- scientific name, strain, source, any pretreatment, acclimation, age, size-range, etc.

Test conditions:

- test procedure used (e.g. flow-through or semi-static);
- type and characteristics of illumination used and photoperiod(s);
- test design (e.g. number and size of test chambers, water volume replacement rate, number of replicates, number of fish per replicate, number of test concentrations, length of uptake and depuration phases, sampling frequency for fish and water samples);

- method of preparation of stock solutions and frequency of renewal (the solubilising agent, its concentration and its contribution to the organic carbon content of test water must be given, when used);
- the nominal test concentrations, the means of the measured values and their standard deviations in the test vessels and the method by which these were attained;
- source of the dilution water, description of any pretreatment, results of any demonstration of the ability of test fish to live in the water, and water characteristics: pH, hardness, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), total organic carbon, suspended solids, salinity of the test medium (if appropriate) and any other measurements made;
- water quality within test vessels, pH, hardness, TOC, temperature and dissolved oxygen concentration;
- detailed information on feeding (e.g. type of food(s), source, composition - at least lipid and protein content if possible, amount given and frequency);
- information on the treatment of fish and water samples, including details of preparation, storage, extraction and analytical procedures (and precision) for the test substance and lipid content (if measured).

#### Results:

- results from any preliminary study performed;
- mortality of the control fish and the fish in each exposure chamber and any observed abnormal behaviour;
- the lipid content of the fish (if determination on testing occasion);
- curves, (including all measured data,) showing the uptake and depuration of the test chemical in the fish, the time to steady-state;
- $C_f$  and  $C_w$  (with standard deviation and range, if appropriate) for all sampling times ( $C_f$  expressed in mg/g wet weight (ppm) of whole body or specified tissues thereof e.g. lipid, and  $C_w$  in mg/ml (ppm).  $C_w$  values for the control series (background should also be reported);
- the steady-state bioconcentration factor, ( $BCF_{ss}$ ), and/or kinetic concentration factor ( $BCF_k$ ) and if applicable, 95% confidence limits for the uptake and depuration (loss) rate constants (all expressed in relation to the whole body and the total lipid content, if measured, of the animal or specified tissues thereof), confidence limits and standard deviation (as available) and methods of computation/data analysis for each concentration of test substance used;
- where radiolabelled substances are used, and if it is required, the accumulation of any detected metabolites may be presented;
- anything unusual about the test, any deviation from these procedures, and any other relevant information.

59. Minimise results reported as "not detected at the limit of detection" by pre-test method development and experimental design, since such results cannot be used for rate constant calculations.

#### LITERATURE

- (1) OECD, Paris (1993). OECD Guidelines for testing of chemicals.
- (2) CEC, Bioaccumulation of chemical substances in fish: the flow-through method - Ring Test Programme, 1984-1985 Final report, March 1987. Authors: P. Kristensen and N. Nyholm.

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## ANNEX 1

**DEFINITIONS AND UNITS**

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 bioconcentration factor ( $BCF_{ss}$  or  $K_B$ ) 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.
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.
5. Bioconcentration factors 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 Guideline 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  $\text{day}^{-1}$ ).
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  $\text{day}^{-1}$ ).

## ANNEX 2

**SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER**

<b>Substance</b>	<b>Limit concentration</b>
Particulate matter	5 mg/l
Total organic carbon	2 mg/l
Un-ionised ammonia	1 µg/l
Residual chlorine	10 µg/l
Total organophosphorous pesticides	50 ng/l
Total organochlorine pesticides plus polychlorinated biphenyls	50 ng/l
Total organic chlorine	25 ng/l
Aluminium	1 µg/l
Arsenic	1 µg/l
Chromium	1 µg/l
Cobalt	1 µg/l
Copper	1 µg/l
Iron	1 µg/l
Lead	1 µg/l
Nickel	1 µg/l
Zinc	1 µg/l
Cadmium	1 µg/l
Mercury	100 ng/l
Silver	100 ng/l
	100 ng/l

## ANNEX 3

**FISH SPECIES RECOMMENDED FOR TESTING**

Recommended species	Recommended range of test temperature (°C)	Recommended total length of test animal (cm)
<u>Danio rerio</u> <sup>(1)</sup> (Teleostei, Cyprinidae) (Hamilton-Buchanan) Zebra-fish	20 - 25	3.0 ± 0.5
<u>Pimephales promelas</u> (Teleostei, Cyprinidae) (Rafinesque) Fathead minnow	20 - 25	5.0 ± 2.0
<u>Cyprinus carpio</u> (Teleostei, Cyprinidae) (Linnaeus) Common carp	20 - 25	5.0 ± 3.0
<u>Oryzias latipes</u> (Teleostei, Poeciliidae) (Temminck and Schlegel) Ricefish	20 - 25	4.0 ± 1.0
<u>Poecilia reticulata</u> (Teleostei, Poeciliidae) (Peters) Guppy	20 - 25	3.0 ± 1.0
<u>Lepomis macrochirus</u> (Teleostei Centrarchidae) (Rafinesque) Bluegill	20 - 25	5.0 ± 2.0
<u>Oncorhynchus mykiss</u> (Teleostei Salmonidae) (Walbaum) Rainbow trout	13 - 17	8.0 ± 4.0
<u>Gasterosteus aculeatus</u> (Teleostei, Gasterosteidae) (Linnaeus) Three-spined stickleback	18 - 20	3.0 ± 1.0

(1) Meyer A., Orti G. (1993) Proc. Royal Society of London, Series B, Vol.252, p.231.

Various estuarine and marine species have been used in different countries, for example:

Spot	( <u>Leiostomus xanthurus</u> )
Sheepshead minnow	( <u>Cyprinodon variegatus</u> )
Silverside	( <u>Menidia beryllina</u> )
Shiner perch	( <u>Cymatogaster aggregata</u> )
English sole	( <u>Parophrys vetulus</u> )
Staghorn sculpin	( <u>Leptocottus armatus</u> )
Three-spined stickleback	( <u>Gasterosteus aculeatus</u> )
Sea bass	( <u>Dicentracus labrax</u> )
Bleak	( <u>Alburnus alburnus</u> )

### COLLECTION

The freshwater fish listed in the table above are easy to rear and/or are widely available throughout the year, whereas the availability of marine and estuarine species is partially confined to the respective countries. They are capable of being bred and cultivated either in fish farms or in the laboratory, under disease-and parasite-controlled conditions, so that the test animal will be healthy and of known parentage. These fish are available in many parts of the world.

## ANNEX 4

**PREDICTION OF THE DURATION OF THE UPTAKE AND DEPURATION PHASES****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  $k_2$  ( $\text{day}^{-1}$ ) may be obtained, for example from the following empirical relationship (1):

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

For other relationships see (2).

If the partition coefficient ( $P_{ow}$ ) is not known, an estimate can be made (3) from a knowledge of the aqueous solubility (s) of the substance using:

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

where s=solubility (moles/l): (n=36)

These relationships apply only to chemicals with  $\log P_{ow}$  values between 2 and 6.5 (4).

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:

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

[equation 3]

When "steady-state" is approached ( $t \rightarrow \infty$ ), equation 3 may be reduced (see (5) (6)) 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" ( $C_{fs}$ ).

Equation 3 may be transcribed to:

or

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

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

[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 (7).

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

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

or

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

[equation 5]

Similarly 95 percent of "steady-state" is:

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

[equation 6]

For example, the duration of the uptake phase (up) for a test substance with  $\log P_{ow} = 4$  would be (using equations 1, 5 and 6):

$$\begin{aligned} \log_{10} k_2 &= -0.414 \cdot (4) + 1.47 \\ k_2 &= 0.652 \text{ days}^{-1} \end{aligned}$$

or

up (80 pct) =  $1.6/0.652$ , i.e. 2.45 days (59 hours)  
 up (95 pct) =  $3.0/0.652$ , i.e. 4.60 days (110 hours)

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

$$\begin{aligned} \log_{10} (P_{ow}) &= -0.862 \cdot (-5.0) + 0.710 = 5.02 \\ \log_{10} k_2 &= -0.414 \cdot (5.02) + 1.47 \\ k_2 &= 0.246 \text{ days}^{-1} \\ \text{up (80 pct)} &= 1.6/0.246, \text{ i.e. } 6.5 \text{ days (156 hours)} \\ \text{or up (95 pct)} &= 3.0/0.246, \text{ i.e. } 12.2 \text{ days (293 hours)} \end{aligned}$$

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 (4).

## 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) (1) (8).

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

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

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

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

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

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 (1)).

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## ANNEX 5

Theoretical example of sampling schedule for bioconcentration tests of substances with  $\log P_{ow} = 4$ .

Fish Sampling	Sample time schedule		No. of water samples	No. of fish per sample
	Minimal required frequency (days)	Additional sampling (days)		
<b>Uptake phase</b>	- 1 0		2* 2	<b>add 45-80 fish</b>
1st	0.3	0.4	2 (2)	4 (4)
2nd	0.6	0.9	2 (2)	4 (4)
3rd	1.2	1.7	2 (2)	4 (4)
4th	2.4	3.3	2 (2)	4 (4)
5th	4.7		2	6
<b>Depuration phase</b>				<b>Transfer fish to water free of test chemical</b>
6th	5.0	5.3		4 (4)
7th	5.9	7.0		4 (4)
8th	9.3	11.2		4 (4)
9th	14.0	17.5		6 (4)

\* Sample water after minimum of 3 "chamber-volumes" have been delivered.

Values in brackets are numbers of samples (water, fish) to be taken if additional sampling is carried out.

Note: Pre-test estimate of  $k_2$  for  $\log P_{ow}$  of 4.0 is  $0.652 \text{ days}^{-1}$ . The total duration of the experiment is set to  $3 \times t_{up} = 3 \times 4.6$  days, i.e. 14 days. For the estimation of  $t_{up}$  refer to Annex 4.

## ANNEX 6

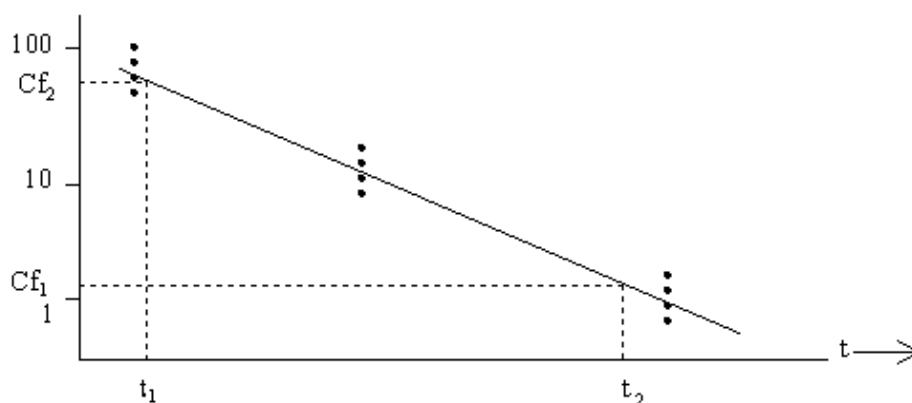
**MODEL DISCRIMINATION**

Most bioconcentration data have been assumed to be "reasonably" well described by a simple two-compartment/two-parameter model, as indicated by the rectilinear curve which approximates to the points for concentrations in fish, during the depuration phase, when these are plotted on semi-log paper. (Where these points cannot be described by a rectilinear curve then more complex models should be employed, see, for example, Spacie and Hamelink, Ref 1 in Annex 4).

**GRAPHICAL METHOD FOR DETERMINATION OF DEPURATION (LOSS) RATE CONSTANT  $k_2$** 

Plot the concentration of the test substance found in each sample of fish against sampling time on semi-log paper. The slope of that line is  $k_2$ .

$$k_2 = \frac{\ln (C_{f1} / C_{f2})}{t_2 - t_1}$$



Note that deviations from a straight line may indicate a more complex depuration pattern than first order kinetics. A graphical method may be applied for resolving types of depuration deviating from first order kinetics.

**GRAPHICAL METHOD FOR DETERMINATION OF UPTAKE RATE CONSTANT  $k_1$** 

Given  $k_2$ , calculate  $k_1$  as follows:

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

[equation 1]

The value of  $C_f$  is read from the midpoint of the smooth uptake curve produced by the data when log concentration is plotted versus time (on an arithmetical scale).

**COMPUTER METHOD FOR CALCULATION OF UPTAKE AND DEPURATION (LOSS) RATE CONSTANTS**

The preferred means for obtaining the bioconcentration factor and  $k_1$  and  $k_2$  rate constants is to use non-linear parameter estimation methods on a computer. These programs find values for  $k_1$  and  $k_2$  given a set of sequential time concentration data and the model:

$$C_f = C_w \cdot \frac{k_1}{k_2} \times (1 - e^{-k_2 t}) \quad 0 < t < t_c \quad \text{[equation 2]}$$

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

where  $t_c$  = time at the end of the uptake phase.

This approach provides standard deviation estimates of  $k_1$  and  $k_2$ .

As  $k_2$  in most cases can be estimated from the depuration curve with relatively high precision, and because a strong correlation exists between the two parameters  $k_1$  and  $k_2$  if estimated simultaneously, it may be advisable first to calculate  $k_2$  from the depuration data only, and subsequently calculate  $k_1$  from the uptake data using non-linear regression.