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OECD GUIDELINES FOR TESTING OF CHEMICALS

Bioaccumulation in Fish: Aqueous and Dietary Exposure

INTRODUCTION

1. The major goal of this revision of Test Guideline 305 is two-fold. Firstly, it is intended to incorporate a dietary bioaccumulation [(1)] test suitable for determining the bioaccumulation potential of substances with very low water solubility. Secondly, it is intended to create a Test Guideline that, when appropriate, utilises fewer fish for animal welfare reasons, and that is more cost-effective.

2. In the years since adoption of the consolidated Test Guideline (TG) in 1996 (1), numerous substances have been tested, and considerable experience has been gained both by laboratories and by regulatory authorities. This has led to the conviction that the complexity of the test can be reduced if specific criteria are met (*cf.* paragraph 88), and that a tiered approach is possible. Experience has also shown that biological factors such as growth and fish lipid content can have a strong impact on the results and may need to be taken into account. In addition, it has been recognised that testing very poorly water soluble substances may not be technically feasible. In addition, for substances with very low water solubility in the aquatic environment, exposure via water may be of limited importance in comparison to the dietary route. This has led to the development of a test method in which fish are exposed via their diet (*cf.* paragraph 7-14 and 97 onwards). Validation (ring test) of the dietary exposure test was conducted in 2010 (51).

The main changes include:

- The testing of only one test concentration can be considered sufficient, when it is likely that the bioconcentration factor (BCF) is independent of the test concentration.
- A minimised aqueous exposure test design in which a reduced number of sample points is possible, if specific criteria are met.
- Fish lipid content should be measured so that BCF can be expressed on a 5% lipid content basis.
- Greater emphasis on kinetic BCF estimation (when possible) next to estimating the BCF at steady state.
- For certain groups of substances, a dietary exposure test will be proposed, where this is considered more suitable than an aqueous exposure test.
- Fish weight should be measured so that BCF_k can be corrected for growth dilution.

3. Before carrying out any of the bioaccumulation tests, the following information about the test substance should be known:

(1) See Annex 1 for definitions and units

(a) Sensitivity of the analytical technique for measuring tissue and aqueous or food concentrations of both the test substance and possible metabolites (*cf.* paragraph 65).

(b) Solubility in water [TG 105; (2)]; this should be determined in accordance with a method that is appropriate for the (estimated) range of the solubility to obtain a reliable value. For hydrophobic substances, this will generally be the column elution method.

(c) n-Octanol-water partition coefficient, K_{OW}[(2)] [TGs 107 (4), 117 (5), 123 (6)]; or other suitable information on partitioning behaviour (*e.g.* sorption to lipids, K_{OC}); this should be determined in accordance with a method that is appropriate for the (estimated) range of the K_{OW} to obtain a reliable value. For hydrophobic substances, this will generally be the slow-stirring method [TG 123 (6)];

(d) Substance stability in water (hydrolysis [TG 111 (7)];

(e) Substance stability in food (specifically when a dietary exposure test approach is chosen);

(f) Information on phototransformation relevant for the irradiation conditions in the test (8);

(g) Surface tension (*i.e.* for substances where the log K_{OW} cannot be determined) [TG 115 (9)];

(h) Vapour pressure [TG 104 (10)];

(i) Any information on biotic or abiotic degradation in water, such as (but not restricted to) ready biodegradability [TGs 301 A to F (11), 310 (12)], where appropriate;

(j) Information on metabolites: structure, log K_{OW}, formation and degradability, where appropriate;

(k) Acid dissociation constant (pK_a) for substances that might ionise. If necessary, the pH of the test water should be adjusted to ensure that the substance is in the unionised form in the test if compatible with fish species.

4. Independent of the chosen exposure method or sampling scheme, this TG describes a procedure for characterising the bioaccumulation potential of substances in fish. Although flow-through test regimes are much to be preferred, semi-static regimes are permissible, provided that the validity criteria (*cf.* paragraphs 24 and 113) are satisfied. In the dietary exposure route, the flow-through system is not necessary to maintain aqueous concentrations of the tested substance, but will help maintain adequate dissolved oxygen concentrations and help ensure clean water and remove influences of *e.g.* excretion products.

5. Independent of the chosen test method, sufficient details are given in this TG 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. The aqueous exposure test is most appropriately applied to stable organic chemicals with log K_{OW} values between 1.5 and 6.0 (13)

(2) Sometimes denoted by P_{OW}; determined by a shake-flask method in TG 107 (3), an HPLC method in TG 117 (4) and a slow-stirring method in TG 123 (5). The generator-column technique is occasionally used for the determination of log K_{OW}. A limited number of studies are available that makes use of this technique, primarily for chlorinated biphenyls and dibenzodioxins (*e.g.* Li and Doucette, 1993) (3). For substances that might ionise, log K_{OW} should refer to the unionised form.

but may still be applied to strongly hydrophobic substances (having log K_{OW} > 6.0), if a stable and fully dissolved concentration of the test substance in water can be demonstrated. If a stable concentration of the test substance in water cannot be demonstrated, an aqueous study would not be appropriate thus the dietary approach for testing the substance in fish would be required (although interpretation and use of the results of the dietary test may depend on the regulatory framework). Pre-estimates of the bioconcentration factor (BCF, sometimes denoted as K_B) for organic chemicals with log K_{OW} values up to about 9.0 can be obtained using the equation of Bintein *et al.* (14). The pre-estimate of the bioconcentration factor for such strongly hydrophobic substances may be higher than the steady-state bioconcentration factor (BCF_{SS}) value expected to be obtained from laboratory experiments, especially when a simple linear model is used for the pre-estimate. Parameters which characterise the bioaccumulation potential include the uptake rate constant (k_1), loss rate constants including the depuration rate constant (k_2), the steady-state bioconcentration factor (BCF_{SS}), the kinetic bioconcentration factor (BCF_K) and the dietary biomagnification factor (BMF)[(3)].

6. Radiolabelled test substances can facilitate the analysis of water, food and fish samples, and may be used to determine whether identification and quantification of metabolites will be necessary. If total radioactive residues are measured alone (*e.g.* by combustion or tissue solubilisation), the BCF or BMF is based on the total of the parent substance, any retained metabolites and also assimilated carbon. BCF or BMF values based on total radioactive residues may not, therefore, be directly comparable to a BCF or BMF derived by specific chemical analysis of the parent substance only. Separation procedures, such as TLC, HPLC or GC [(4)] may be employed before analysis in radiolabelled studies in order to determine BCF or BMF based on the parent substance. When separation techniques are applied, identification and quantification of parent substance and relevant metabolites should be performed [(5)] (*cf.* paragraph 65) if BCF or BMF is to be based upon the concentration of the parent substance in fish and not upon total radiolabelled residues. It is also possible to combine a fish metabolism or *in vivo* distribution study with a bioaccumulation study by analysis and identification of the residues in tissues. The possibility of metabolism can be predicted by suitable tools (*e.g.* OECD QSAR toolbox (15) and proprietary QSAR programs).

7. The decision on whether to conduct an aqueous or dietary exposure test, and in what set-up, should be based on the factors in paragraph 3 considered together with the relevant regulatory framework. For example, for substances, which have a high log K_{OW} but still show appreciable water solubility with respect to the sensitivity of available analytical techniques, an aqueous exposure test should be considered in the first instance. However it is possible that information on water solubility is not definitive for these hydrophobic types of chemicals, so the possibility of preparing stable, measurable dissolved aqueous concentrations (stable emulsions are not allowed) applicable for an aqueous exposure study should be investigated before a decision is made on which test method to use (16). It is not possible to give exact prescriptive guidance on the method to be used based on water solubility and octanol-water partition coefficient "cut off" criteria, as other factors (analytical techniques, degradation, adsorption, etc.) can have a marked influence on method applicability for the reasons given above. However, a log K_{OW} above 5 and a water solubility below ~0.01 – 0.1 mg/L mark the range of substances where testing via aqueous exposure may become increasingly difficult.

8. Other factors that may influence test choice should be considered, including the substance's potential for adsorption to test vessels and apparatus, its stability in aqueous solution versus its stability in fish food (17) (18), etc.

(3) See Annex 1 for definitions and units

(4) TLC: thin layer chromatography; HPLC: high pressure liquid chromatography; GC: gas chromatography

(5) In some regulatory frameworks analysis of metabolites may be obligatory when certain conditions are met (*cf.* paragraph 65).

9. Information on such practical aspects may be available from other completed aqueous studies. Further information on the evaluation of aspects relating to the performance of bioaccumulation studies are available in the literature (*e.g.* (19)).

10. For substances where the solubility or the maintenance of the aqueous concentration as well as the analysis of these concentrations do not pose any constraints to the realization of an aqueous exposure method, this method is preferred to determine the bioconcentration potential of the substance. In any case, it should be verified that the aqueous exposure concentration(s) to be applied are within the aqueous solubility in the test media. Different methods for maintaining stable concentrations of the dissolved test substance can be used, such as the use of stock solutions or passive dosing systems (*e.g.* column elution method), as long as it can be demonstrated that stable concentrations can be maintained and the test media are not altered from that recommended in paragraph 27.

11. For strongly hydrophobic substances (log K_{OW} > 5 and a solubility below ~ 0.01-0.1 mg/L), testing via aqueous exposure may become increasingly difficult. Reasons for constraints may be that the aqueous concentration cannot be maintained at a level that is considered to be sufficiently constant (*e.g.* due to sorption to the glass of exposure containers or rapid uptake by the fish) or that the aqueous concentrations to be applied are so low that they are in the same range as or below the analytical limit of quantification [(6)]. For these highly hydrophobic substances the dietary test is recommended, provided that the test is consistent with the relevant regulatory framework and risk assessment needs.

12. For surfactants it should be considered whether the aqueous bioconcentration test is feasible, given the substance properties, otherwise the dietary study is probably more appropriate. Surfactants are surface acting agents, which lower the interfacial tension between two liquids. Their amphiphilic nature (*i.e.* they contain both a hydrophilic and a hydrophobic part) causes them to accumulate at interfaces such as the water-air interface, the water-food interface, and glass walls, which hampers the determination of their aqueous concentration.

13. The dietary test can circumvent some of the exposure aspects for complex mixtures with components of differing water solubility limits, in that comparable exposure to all components of the mixture is more likely than in the aqueous method (*cf.* (20)).

14. It should be noted that the dietary approach yields a dietary biomagnification factor (BMF) rather than a bioconcentration factor (BCF)[(7)]. Approaches are available to estimate a kinetic bioconcentration factor (BCF_K) from data generated in the dietary study (as discussed in Annex 8, but these approaches should be used with caution. In general, these approaches assume first order kinetics, and are only applicable to certain groups of compounds. It is unlikely that such approaches can be applied for surfactants (see paragraph 12).

15. A minimised aqueous exposure test set-up with fewer sampling points to reduce the number of animals and/or resources (*cf.* paragraph 83 onwards) should only be applied to those substances where there is reason to expect that uptake and depuration will follow approximately first order kinetics (*i.e.* in general non-ionized organic substances, *cf.* paragraph 88).

(6) In general, measured concentrations in water during the uptake phase should be at least an order of magnitude above the limit of quantification so that more than one half-life of body burden can be measured in the depuration phase of the study.

(7) See Annex 1 for definitions and units

305-I: Aqueous Exposure Bioconcentration Fish Test

PRINCIPLE OF THE TEST

16. The test consists of two phases: the exposure (uptake) and post-exposure (depuration) phases. During the uptake phase, a group of fish of one species is exposed to the test substance at one or more chosen concentrations, depending on the properties of the test substance (*cf.* paragraph 49). 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. 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 exposed group, 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[8].

17. In the aqueous exposure test, the uptake phase is usually run for 28 days. The duration can be lengthened if necessary (*cf.* paragraph 18), or shortened if it is demonstrated that steady-state has been reached earlier (see Annex 1, definitions and units). A prediction of the length of the uptake phase and the time to steady-state can be made from equations in Annex 5. The depuration period is then begun when the fish are no longer exposed to the test substance, by transferring the fish to the same medium but without the test substance in a clean vessel. Where possible the bioconcentration factor is calculated preferably both as the ratio of concentration in the fish (C_f) and in the water (C_w) at steady-state (BCF_{SS}; see Annex 1, definition) and as a kinetic bioconcentration factor (BCF_K; see Annex 1, definitions and units), which is estimated as the ratio of the rate constants of uptake (k_1) and depuration (k_2) assuming first order kinetics [(9)].

18. If a steady-state is not achieved within 28 days, either the BCF is calculated using the kinetic approach (*cf.* paragraph 38) or the uptake phase can be extended. Should this lead to an impractically long uptake phase to reach steady-state (*cf.* paragraphs 37 and 38, Annex 5), the kinetic approach is preferred. Alternatively, for highly hydrophobic substances the conduction of a dietary study should be considered [(10)], provided that the dietary test is consistent with the relevant regulatory framework.

19. The uptake rate constant, the depuration (loss) rate constant (or constants, where more complex models are involved), the bioconcentration factor (steady-state and/or kinetic), 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 (*cf.* Annex 5).

20. The increase in fish mass during the test will result in a decrease of test substance concentration in growing fish (so-called growth dilution), and thus the kinetic BCF will be underestimated if not corrected for growth (*cf.* paragraphs 72 and 73).

(8) For most test substances, there should ideally be no detections in the control water. Background concentrations should only be relevant to naturally occurring materials (*e.g.*, some metals) and substances that are ubiquitous in the environment.

(9) If first order kinetics is obviously not obeyed, more complex models should be employed (see references in *Annex 5* and advice from a biostatistician sought.

(10) Uptake may be limited by low exposure concentrations because of low water solubility in the bioconcentration test, whereas far higher exposure concentrations can be achieved with the dietary test.

21. The BCF is based on the total concentration in the fish (*i.e.* per 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 hydrophobicity, 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 K_{OW} > 3), bioconcentration should be expressed as normalised to a fish with a 5% lipid content (based on whole body wet weight) in addition to that derived directly from the study. This is necessary to provide a basis from which results for different substances and/or test species can be compared against one another. The figure of 5% lipid content has been widely used as this represents the average lipid content of fish commonly used in this TG (21).

INFORMATION ON THE TEST SUBSTANCE

22. 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) and/or toxicity estimated from long-term fish tests (*e.g.* OECD TGs 210 (22), 212 (23), 215(24)).

23. An appropriate analytical method, of known accuracy, precision, and sensitivity, for the quantification of the substance in the test solutions and in biological material should be available, together with details of sample preparation and storage. The analytical quantification limit of the test substance in both water and fish tissues should also be known. When a radiolabelled test substance is used, it should be of the highest purity (*e.g.* preferably > 98%) and the percentage of radioactivity associated with impurities should be known.

VALIDITY OF THE TEST

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

- The water temperature variation is less than ± 2°C, because large deviations can affect biological parameters relevant for uptake and depuration as well as cause stress to animals;
- The concentration of dissolved oxygen does not fall below 60% saturation;
- The concentration of the test substance in the chambers is maintained within ± 20% of the mean of the measured values during the uptake phase;
- The concentration of the test substance is below its limit of solubility in water, taking into account the effect that the test water may have on effective solubility [(11)];
- 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. Significant differences in average growth between the test and the control groups of sampled fish could be an indication of a toxic effect of the test chemical.

(11) For multi-constituent substances such as UVCBs, the water solubility of each relevant component should be considered to determine the appropriate exposure concentrations.

REFERENCE SUBSTANCES

25. The use of reference substances of known bioconcentration potential and low metabolism would be useful in checking the experimental procedure, when required (*e.g.* when a laboratory has no previous experience with the test or experimental conditions have been changed).

DESCRIPTION OF THE METHOD

Apparatus

26. 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 (*cf.* paragraph 43), can be used. The use of soft plastic tubing should be minimised. Teflon®, stainless steel and/or glass tubing should be used. Experience has shown that for test substances with high adsorption coefficient, such as the synthetic pyrethroids, silanized glass may be required. In such situations the equipment should be discarded after use. It is preferable to expose test systems to concentrations of the test substance to be used in the study for as long as is required to demonstrate the maintenance of stable exposure concentrations prior to the introduction of test organisms.

Water

27. Natural water is generally used in the test and should be obtained from uncontaminated and uniform quality source. Yet, reconstituted water (*i.e.* demineralized water with specific nutrients added in known amounts) may be more suitable to guarantee uniform quality over time. The dilution water, which is the water that is mixed with the test substance before entering the test vessel (*cf.* paragraph 30), should 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 dilution water should be characterised at least by pH, hardness, total solids, total organic carbon (TOC [(12)]) 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.

28. The dilution 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 at test start, but during a given test it should be within a range of ± 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, at least at the beginning and end of the test. Determination of heavy metals (*e.g.* Cu, Pb, Zn, Hg, Cd, and Ni), major anions and cations (*e.g.* Ca, Mg, Na, K, Cl, and SO4), pesticides (*e.g.* total organophosphorous and total organochlorine pesticides), total organic carbon and suspended solids should be conducted, for example, every three months where dilution water is known to be relatively constant in quality. If dilution 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).

29. The natural particle content as well as the total organic carbon 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 and therewith result in an underestimation of the BCF. The maximum acceptable value is 5 mg/L for particulate matter (dry matter, not passing a 0.45 µm filter) and 2 mg/L for total organic carbon (*cf.* Annex 2). If necessary, the dilution water should be filtered before use. The contribution to

(12) TOC includes organic carbon from particles and dissolved organic carbon, *i.e.* TOC = POC + DOC.

the organic carbon content in test water from the test fish (excreta) and from the food residues should be kept as low as possible (*cf.* paragraph 46).

Test Solutions

30. 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. An alternative that may be appropriate in some cases is the use of a solid phase desorption dosing system. The use of solvents and dispersants (solubilising agents) is not generally recommended (*cf.* (25)); however the use of these materials may be acceptable in order to produce a suitably concentrated stock solution, but every effort should be made to minimise the use of such materials and their critical micelle concentration should not be exceeded (if relevant). Solvents which may be used are acetone, ethanol, methanol, dimethyl formamide and triethylene glycol; dispersants that have been used are Tween 80, methylcellulose 0.01% and HCO-40. The solvent concentration in the final test medium should be the same in all treatments (i.e. regardless of test substance concentration) and should not exceed the corresponding toxicity thresholds determined for the solvent under the test conditions. The maximum level is a concentration of 100 mg/L (or 0.1 mL/L). It is unlikely that a solvent concentration of 100 mg/L will significantly alter the maximum dissolved concentration of the test substance which can be achieved in the medium (25). The solvent's contribution (together with the test substance) to the overall content of organic carbon in the test water should be known. Throughout the test, the concentration of total organic carbon in the test vessels should not exceed the concentration of organic carbon originating from the test substance, and solvent or solubilising agent [(13)], if used, by more than 10 mg/L (± 20%). Organic matter content can have a significant effect on the amount of freely dissolved test substance during flow-through fish tests, especially for highly lipophilic substances. Solid-phase microextraction (*cf.* paragraph 60) can provide important information on the ratio between bound and freely dissolved compounds, of which the latter is assumed to represent the bioavailable fraction. The test substance concentration should be below the solubility limit of the test substance in the test media in spite of the use of a solvent or solubilising agent. Care should be taken when using readily biodegradable solvents as these can cause problems with bacterial growth in flow-through tests. If it is not possible to prepare a stock solution without the use of a solubilising agent, consideration should be given to the appropriateness of an aqueous exposure study as opposed to a dietary exposure study.

31. 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) or a solid phase desorption dosing 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 (*cf.* paragraph 24). 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

32. 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

(13) Although not generally recommended, if a solvent or solubilising agent is used the organic carbon originating from this agent should be added to the organic carbon from the test substance to evaluate the concentration of organic carbon in the test vessels.

rationale for the selection of the species and the experimental method should be reported in this case. In general, the use of smaller fish species will shorten the time to steady-state, but more fish (samples) may be needed to adequately analyse lipid content and test substance concentrations in the fish. In addition it is possible that differences in respiration rate and metabolism between young and older fish may hamper comparisons of results between different tests and test species. It should be noted that fish species tested during a (juvenile) life-stage with rapid growth can complicate data interpretation.

Holding of fish (relevant for aqueous and dietary exposure)

33. The stock population of fish should be acclimated for at least two weeks in water (*cf.* paragraph 28) at the test temperature and feed throughout on a sufficient diet (*cf.* paragraph 45). Both water and diet should be of the same type as those to be used during the test.

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

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

35. Fish used in tests should be free from observable diseases and abnormalities. Any diseased fish should be discarded. Fish should not receive treatment for disease in the two weeks preceding the test, or during the test.

PERFORMANCE OF THE TEST

Preliminary test

36. 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, or to determine whether a full test need be conducted. The design of the preliminary test should be such as to obtain the information required. It can be considered if a minimised test may be sufficient to derive a BCF, or if a full study is needed (*cf.* paragraphs 83-95 on the minimised test).

Conditions of Exposure

Duration of uptake phase

37. 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 (provided that uptake follows first order kinetics, *cf.* Annex 5).

38. The uptake phase should be run for 28 days unless it can be demonstrated that steady-state has been reached earlier (see Annex 1, definitions and units). A steady-state is reached in the plot of test substance in fish (C_f) against time when the curve becomes parallel to the time axis and three successive analyses of C_f made on samples taken at intervals of at least two days are within ± 20% of each other, and there is no significant increase of C_f in time between the first a last successive analysis.

When pooled samples are analysed, at least four successive analyses are required. For test substances which are taken up slowly the intervals would more appropriately be seven days. If steady-state has not been reached by 28 days, either the BCF is calculated using only the kinetic approach, which is not reliant on steady-state being reached, or the uptake phase can be extended, taking further measurements, until steady-state is reached or for 60 days, whichever is shorter. Also, the test substance concentration in the fish at the end of the uptake phase needs to be sufficiently high to ensure a reliable estimation of k_2 from the depuration phase. If no significant uptake is shown after 28 days, the test can be stopped.

Duration of the depuration phase

39. For substances following first order kinetics, 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 (*cf.* Annex 5 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, longer depuration periods may be necessary for substances having more complex patterns of uptake and depuration than are represented by a one-compartment fish model that yields first order kinetics. If such complex patterns are observed and/or anticipated, it is advised to seek advice from a biostatistician and/or pharmacokineticist to ensure a proper test set-up. As the depuration period is extended, numbers of fish to sample may become limiting and growth differences between fish can influence the results. The period will also be governed by the period over which the concentration of the test substance in the fish remains above the analytical limit of quantification.

Numbers of test fish

40. Select the numbers of fish per test concentration such that a minimum of four fish are available at each sampling point. Fish should only be pooled if analysis of single fish is not feasible. If higher precision in curve fitting (and derived parameters) is intended or if metabolism studies are required (*e.g.* to distinguish between metabolites and parent substance when using radiolabelled test substances), more fish per sampling point will be necessary. The lipid content should be determined on the same biological material as is used to determine the concentration of the test substance. Should this not be feasible, additional fish may be needed (*cf.* paragraphs 56 and 57).

41. If adult (*i.e.* sexually mature) fish are used, they should not be in a spawning state or recently spent (*i.e.* already spawned) either before or during the test. It should also be reported whether male or female, or both are used in the experiment. If both sexes are used, differences in growth and lipid content between sexes should be documented to be non-significant before the start of the exposure, in particular if it is anticipated that pooling of male and female fish will be necessary to ensure detectable substance concentrations and/or lipid content.

42. 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 may have a significant effect on BCF values (12) these details should be recorded accurately. It is recommended that a sub-sample of the stock of fish is weighed shortly before the start of the test in order to estimate the mean weight (*cf.* paragraph 61).

Loading

43. High water-to-fish ratios should be used in order to minimise the reduction in the concentration of the test compound in water 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 fish-to-water loading rate of 0.1-1.0 g of fish (wet

weight) per litre of water per day is normally recommended. Higher fish-to-water loading rates can be used if it is shown that the required concentration of test substance can be maintained within ± 20% limits, and that the concentration of dissolved oxygen does not fall below 60% saturation (*cf.* paragraph 24).

44. In choosing appropriate loading regimes, take into account 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 compared to pelagic fish species.

Feeding

45. 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 (some growth is allowed). Feed daily throughout the acclimation and test periods at a set level depending on the species used, experimental conditions and calorific value of the food (for example for rainbow trout between approximately 1 to 2% of body weight per day). The feeding rate should be selected such that fast growth and large increase of lipid content are avoided. To maintain the same feeding rate, the amount of feed should be re-calculated as appropriate, for example once per week. 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.

46. Uneaten food and faeces should be siphoned daily from the test chambers shortly after feeding (30 minutes to one hour). The chambers should be kept as clean as possible throughout the test to keep the concentration of organic matter as low as possible (*cf.* paragraph 29), since the presence of organic carbon may limit the bioavailability of the test substance (12).

47. Since many feeds are derived from fishmeal, it should be ensured that the feed will not influence the test results or induce adverse effects, *e.g.* by containing (traces of) pesticides, heavy metals and/or the test substance itself.

Light and temperature

48. A 12 to 16 hour photoperiod is recommended and the temperature (± 2°C) should be appropriate for the test species (*cf.* 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

49. The test was originally designed for non-polar organic substances. For this type of substance, the exposure of fish to a single concentration is expected to be sufficient, as no concentration effects are expected, although two concentrations may be required for the relevant regulatory framework. If substances outside this domain are tested, or other indications of possible concentration dependence are known, the test should be run with two or more concentrations. If only one concentration is tested, justification for the use of one concentration should be given (*cf.* paragraph 79). Also, the tested concentration should be as low as is practical or technically possible (*i.e.* not close to the solubility limit).

50. In some cases it can be anticipated that the bioconcentration of a substance is dependent on the water concentration (*e.g.* for metals, where the uptake in fish may be at least partly regulated). In such a case it is necessary that at least two, but preferably more, concentrations are tested

(*cf.* paragraph 49) which are environmentally relevant. Also for substances where the concentrations tested have to be near the solubility limit for practical reasons, testing at least two concentrations is recommended, because this can give insight into the reliability of the exposure concentrations. The choice of the test concentrations should incorporate the environmentally realistic concentration as well as the concentration that is relevant to the purpose of the specific assessment.

51. The concentration(s) of the test substance should be selected to be below its chronic effect level or 1% of its acute asymptotic LC_{50}, within an environmentally relevant range and at least an order of magnitude above its limit of quantification in water by the analytical method used. The highest permissible test concentration can also be determined by dividing the acute 96 h LC_{50} by an appropriate acute/ chronic ratio (*e.g.* appropriate ratios for some chemicals are about three, but a few are above 100). If a second concentration is used, it should differ from the one above by a factor of ten. If this is not possible because of the toxicity criterion (that limits the upper test concentration) and the analytical limit (that limits the lower test concentration), a lower factor than ten can be used and use of radiolabelled test substance (of the highest purity, *e.g.* preferably > 98%) should be considered. Care should be taken that no concentration used is above the solubility limit of the test substance in the test media.

Controls

52. One dilution water control or if relevant (*cf.* paragraphs 30 and 31), one control containing the solvent should be run in addition to the test series.

Frequency of Water Quality Measurements

53. During the test, dissolved oxygen, TOC, pH and temperature should be measured in all test and control vessels. Total hardness and salinity (if relevant) should be measured in the control(s) and one vessel. If two or more concentrations are tested, measure these parameters 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 (24 h and 48 h 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 and recorded 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

54. Water should be sampled from the test chambers for the determination of test substance concentration before addition of the fish and during both uptake and depuration phases. The water should be sampled the before feeding, at the same time as the fish sampling. More frequent sampling may be useful to ensure stable concentrations after introduction of the fish. During the uptake phase, the concentrations of test substance should be determined in order to check compliance with the validity criteria (paragraph 24). If water sample analyses at the beginning of the depuration phase show that the test substance is not detected, this can be used as a justification not to measure test and control water for the test substance for the remainder of the depuration phase.

55. Fish should be sampled on at least five occasions during the uptake phase and on at least four occasions during the depuration phase for test substance. 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 uptake and depuration kinetics are indicated), it may be advisable to take samples at a higher frequency in both periods (*cf.* Annex 4).

56. The lipid content should be determined on the same biological material as is used to determine the concentration of the test substance at least at the start and end of the uptake phase and at the end of the depuration phase. Should this not be feasible, at least three independent fish should be sampled to determine lipid content at each of the same three time-points. The number of fish per tank at the start of the experiment should be adjusted accordingly[14]. Alternatively, if no significant amounts of the test substance are detected in control fish (*i.e.* fish from the stock population), the control fish from the test can be analysed for lipid content only and test substance analysis in the test group(s) (and the related uptake rate constant, depuration rate constant and BCF values) can be corrected for changes according to control group lipid content during the test [(15)].

57. Dead or diseased fish should not be analysed for test substance or lipid concentration.

58. An example of an acceptable sampling schedule is given in Annex 4. Other schedules can readily be calculated using other assumed values of K_{OW} to calculate the exposure time for 95% uptake (refer to Annex 5 for calculations).

59. Sampling should be continued during the uptake phase until a steady-state has been established (see Annex 1, definitions and units) or the uptake phase is otherwise terminated (after 28 or 60 days, *cf.* paragraphs 37 and 38). Before beginning the depuration phase, the fish should be transferred to clean vessels.

Sampling and sample preparation

60. Water samples should be obtained for analysis *e.g.* by siphoning through inert tubing from a central point in the test chamber. Neither filtration nor centrifuging appears always to separate the non-bioavailable fraction of the test substance from that which is bioavailable. If a separation technique is applied, a justification for, or validation of, the separation technique should always be provided in the test report given the bioavailability difficulties (25). Especially for highly hydrophobic chemicals (*i.e.* those chemicals with a log $K_{OW} > 5$) (12) (26), where adsorption to the filter matrix or centrifugation containers could occur, samples should not be subjected to those treatments. Instead, measures should be taken to keep the tanks as clean as possible (*cf.* paragraph 46) and the content of total organic carbon should be monitored during both the uptake and depuration phases (*cf.* paragraph 53). To avoid possible issues with reduced bioavailability, sampling by solid phase microextraction techniques may be used for poorly soluble and highly hydrophobic substances.

61. The sampled fish should be euthanized instantly, using the most appropriate and humane method (for whole fish measurements, no further processes than rinsing with water (*cf.* paragraph 28) and blot drying the fish should be done). Weigh and measure total length [(16)]. In each individual fish, the measured weight and length should be linked to the analysed chemical concentration (and lipid content, if applicable), for example using a unique identifier code for each sampled fish.

62. 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 rate constants as the test proceeds. Immediate analysis also avoids delay in determining when a plateau (steady-state) has been reached.

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(14) If the lipid content is not analysed in the same fish as the test substance, fish should at least be of the similar weight, and (if relevant) the same sex.

(15) This alternative is only valid if the fish in all test groups are held in similar group sizes, fish are removed according to the same pattern and fed in the same way. This ensures that fish growth in all test groups is similar, if the tested concentration is below the toxic range. If the growth is similar, also the lipid content is expected to be similar. A different growth in the control would indicate a substance effect and invalidate the study.

(16) In addition to weight, total length should be recorded because comparison of the extent of length increase during the test is a good indicator of whether an adverse effect has occurred.

63. Failing immediate analysis, the samples should be stored by an appropriate method. Before the beginning of the study, information should be obtained on the proper method of storage for the particular test substance – for example, deep-freezing, holding at 4°C, extraction, etc. The duration of storage should be selected to ensure that the chemical has not degraded while in storage.

Quality of analytical method

64. 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 accuracy, 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. This should be part of preliminary tests. Also, check that the test substance is not detectable in the dilution water used. If necessary, correct the values of test substance concentration in water and fish obtained from the test for the recoveries and background values of controls. The fish and water samples should be handled throughout in such a manner as to minimise contamination and loss (*e.g.* resulting from adsorption by the sampling device).

Analysis of fish samples

65. If radiolabelled materials are used in the test, it is possible to analyse for total radiolabel (*i.e.* parent and metabolites) or the samples may be cleaned up so that the parent substance can be analysed separately. If the BCF is to be based on the parent substance, the major metabolites should be characterised, as a minimum at the end of the uptake phase (*cf.* paragraph 6). Major metabolites are those representing ≥ 10% of total residues in fish tissues, those representing≥ 5% at two consecutive sampling points, those showing increasing levels throughout the uptake phase, and those of known toxicological concern. If the BCF for the whole fish in terms of total radiolabelled residues is ≥ 500, it may be advisable – and for certain categories of chemicals such as pesticides strongly recommended – identifying and quantifying major metabolites. Quantification of such metabolites may be required by some regulatory authorities. If degradates representing ≥ 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. Should this not be feasible, this should be explained in the report.

66. 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, so an adequate number of fish to accommodate the desired pooling, statistical procedure and power should be included in the test. References (27) and (28) may be used as an introduction to relevant pooling procedures.

67. BCF should be expressed as normalised to a fish with a 5% lipid content (based on wet weight) in addition to that derived directly from the study (*cf.* paragraph 21), unless it can be argued that the test substance does not accumulate primarily in lipids. The lipid content of the fish should be determined on each sampling occasion if possible, preferably 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. However, analysis of test substances often requires specific extraction procedures which might be in contradiction to the guidelines for lipid determination. In this case (until suitable non-destructive instrumental methods are available), it is recommended to employ a different strategy to determine the fish lipid content (*cf.* paragraph 56). Suitable methods should be used for determination of lipid content (20). The chloroform/methanol extraction technique (29) may be recommended as standard method (30), but the Smedes-method (31) is recommended as an alternative technique. This latter method is characterised by a comparable efficiency of extraction, high accuracy, the use of less toxic organic solvents and ease of performance. Other methods for which accuracy compares favourably to the recommended methods could be used if properly justified. It is important to give details of the method used.

Fish growth measurement

68. At the start of the test, five to ten fish from the stock population need to be weighed individually and their total length measured. These can be the same fish used for lipid analysis (*cf.* paragraph 56). The weight and length of fish used for each sampling event from both test and control groups should be measured before chemical or lipid analysis is conducted. The measurements of these sampled fish can be used to estimate the weight and length of fish remaining in the test and control tanks (*cf.* paragraph 45).

DATA AND REPORTING

Treatment of results

69. The uptake curve of the test substance should be obtained 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, the steady-state BCF (BCF_{SS}) should be calculated from:

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

The development of C_f may be influenced by fish growth (*cf.* paragraphs 72 and 73). The mean exposure concentration (C_w) is influenced by variation over time. It can be expected that a time-weighted average concentration is more relevant and precise for bioaccumulation studies, even if variation is within the appropriate validity range (*cf.* paragraph 24). A time weighted average (TWA) water concentration can be calculated according to Annex 5, section 1.

70. The kinetic bioconcentration factor (BCF_K) should be determined as the ratio k_1/k_2, the two first order kinetic rate constants. Rate constants k_1 and k_2 and BCF_K can be derived by simultaneously fitting both the uptake and the depuration phase. Alternatively, k_1 and k_2 can be determined sequentially (see Annex 5 for a description and comparison of these methods). The depuration rate constant (k_2) may need correction for growth dilution (*cf.* paragraphs 72 and 73). If the uptake and/or depuration curve is obviously not first order, then more complex models should be employed (see references in Annex 5 and advice from a biostatistician and/or pharmacokineticist sought.

Fish weight/length data

71. Individual fish wet weights and total lengths for all sampling intervals are tabulated separately for test and control groups during the uptake (including stock population for start of uptake) and depuration phases. In each individual fish the measured weight and length should be linked to the analysed chemical concentration, for example using a unique identifier code for each sampled fish. Weight is the preferred measure of growth for the purposes of correcting kinetic BCF values for growth dilution (see paragraph 73 and Annex 5 for the method used to correct data for growth dilution).

Growth-Dilution Correction and Lipid Normalisation

72. Fish growth during the depuration phase can lower measured chemical concentrations in the fish with the effect that the overall depuration rate constant (k_2) is greater than would arise from removal processes (*e.g.* respiration, metabolism, egestion) alone. Kinetic bioconcentration factors should be corrected for growth dilution. A BCF_{SS} will also be influenced by growth, but no agreed procedure is available to correct a BCF_{SS} for growth. In cases of significant growth, the BCF_K, corrected for growth (BCF_{Kg}), should also be derived as it may be a more relevant measure of the bioconcentration factor. Lipid contents of test fish (which are strongly associated with the

bioaccumulation of hydrophobic chemicals) can vary enough in practice such that normalisation to a set fish lipid content (5% w/w) is necessary to present both kinetic and steady-state bioconcentration factors in a meaningful way, unless it can be argued that the test substance does not primarily accumulate in lipid (*e.g.* some perfluorinated substances may bind to proteins). Equations and examples for these calculations can be found in Annex 5.

73. To correct a kinetic BCF for growth dilution, the depuration rate constant should be corrected for growth. This growth-corrected depuration rate constant (k_{2g}) is calculated by subtracting the growth rate constant (k_g, as obtained from the measured weight data) from the overall depuration rate constant (k_2). The growth-corrected kinetic bioconcentration factor is then calculated by dividing the uptake rate constant (k_1) by the growth-corrected depuration rate constant (k_{2g}) (*cf.* Annex 5). In some cases this approach is compromised. For example, for very slowly depurating substances tested in fast growing fish, the derived k_{2g} may be very small and so the error in the two rate constants used to derive it becomes critical, and in some cases kg estimates can be larger than k_2. An alternative approach that circumvents the need for growth dilution correction involves using mass of test chemical per fish (whole fish basis) depuration data rather than the usual mass of test chemical per unit mass of fish (concentration) data. This can be easily achieved as tests according to this TG should link recorded tissue concentrations to individual fish weights. The simple procedure for doing this is outlined in Annex 5. Note that k_2 should still be reported even if this alternative approach is used.

74. Kinetic and steady-state bioconcentration factors should also be reported relative to a default fish lipid content of 5% (w/w), unless it can be argued that the test substance does not primarily accumulate in lipid. Fish concentration data, or the BCF, are normalised according to the ratio between 5% and the actual (individual) mean lipid content (in % wet weight) (*cf.* Annex 5).

75. If chemical and lipid analyses have been conducted on the same fish, then individual fish lipid normalised data should be used to calculate a lipid-normalised BCF. Alternatively, if the growth in control and exposed fish is similar, the lipid content of control fish alone may be used for lipid-correction (*cf.* paragraph 56). A method for calculating a lipid-normalised BCF is described in Annex 5.

Interpretation of results

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

77. Average growth in both test and control groups should in principle not be significantly different to exclude toxic effects. The growth rate constants or the growth curves of the two groups should be compared by an appropriate procedure [(17)]).

78. Clearly defined uptake and depuration curves are an indication of good quality bioconcentration data. For the rate constants, the result of a χ^2 goodness-of-fit-test should show a good fit (*i.e.* small measurement error percentage (32)) for the bioaccumulation model, so that the rate constants can be considered reliable (*cf.* Annex 5). If more than one test concentration is used, the variation in uptake/depuration constants between the test concentrations should be less than 20 %[(18)]. If not, concentration dependence could be indicated. Observed significant differences in uptake/ depuration rate constants between the applied test concentrations should be recorded and possible

(17) A *t*-test on growth rate constants can be performed, to test whether growth differs between control and test groups, or an *F*-test in case of analysis of variance. If needed, an *F*-test or likelihood ratio test can be used to assist in the choice of the appropriate growth model (OECD monograph 54, (32).

(18) These percentages assume that the analytical methods are reliable and the half life is < 14 days. If the analytical methods are less reliable or the half life is (greatly) increased these numbers will become larger.

explanations given. Generally the 95% confidence limit of BCFs from well-designed studies approach ± 20% of the derived BCF.

79. If two or more concentrations are tested, the results of both or all concentrations are used to examine whether the results are consistent and to show whether there is concentration dependence. If only one concentration is tested to reduce the use of animals and/or resources, justification of the use of one concentration should be given.

80. The resulting BCF_{SS} is doubtful if the BCF_K is significantly larger than the BCF_{SS}, as this can be an indication that steady-state has not been reached or growth dilution and loss processes have not been taken into account. In cases where the BCF_{SS} is very much higher than the BCF_K, the derivation of the uptake and depuration rate constants should be checked for errors and re-evaluated. A different fitting procedure might improve the estimate of BCF_K (*cf.* Annex 5).

Test Report

81. Apart from the test substance information indicated in paragraph 3, the test report includes the following information:

Test substance:

- Physical nature and, where relevant, physicochemical properties;
    - Chemical identification data, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate).
    - For multi-constituent substances and UVCB (chemical substances of Unknown or Variable composition, Complex reaction products and Biological materials) description, as far as possible, of the chemical identity of the individual constituents and, for each, of its percentage of the total mass of the substance. How the analytical method used in the test reflects a measure of the concentration of the substance should be summarised; all analytical procedures should be described including the accuracy of the method, method detection limit, and limit of quantification.
    - If radiolabelled, the precise position of the labelled atom(s) and the percentage of radioactivity associated with impurities.
    - Information on the test substance toxicity to fish (ideally the test species). The toxicity should be reported as an acute 96-h LC_{50} and a NOAEC & LOAEC from a chronic study (*i.e.*, an early life stage test or a full life cycle test, if available).
    - Storage conditions of the test chemical or test substance and stability of the test chemical or test substance under storage conditions if stored prior to use.

Test species:

- Scientific name, strain, source, any pre-treatment, acclimation, age, sex (if relevant), size-range (weight and length), etc.

Test conditions:

- Test procedure used (*e.g.* flow-through or semi-static); regular study or minimised design (including rationale and justification).
- Type and characteristics of illumination used and photoperiod(s).
- Test design (*e.g.* number and size of test chambers, water volume replacement rate, loading 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 solvent, its concentration and its contribution to the organic carbon content of test water should be given, when used) or description of alternative dosing system.
- The nominal test concentrations, the means of the measured values and their standard deviations in the test vessels and the method and frequency by which these were attained.
- Source of the dilution water, description of any pre-treatment, 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; methods used and frequency of measurements.
- Detailed information on feeding, *e.g.* type of food(s), source, composition (at least lipid and protein content if possible), selected feeding rate, 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.
- Methods used for treatment randomization and assignment of fish to test vessels.
- Date of introduction of test organisms to test solutions and test duration.
- Description of range-finding tests and results, if available.

Results:

- Results from any preliminary study performed.
- Mortality of the control fish and the fish in each exposure chamber and any observed abnormal behaviour.
- Information on any adverse effects observed.
- Complete description of all chemical analysis procedures employed including limits of detection and quantification, variability and recovery.
- The lipid content of the fish, including the method used, and if derived, lipid normalisation factor (L_n, factor to express results relative to fish lipid content of 5%).

- Tabulated fish weight (and length) data, linked to individual fish chemical concentrations (and lipid content, if applicable), both for control and exposure groups (for example using unique identifiers for each sampled fish) and calculations for derived growth rate constant(s).

- Tabulated test substance concentration data in fish (C_f, linked to individual fish) and water (C_w) (with mean values for test group and control, standard deviation and range, if appropriate) for all sampling times (C_f expressed in mg/kg wet weight of whole body or specified tissues thereof, *e.g.* lipid, and C_w in mg/L). C_w values for the control series (background should also be reported).

- Curves (including all measured data), showing the following (if applicable, concentrations may be expressed in relation to the whole body and the lipid content normalised to 5% of the animal or specified tissues thereof):

    - growth, *i.e.* fish weight vs. time or natural logarithm transformed weight vs. time (including the derived growth rate constant, k_g);

    - the uptake and depuration of the test chemical in the fish (on one graph);.

    - the time to steady-state (if achieved);

    - natural logarithm transformed concentration vs. uptake time (including the derived uptake rate constant k_1);

    - natural logarithm transformed concentration (ln concentration) vs. depuration time (including the derived depuration rate constant k_2); and

    - both uptake and depuration phase curves, showing both the data and the fitted model.

- If a visual inspection of a plot shows obvious outliers, a statistically valid outlier test may be applied to remove spurious data points as well as documented justification for their omission.

- The steady-state bioconcentration factor, (BCF_{SS}), if steady-state is (almost) achieved.

- Kinetic bioconcentration factor (BCF_K) and derived uptake and depuration rate constants k_1 and k_2, together with the variances in k_2 (slope and intercept) if sequential fitting is used.

- Confidence limits, standard deviation (as available) and methods of computation/data analysis for each parameter for each concentration of test substance used.

    - Any information concerning radiolabelled test chemical metabolites and their accumulation.
    - Growth rate constant(s) (including 95% confidence interval(s)) and calculated growth-corrected depuration rate constant (k_{2g}), half-life and BCF (BCF_{Kg}) values.
    - Anything unusual about the test, any deviation from these procedures, and any other relevant information.
    - A summary table of relevant measured and calculated data, as hereafter:

Substance Uptake and Depuration Rate Constants and Bioconcentration Factors (BCF)	
k_g (growth rate constant; day^{-1}):	Insert Value (95% CI)(1)
k_1 (overall uptake rate constant; L kg^{-1} day^{-1}):	Insert Value (95% CI)(1)
k_2 (overall depuration rate constant; day^{-1}):	Insert Value (95% CI)(1)
k_{2g} (growth-corrected depuration rate constant; day^{-1}):	Insert Value (95% CI)(1)
C_f (chemical concentration in the fish at steady-state; mg kg^{-1}):	Insert Value ± SD(2)
C_w (chemical concentration in the water; mg L^{-1}):	Insert Value ± SD(2)
L_n (lipid normalisation factor):	Insert Value(3)
BCF_{SS} (steady-state BCF; L kg^{-1})	Insert Value ± SD(2)
BCF_{SSL} (lipid normalised steady-state BCF; L kg^{-1}):	Insert Value(3) ± SD(2)
BCF_K (kinetic BCF; L kg^{-1})	Insert Value (95% CI)(1)
BCF_{Kg} (growth-corrected kinetic BCF; L kg^{-1})	Insert Value (95% CI)(1)
$t_{1/2g}$ (growth-corrected half-life; day):	Insert Value (95% CI)(1)
BCF_{KL} (lipid-normalised kinetic BCF; L kg^{-1}):	Insert Value
BCF_{KLG} (lipid-normalised growth corrected kinetic BCF; L kg^{-1}):	Insert Value

(1) CI: confidence interval (where possible to estimate)

(2) SD: Standard deviation (where possible to estimate)

82. Results reported as "not detected/quantified at the limit of detection/quantification" by pre-test method development and experimental design should be avoided, since such results cannot be used for rate constant calculations.

305-II: Minimised Aqueous Exposure Fish Test

INTRODUCTION

83. The growing experience that has been gained in conducting and interpreting the full test, both by laboratories and regulatory bodies, shows that – with some exceptions – first order kinetics apply for estimating uptake and depuration rate constants. Thus, uptake and depuration rate constants can be estimated with a minimum of sampling points, and the kinetic BCF derived.

84. The initial purpose of examining alternative designs for BCF studies was to develop a small test to be used in an intermediate testing step to refute or confirm BCF estimates based on K_{OW} and QSARs and so eliminate the need for a full study for many chemicals, and to minimize cost and animal use via reduction in sampling and in the number of analytical sequences performed. While following the main design of the previous OECD TG to allow integration of test results with existing BCF data, and to ease performance of testing and data interpretation, the aim was to provide BCF estimates of adequate accuracy and precision for risk assessment decisions. Many of the same considerations apply as in the full test, *e.g.* validity criteria (*cf.* paragraph 24) and stopping a test if insignificant uptake is seen at the end of the uptake phase (*cf.* paragraphs 16 and 38).

85. Substances that would be eligible for the minimised test design should belong to the general domain that this Test Guideline (TG) was developed for, *i.e.* non-polar organic substances (*cf.* paragraph 49). If there is any indication that the substance to be tested might show a different behaviour (*e.g.* a clear deviation from first-order kinetics), a full test should be conducted for regulatory purposes.

86. Typically, the minimised test is not run over a shorter period than the standard BCF test, but comprises less fish sampling (see Annex 6 for the rationale). However, the depuration period may be shortened for rapidly depurating chemicals to avoid concentrations in the fish falling below the limit of detection/quantification before the end of the test. A minimised exposure fish test with a single concentration can be used to determine the need for a full test, and if the resulting data used to calculate rate constants and BCF are robust (*cf.* paragraph 93), the full test may be waived if the resulting BCF is far from regulatory values of concern.

87. In some cases it may be advantageous to perform the minimised test design with more than one test concentration as a preliminary test to determine whether BCF estimates for a chemical are concentration dependent. If the BCF estimates from the minimised test show concentration dependence, the performance of the full test will be necessary. If, based on such a minimised test, BCF estimates are not concentration dependent but the results are not considered definitive, then any subsequent full test could be performed at a single concentration, thereby reducing animal use in comparison to a two (or more) concentration full test.

88. Substances potentially eligible for the minimised test should:

- Be likely to exhibit approximate first order uptake and depuration kinetics, *e.g.* derived from read-across with similar substances;
- Have a log $K_{OW} < 6$ unless rapid metabolism is expected[(19)];

(19) The minimized test may in fact be used to demonstrate rapid metabolism when it is known that rapid metabolism is likely.

- Be sufficiently water-soluble with respect to the analytical technique (*cf.* paragraph 24);

- Be clearly quantifiable (*i.e.* concentrations should be at least one order of magnitude above the limit of quantification), both in fish and water, radioactive labelling is recommended (*cf.* paragraph 23); and

- Have a depuration period greater than its predicted half-life (*cf.* Annex 5 for calculations), or the duration of depuration should be adjusted accordingly (*cf.* paragraph 91). An exception to this rule is allowed if rapid metabolism of the chemical is expected.

SAMPLING SCHEDULE FOR STUDIES FOLLOWING THE MINIMISED DESIGN

Fish sampling

89. Fish sampling is reduced to four sampling points:

- At the middle and end of the uptake phase (the latter being the beginning of depuration as well), *e.g.* after 14 and 28 days (33).

- At the middle of the depuration phase and at termination of the study (where substance concentration is < 10% of the maximum concentration, or at least clearly past one half-life of the substance), *e.g.* after 7 and 14 days of depuration (33). If rapid depuration is expected or observed, it may be necessary to shorten the depuration period to avoid concentrations in the fish falling below the limit of quantification.

- Lipid measurement as in full study.

- Growth correction as in full study.

The BCF is calculated as a kinetic BCF.

Water sampling

90. For the minimised design, water is sampled as in full study (*cf.* paragraph 54) or at least five times equally divided over the uptake phase, and weekly in the depuration phase.

Design modifications

91. Taking into account the test substance properties, valid QSAR predictions and the specific purpose of the study, some modifications in the design of the study can be considered:

- If greater precision is needed, more fish (6 or 8 instead of 4) could be used for the sample at the end of the uptake phase.

- Inclusion of an 'extra' group of fish to be used if depuration at 14 days (or the predicted end of the depuration phase) has not been sufficient for adequate depuration (*i.e.* > 50%). If the predicted duration of the depuration phase is shorter or longer than 14 days, the sampling schedule should be adapted (*i.e.* one group of fish at the predicted end of the depuration phase, and one group after half that time).

- Use of two test concentrations to explore possible concentration dependence. If the results of the minimised test, conducted with two test concentrations, show that the BCF is not concentration dependent (*i.e.* differ less than 20%), one test concentration may be considered sufficient in a full test, if it is conducted.

- It seems likely that models of bioaccumulation processes such as those proposed by Arnot *et al.* (35) can be used to assist in planning the length of uptake and depuration phases (see also Annex 5).

Calculations

92. The rationale for this approach is that the bioconcentration factor in a full test can either be determined as a steady-state bioconcentration factor (BCF_{SS}) by calculating the ratio of the concentration of the test substance in the fish's tissue to the concentration of the test substance in the water, or by calculating the kinetic bioconcentration factor (BCF_K) as the ratio of the uptake rate constant k_1 to the depuration rate constant k_2. The BCF_K is valid even if a steady-state concentration of a chemical is not achieved during uptake, provided that uptake and depuration act approximately according to first order kinetic processes. As an absolute minimum two data points are required to estimate uptake and depuration rate constants, one at the end of the uptake phase (*i.e.* at the beginning of the depuration phase) and one at the end (or after a significant part) of the depuration phase. The intermediate sampling point is recommended as a check on the uptake and depuration kinetics[(20)]. For calculations, see Annexes 5 and 6.

Interpretation of the results

93. To assess the validity and informative value of the test, verify that the depuration period exceeds one half-life. Also, the BCF_{Km} (kinetic BCF derived from a minimised test) should be compared to the minimised BCF_{SS} value (which is the BCF_{SS} calculated at the end of the uptake phase, assuming that steady-state has been reached. This can only be assumed, as the number of sampling points is not sufficient for proving this). If the BCF_{Km} < minimised BCF_{SS}, the minimised BCF_{SS} should be the preferred value. If BCF_{Km} is less than 70 % of the minimised BCF_{SS}, the results are not valid, and a full test should be conducted.

94. If the minimised test gives a BCF_{Km} in the region of any value of regulatory concern, a full test should be conducted. If the result is far from any regulatory value of concern (well above or below), a full test may not be necessary, or a single concentration full test may be conducted if required by the relevant regulatory framework.

95. If a full test is found to be necessary after a minimised test at one concentration, this can be conducted at a second concentration. If the results are consistent, a further full test at a different concentration can be waived, as the bioconcentration of the substance is not expected to be concentration dependent. If the minimised test has been conducted at two concentrations, and the results show no concentration dependence, the full test may be conducted with only one concentration (*cf.* paragraph 87).

Test report

96. The test report for the minimised test should include all the information demanded for the full test (*cf.* paragraph 81), except that which is not possible to elaborate (*i.e.* a curve showing the time to steady-state and the steady-state bioconcentration factor; for the latter the minimised BCF_{ss} should be given instead). Additionally, it should also include the reasoning for using the minimised test and the resulting BCF_{Km}.

(20) When only two data points are measured, estimates of the confidence limits for BCF_{Km} can be made using bootstrap methods. When intermediate data points are also available confidence limits for BCF_{Km} can be calculated as in the full test.

305-III: Dietary Exposure Bioaccumulation Fish Test

INTRODUCTION

97. The method described in this section should be used for substances where the aqueous exposure methodology is not practicable (for example because stable, measurable water concentrations cannot be maintained, or adequate body burdens cannot be achieved within 60 days of exposure; see previous sections on the aqueous exposure method). It should be realised though that the endpoint from this test will be a dietary biomagnification factor (BMF) rather than a bioconcentration factor (BCF)[(21)].

98. In May 2001 a new method for the bioaccumulation testing of poorly water soluble organic chemicals was presented at the SETAC Europe conference held in Madrid (36). This work built on various reported bioaccumulation studies in the literature using a dosing method involving spiked feed (*e.g.* (37)). Early in 2004 a draft protocol (38), designed to measure the bioaccumulation potential of poorly water soluble organic chemicals for which the standard water exposure bioconcentration method was not practicable, together with a supporting background document (39), was submitted to an EU PBT working group. Further justification given for the method was that potential environmental exposure to such poorly soluble substances (*i.e.* log K_{OW} >5) may be largely via the diet (*cf.* (40) (41) (42) (43) (44)). For this reason, dietary exposure tests are referred to in some published chemicals regulations [(22)]. It should be realized however, that in the method described in this Guideline exposure via the aqueous phase is carefully avoided and thus a BMF value from this test method cannot directly be compared to a BMF value from a field study (in which both water and dietary exposure may be combined).

99. This section of the present Test Guideline (TG) is based on this protocol (38) and is a new method that did not appear in the previous version of OECD TG 305 (adopted 14.06.96). This alternative test allows the dietary exposure pathway to be directly investigated under controlled laboratory conditions.

100. Potential investigators should refer to paragraphs 1 to 14 of this TG for information on when the dietary exposure test may be preferred over the aqueous exposure test. Information on the various substance considerations is laid out, and should be considered before a test is conducted.

101. The use of radiolabelled test substances can be considered with similar considerations as for the aqueous exposure method (*cf.* paragraphs 6 and 65).

102. The dietary method can be used to test more than one substance in a single test, so long as certain criteria are fulfilled; these are explored further in paragraph 112. For simplicity the methodology here describes a test using only one test substance.

103. The dietary test is similar to the aqueous exposure method in many respects with the obvious exception of the exposure route. Hence many aspects of the method described here overlap with the aqueous exposure method described in the previous section. Cross-reference to relevant paragraphs in the previous section has been made as far as possible, but in the interests of readability and understanding a certain amount of duplication is unavoidable.

(21) See Annex 1 for definitions and units

(22) For example Regulation (EC) No 1907/2006 of the European Parliament and Council, REACH Technical Guidance: "Guidance on Information Requirements and Chemical Safety Assessment", chapter R.7c, R.7.10.3.1, pp 13; R.7.10.4.1, pp 31-32; and figure R7.10-2, pp 50.

PRINCIPLE OF THE TEST

104. Flow-through or semi-static conditions can be employed (*cf.* paragraph 4); flow-through conditions are recommended to limit potential exposure of test substance via water as a result of any desorption from spiked food or faeces. The test consists of two phases: uptake (test substance-spiked feed) and depuration (clean, untreated feed) (*cf.* paragraph 16). In the uptake phase, a "test" group of fish are fed a set diet of a commercial fish food of known composition, spiked with the test substance, on a daily basis. Fish ideally should consume all of the offered food (*c.f.* paragraph 141). Fish are then fed the pure, untreated commercial fish food during the depuration phase. As for the aqueous exposure method, more than one test group with different spiked test substance concentrations can be used if necessary, but for the majority of highly hydrophobic organic test substances one test group is sufficient (*cf.* paragraphs 49 and 107). If semi-static conditions are used fish should be transferred to a new medium and/or a new test chamber at the end of the uptake phase (in case the medium and/or apparatus used in the uptake phase has been contaminated with the test substance through leaching). The concentrations of the test substance in the fish are measured in both phases of the test. In addition to the group of fish fed the spiked diet (the test group), a control group of fish is held under identical conditions and fed identically except that the commercial fish food diet is not spiked with test substance. This control group allows background levels of test substance to be quantified in unexposed fish and serves as a comparison for any treatment-related adverse effects noted in the test group(s) [23]. It also allows comparison of growth rate constants between groups as a check that similar quantities of offered diet have been consumed (potential differences in palatability between diets should also be considered in explaining different growth rate constants; *cf.* paragraph 138). It is important that during both the uptake and depuration phases, diets of nutritional equivalency are fed to the test and control groups.

105. An uptake phase that lasts 7-14 days is generally sufficient, based on experience from the method developers (38) (39). This range should minimise the cost of undertaking the test whilst still ensuring sufficient exposure for most substances. However, in some cases the uptake phase may be extended (*cf.* paragraph 127). During the uptake phase the chemical concentration in the fish may not reach steady-state so data treatment and results from this method are usually based on a kinetic analysis of tissue residues. (Note: Equations for estimating time to steady-state can be applied here as for the aqueous exposure test – see Annex 5). The depuration phase begins when the fish are first fed unspiked diet and typically lasts for up to 28 days or until the test substance can no longer be quantified in whole fish, whichever is the sooner. The depuration phase can be shortened or lengthened beyond 28 days, depending on the change with time in measured chemical concentrations and fish size.

106. This method allows the determination of the substance-specific half-life ($t_{1/2}$, from the depuration rate constant, k_2), the assimilation efficiency (absorption across the gut; α), the kinetic dietary biomagnification factor (BMF_K), the growth-corrected kinetic dietary biomagnification factor (BMF_{Kg}), and the lipid-corrected[(24)] kinetic dietary biomagnification factor (BMF_{KL}) (and/or the growth- and lipid-corrected kinetic dietary biomagnification factor, BMF_{KgL}) for the test substance in fish. As for the aqueous exposure method, increase in fish mass during the test will result in dilution of test substance in growing fish and thus the (kinetic) BMF will be underestimated if not corrected for growth (*cf.* paragraphs 162 and 163). In addition, if it is estimated that steady-state was reached in the uptake phase an indicative steady-state BMF can be calculated. Approaches are available that make it

23 For most test substances, there should ideally be no detections in the control water. Background concentrations should only be relevant to naturally occurring materials (*e.g.*, some metals) and substances that are ubiquitous in the environment.

(24) As the BMF is defined as the ratio of the concentration of a substance in an organism to that in the organism's food at steady-state, lipid is taken into account by correcting for the contents of lipid in the organism and in the food, hence it is described more accurately as a "correction". This approach differs from "normalisation" to a set organism lipid content as is done in the aqueous exposure bioconcentration test.

feasible to estimate a kinetic bioconcentration factor (BCF_K) from data generated in the dietary study (*e.g.* (44) (45) (46) (47) (48). Pros and cons of such approaches are discussed in Annex 8.

107. The test was designed primarily for poorly soluble non-polar organic substances that follow approximately first order uptake and depuration kinetics in fish. In case a substance is tested that does not follow approximately first order uptake and depuration kinetics, then more complex models should be employed (see references in Annex 5) and advice from a biostatistician and/or pharmacokineticist sought.

108. The BMF is normally determined using test substance analysis of whole fish (wet weight basis). If relevant for the objectives of the study, specific tissues (*e.g.* muscle, liver) can be sampled if the fish is divided into edible and non-edible parts (*cf.* paragraph 21). Furthermore, removal and separate analysis of the gastrointestinal tract may be employed to determine the contribution to whole fish concentrations for sample points at the end of the uptake phase and near the beginning of the depuration phase, or as part of a mass balance approach.

109. Lipid content of sampled whole fish should be measured so that concentrations can be lipid-corrected, taking account of lipid content of both the diet and the fish (*cf.* paragraphs 56 and 57, and Annex 7).

110. Fish weight of sampled individuals should be measured and recorded, and be linked to the analysed chemical concentration for that individual (*e.g.* reported using a unique identifier code for each fish sampled), for the purpose of calculating growth that may occur during the test. Fish total length should also be measured where possible [(25)]. Weight data are also necessary for estimating BCF using depuration data from the dietary test.

INFORMATION ON THE TEST SUBSTANCE

111. Information on the test substance as described in paragraphs 3 and 22 should be available. An analytical method for test substance concentrations in water is not usually necessary; methods with suitable sensitivity for measuring concentrations in fish food and fish tissue are required.

112. The method can be used to test more than one substance in a single test. However, test substances should be compatible with one another such that they do not interact or change their chemical identity upon spiking into fish food. The aim is that measured results for each substance tested together should not differ greatly from the results that would be given if individual tests had been run on each test substance. Preliminary analytical work should establish that each substance can be recovered from a multiply-spiked food and fish tissue sample with i) high recoveries (*e.g.* > 85% of nominal) and ii) the necessary sensitivity for testing. The total dose of substances tested together should be below the combined concentration that might cause toxic effects (*cf.* paragraph 51). Furthermore, possible adverse effects in fish and the potential for interactive effects (*e.g.* metabolic effects) associated with testing multiple substances simultaneously should be taken into consideration in the experimental design. Simultaneous testing of ionisable substances should be avoided. In terms of exposure, the method is also suitable for complex mixtures (*cf.* paragraph 13, although the same limitations in analysis as for any other method will apply).

VALIDITY OF THE TEST

113. For a test to be valid the following conditions apply (*cf.* paragraph 24):

- Water temperature variation is less than ± 2 ºC in treatment or control groups

(25) Total length should also be recorded during the test as it is a good indicator of whether an adverse effect has occurred.

- Concentration of dissolved oxygen does not fall below 60% of the air saturation value
- The concentration of the test substance in fish food before and at the end of the uptake phase is within a range of ± 20% (based on at least three samples at both time points)
- A high degree of homogeneity of substance in food should be demonstrated in preliminary analytical work on the spiked diet; at least three sample concentrations for the substance taken at test start should not vary more than ± 15% from the mean
- Concentrations of test substance are not detected, or are present only at typical trace levels, in un-spiked food or control fish tissues relative to treated samples
- Mortality or other adverse effects/disease in both control and test group fish should be ≤10% at the end of the test; if the test is extended for any reason, adverse effects in both groups are ≤ 5% per month, and ≤ 30% cumulatively. Significant differences in average growth between the test and the control groups of sampled fish could be an indication of a toxic effect of the test chemical.

REFERENCE SUBSTANCES

114. If a laboratory has not performed the assay before or substantial changes (*e.g.* change of fish strain or supplier, different fish species, significant change of fish size, fish food or spiking method, etc.) have been made, it is advisable that a technical proficiency study is conducted, using a reference substance. The reference substance is primarily used to establish whether the food spiking technique is adequate to ensure maximum homogeneity and bioavailability of test substances. One example that has been used in the case of non-polar hydrophobic substances is hexachlorobenzene (HCB), but other substances with existing reliable data on uptake and biomagnification should be considered due to the hazardous property of HCB[26]. If used, basic information on the reference substance should be presented in the test report, including name, purity, CAS number, structure, toxicity data (if available) as for test substances (*cf.* paragraphs 3 and 22).

DESCRIPTION OF THE METHOD

Apparatus

115. Materials and apparatus should be used as described in the aqueous exposure method (*cf.* paragraph 26). A flow-through or static renewal test system that provides a sufficient volume of dilution water to the test tanks should be used. The flow rates should be recorded.

Water

116. Test water should be used as described in the aqueous exposure method (*cf.* paragraphs 27-29). The test medium should be characterised as described and its quality should remain constant during the test. The natural particle content and total organic carbon should be as low as possible (≤ 5 mg/L particulate matter; ≤ 2 mg/L total organic carbon) before test start. TOC need only be measured before the test as part of the test water characterisation (*cf.* paragraph 53).

Diet

117. A commercially available fish food (floating and/or slow sinking pelletized diet) that is characterised in terms of at least protein and fat content is recommended. The food should have a uniform pellet size to increase the efficiency of the feed exposure, *i.e.* the fish will eat more of the

(26) HCB is listed in Annexes A and C to the Stockholm Convention

food instead of eating the larger pieces and missing the smaller ones. The pellets should be appropriately sized for the size of the fish at the start of the test (*e.g.* pellet diameters roughly 0.6-0.85 mm for fish between 3 and 7 cm total length, and 0.85-1.2 mm for fish between 6 and 12 cm total length may be used). Pellet size may be adjusted depending on fish growth at the start of the depuration phase. An example of a suitable food composition, as commercially supplied, is given in Annex 7. Test diets with total lipid content between 15 and 20% (w/w) have commonly been used in the development of this method. Fish food with such a high lipid concentration may not be available in some regions. In such cases studies could be run with a lower lipid concentration in the food, and if necessary the feeding rate adjusted appropriately to maintain fish health (based on preliminary testing). The total lipid content of the test group and control group diets needs to be measured and recorded before the start of the test and at the end of the uptake phase. Details provided by the commercial feed supplier of analysis for nutrients, moisture, fibre and ash, and if possible minerals and pesticide residues (*e.g.* "standard" priority pollutants), should be presented in the study report.

118. When spiking the food with test substance, all possible efforts should be made to ensure homogeneity throughout the test food. The concentration of test substance in the food for the test group should be selected taking into account the sensitivity of the analytical technique, the test substance's toxicity (NOEC if known) and relevant physicochemical data. If used, the reference substance should preferably be incorporated at a concentration around 10% of that of the test substance (or in any case as low as is practicable), subject to analysis sensitivity (*e.g.* for hexachlorobenzene a concentration in the food of 1-100 µg/g has been found to be acceptable; *cf.* (47) for more information on assimilation efficiencies of HCB).

119. The test substance can be spiked to the fish food in several ways depending on its physical characteristics and solubility (see Annex 7 for more details on spiking methods):

- If the substance is soluble and stable in triglycerides, the chemical should be dissolved in a small amount of fish oil or edible vegetable oil before mixing with fish food. In this instance, care should be taken to avoid producing a ration that is too high in lipid, taking into account the natural lipid content of the spiked feed, by adding the minimum known quantity of oil required to achieve distribution and homogeneity of the test substance in the food, or;

- The food should be spiked using a suitable organic solvent, so long as homogeneity and bioavailability are not compromised (it is possible that (micro)crystals of the test substance may form in the food as a consequence of solvent evaporation and there is no easy way to prove this has not occurred; *cf.* (49)), or;

- Non-viscous liquids should be added directly to fish food but they should be well mixed to promote homogeneity and facilitate good assimilation. The technique for mixing should ensure homogeneity of the spiked feed.

In few cases, *e.g.* less hydrophobic test substances more likely to desorb from the food, it may be necessary to coat prepared food pellets with a small quantity of corn/fish oil (see paragraph 142). In such cases, control food should be treated similarly and the final prepared feed used for lipid measurement.

120. If used, the results of the reference substance should be comparable with literature study data carried out under similar conditions with a comparable feeding rate (*cf.* paragraph 45) and reference substance-specific parameters should meet the relevant criteria in paragraph 113 (3rd, 4th and 5th points).

121. If an oil or carrier solvent is used as a vehicle for the test chemical, an equivalent amount of the same vehicle (excluding test substance) should be mixed with the control diet in order to maintain

equivalency with the spiked diet. It is important that during both the uptake and depuration phases diets of nutritional equivalency are fed to the test and control groups.

122. The spiked diet should be stored under conditions that maintain stability of the test chemical within the feed mix (*e.g.* refrigeration) and these conditions reported.

Selection of fish species

123. Fish species as specified for the aqueous exposure may be used (*cf.* paragraph 32 and Annex 3). Rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus carpio*) and fathead minnow (*Pimephales promelas*) have been commonly used in dietary bioaccumulation studies with organic chemicals before the publication of this TG. The test species should have a feeding behaviour that results in rapid consumption of the administered food ration to ensure that any factor influencing the concentration of the test substance in food (*e.g.* leaching into the water and the possibility of aqueous exposure) is kept to a minimum. Fish within the recommended size/weight range (*cf.* Annex 3) should be used. Fish should not be so small as to hamper ease of analyses on an individual basis. Species tested during a life-stage with rapid growth can complicate data interpretation, and high growth rates can influence the calculation of assimilation efficiency [(27)].

Holding of fish

124. Acclimatisation, mortality and disease acceptance criteria are the same as for the aqueous exposure method prior to test conductance (*cf.* paragraphs 33-35).

PERFORMANCE OF THE TEST

Pre-study work and range-finding test

125. Pre-study analytical work is necessary to demonstrate recovery of the substance from spiked food/spiked fish tissue. A range-finding test to select a suitable chemical concentration in the food is not always necessary. For the purposes of showing that no adverse effects are observed and evaluating the palatability of spiked diet, sensitivity of analytical method for fish tissue and food, and selection of suitable feeding rate and sampling intervals during depuration phase etc., preliminary feeding experiments may be undertaken but are not obligatory. A preliminary study may be valuable to estimate numbers of fish needed for sampling during the depuration phase. This can result in significant reduction in the number of fish used, especially for test substances that are particularly susceptible to metabolism.

Conditions of exposure

Uptake Phase duration

126. An uptake phase of 7-14 days is usually sufficient, during which one group of fish are fed the control diet and another group of fish the test diet daily at a fixed ration dependent on the species tested and the experimental conditions, *e.g.* between 1-2 % of body weight (wet weight) in the case of rainbow trout. The feeding rate should be selected such that fast growth and large increase of lipid content are avoided. If needed the uptake phase may be extended based on practical experience from previous studies or knowledge of the test substance's (or analogue's) uptake/depuration in fish. The start of the test is defined as the time of first feeding with spiked food. An experimental day runs from the time of feeding to shortly before the time of next feeding (*e.g.* one hour). Thus the first experimental day of uptake runs from the time of first feeding with spiked food and ends shortly

(27) For rapid growth during the uptake phase the true feeding rate will decrease below that set at the beginning of exposure.

before the second feeding with spiked food. In practice the uptake phase ends shortly before (*e.g.* one hour) the first feeding with unspiked test substance as the fish will continue to digest spiked food and absorb the test substance in the intervening 24 hours. It is important to ensure that a sufficiently high (non-toxic) body burden of the test substance is achieved with respect to the analytical method, so that at least an order of magnitude decline can be measured during the depuration phase. In special cases an extended uptake phase (up to 28 days) may be used with additional sampling to gain an insight into uptake kinetics. During uptake the concentration in the fish may not reach steady-state. Equations for estimating time to steady-state, as an indication of the likely duration needed to achieve appreciable fish concentrations, can be applied here as for the aqueous exposure test (*cf.* Annex 5).

127. In some cases it may be known that uptake of chemical in the fish over 7-14 days will be insufficient for the food concentration used to reach a high enough fish concentration to analyse at least an order of magnitude decline during depuration, either due to poor analytical sensitivity or to low assimilation efficiency. In such cases it may be advantageous to extend the initial feeding phase to longer than 14 days, or, especially for highly metabolisable substances, a higher dietary concentration should be considered. However, care should be taken to keep the body burden during uptake below the (estimated) chronic no effect concentration (NOEC) in fish tissue (*cf.* paragraph 138).

Duration of the depuration phase

128. Depuration typically lasts for up to 28 days, beginning once the test group fish are fed pure, untreated diet after the uptake phase. Depuration begins with the first feeding of "unspiked" food rather than straight after the last "spiked" food feeding as the fish will continue to digest the food and absorb the test substance in the intervening 24 hours, as noted in paragraph 126. Hence the first sample in the depuration phase is taken shortly before the second feeding with unspiked diet. This depuration period is designed to capture substances with a potential half-life of up to 14 days, which is consistent with that of bioaccumulative substances[(28)], so 28 days comprises two half-lives of such substances. In cases of very highly bioaccumulating substances it may be advantageous to extend the depuration phase (if indicated by preliminary testing).

129. If a substance is depurated very slowly such that an exact half-life may not be determined in the depuration phase, the information may still be sufficient for assessment purposes to indicate a high level of bioaccumulation. Conversely, if a substance is depurated so fast that a reliable time zero concentration (concentration at the end of uptake/start of depuration, $C_{0,d}$) and k_2 cannot be derived, a conservative estimate of k_2 can be made (*cf.* Annex 7).

130. If analyses of fish at earlier intervals (*e.g.* 7 or 14 days) show that the chemical has depurated below quantification levels before the full 28-day period, then subsequent sampling may be discontinued and the test terminated.

131. In few cases no measurable uptake of the test substance may have occurred at the end of the uptake period (or with the second depuration sample). If it can be demonstrated that: i) the validity criteria in paragraph 113 are fulfilled; and ii) lack of uptake is not due to some other shortcoming of the test (*e.g.* uptake duration not long enough, deficiency in food spiking technique leading to poor bioavailability, lack of sensitivity of the analytical method, fish not consuming food, etc.); it may be possible to terminate the study without the need to re-run it with a longer uptake duration. If preliminary work has indicated that this may be the case, analysis of faeces, if possible, for undigested test substance may be advisable as part of a "mass balance" approach.

(28) In an aqueous exposure study, a 14-day half-life would correspond to a BCF of ca. 10,000 L/kg using fish of 1 g with a corresponding uptake rate of about 500 L/kg/d (according to the equation of Sijm *et al* (46)).

Numbers of test fish

132. Similar to the aqueous exposure test, fish of similar weight and length should be selected, with the smallest fish being no less than two-thirds of the weight of the largest (*cf.* paragraphs 40-42).

133. The total number of fish for the study should be selected based on the sampling schedule (a minimum of one sample at the end of the uptake phase and four to six samples during the depuration phase, but depending on the phases' durations), taking into account the sensitivity of the analytical technique, the concentration likely to be achieved at the end of the uptake phase (based on prior knowledge) and the depuration duration (if prior knowledge allows estimation). Five to ten fish should be sampled at each event, with growth parameters (weight and total length) being measured before chemical or lipid analysis.

134. Owing to the inherent variability in the size, growth rate, and physiology among fish and the likely variation in the quantity of administered diet that each fish consumes, at least five fish should be sampled at each interval from the test group and five from the control group in order to adequately establish the average concentration and its variability. The variability among the fish used is likely to contribute more to the overall uncontrolled variability in the test than the variability inherent in the analytical methodologies employed, and thus justifies the use of up to ten fish per sample point in some cases. However, if background test substance concentrations in control fish are not measurable at the start of depuration, chemical analysis of two-three control fish at the final sampling interval only may be sufficient so long as the remaining control fish at all sample points are still sampled for weight and total length (so that the same number are sampled from test and control groups for growth). Fish should be stored, weighed individually (even if it proves necessary for the sample results to be combined subsequently) and total length measured.

135. For a standard test with, for example, a 28-day depuration duration including five depuration samples, this means a total of 59-120 fish from test and 50-110 from control groups, assuming that the substance's analytical technique allows lipid content analysis to be carried out on the same fish. If lipid analysis cannot be conducted on the same fish as chemical analysis, and using control fish only for lipid analysis is also not feasible (*cf.* paragraph 56), an additional 15 fish would be required (three from the stock population at test start, three each from control and test groups at the start of depuration and three each from control and test groups at the end of the experiment). An example sampling schedule with fish numbers can be found in Annex 4.

Loading

136. Similarly high water-to-fish ratios should be used as for the aqueous exposure method (*cf.* paragraphs 43 and 44). Although fish-to-water loading rates do not have an effect on exposure concentrations in this test, a loading rate of 0.1-1.0 g of fish (wet weight) per litre of water per day is recommended to maintain adequate dissolved oxygen concentrations and minimise test organism stress.

Test diet and Feeding

137. During the acclimatisation period, fish should be fed an appropriate diet as described above (paragraph 117). If the test is being conducted under flow-through conditions, the flow should be suspended while the fish are fed.

138. During the test, the diet for the test group should adhere to that described above (paragraphs 116-121). In addition to consideration of substance-specific factors, analytical sensitivity, expected concentration in the diet under environmental conditions and chronic toxicity levels/body burden, selection of the target spiking concentration should take into account palatability of the food (so that fish do not avoid eating). Nominal spiking concentration of the test substance should be

documented in the report. Based on experience, spiking concentrations in the range of 1-1000 µg/g provide a practical working range for test substances that do not exhibit a specific toxic mechanism. For chemicals acting via a non-specific mechanism, tissue residue levels should not exceed 5 µmol/g lipid since residues above this level are likely to pose chronic effects (19) (48) (50)[29]. For other substances care should be taken that no adverse effects occur from the accumulated exposure (*cf.* paragraph 127). This is especially true if more than one substance is being tested simultaneously (*cf. paragraph* 112).

139. The appropriate amount of the test substance can be spiked to the fish food in one of three ways, as described in paragraph 119 and Annex 7. The methods and procedures for spiking the feed should be documented in the report. Untreated food is fed to the control fish, containing an equivalent quantity of unspiked oil vehicle if this has been used in the spiked feed for the uptake phase, or having been treated with "pure" solvent if a solvent vehicle was used for test group diet preparation. The treated and untreated diets should be measured analytically at least in triplicate for test substance concentration before the start and at the end of the uptake phase. After exposure to the treated feed (uptake phase), fish (both groups) are fed untreated food (depuration phase).

140. Fish are fed at a fixed ration (dependent on species; *e.g.* approximately 1-2 % of wet body weight per day in the case of rainbow trout). The feeding rate should be selected such that fast growth and large increase of lipid content are avoided. The exact feeding rate set during the experiment should be recorded. Initial feeding should be based on the scheduled weight measurements of the stock population just prior to the start of the test. The amount of feed should be adjusted based on the wet weights of sampled fish at each sampling event to account for growth during the experiment. Weights and lengths of fish in the test and control tanks can be estimated from the weights and total lengths of fish used at each sampling event; do not weigh or measure the fish remaining in the test and control tanks. It is important to maintain the same set feeding rate throughout the experiment.

141. Feeding should be observed to ensure that the fish are visibly consuming all of the food presented in order to guarantee that the appropriate ingestion rates are used in the calculations. Preliminary feeding experiments or previous experience should be considered when selecting a feeding rate that will ensure that all food from once-daily feeding is consumed. In the event that food is consistently being left uneaten, it may be advisable to spread the dose over an extra feeding period in each experimental day (*e.g.* replace once-daily feeding with feeding half the amount twice daily). If this is necessary, the second feeding should occur at a set time and be timed so that the maximum period of time possible passes before fish sampling (*e.g.* time for second feeding is set within the first half of an experimental day).

142. Although fish generally rapidly consume the food, it is important to ensure that the chemical remains adsorbed to the food. Efforts should be made to avoid the test substance becoming dispersed in water from the food, thereby exposing the fish to aqueous concentrations of the test substance in addition to the dietary route. This can be achieved by removing any uneaten food (and faeces) from the test and control tanks within one hour of feeding, but preferably within 30 minutes. In addition, a system where the water is continuously cleaned over an active carbon filter to absorb any 'dissolved' contaminant may be used. Flow-through systems may help to flush away food particles and dissolved substances rapidly[30]. In some cases, a slightly modified spiked food preparation technique can help to alleviate this problem (see paragraph 119).

(29) Since the actual internal concentrations can only be determined after the test has been performed, an estimate of the expected internal concentration is needed (*e.g.* based on the expected BMF and the concentration in the food; cf. Equation A5.8 in Annex 5).

(30) The presence of the test substance in the test medium as a result of excretion by the fish or leaching from food may not be totally avoidable. Therefore one option is to measure the substance concentration in water at the end of the uptake period, especially if a semi-static set up is used, to help to establish whether any aqueous exposure has occurred.

Light and Temperature

143. As for the aqueous exposure method (*cf.* paragraph 48), a 12 to 16 hour photoperiod is recommended and temperature (± 2 ºC) appropriate for the test species used (*cf.* Annex 3). Type and characteristics of illumination should be known and documented.

Controls

144. One control group should be used, with fish fed the same ration as the test group but without the test substance present in the feed. If an oil or solvent vehicle has been used to spike the feed in the test group, the control group food should be treated in exactly the same way but with the absence of test substance so that the diets of the test group and control group are equivalent (*cf.* paragraphs 121 and 139).

Frequency of Water Quality Measurements

145. The conditions described in the aqueous exposure method apply here also, except that TOC need only be measured before the test as part of the test water characterisation (*cf.* paragraph 53).

Sampling and Analysis of Fish and Diet

Analysis of Diet Samples

146. Samples of the test and control diets should be analysed at least in triplicate for the test substance and for lipid content at least before the beginning and at the end of the uptake phase. The methods of analysis and procedures for ensuring homogeneity of the diet should be included in the report.

147. Samples should be analysed for the test substance by the established and validated method. Pre-study work should be conducted to establish the limit of quantification, percent recovery, interferences and analytical variability in the intended sample matrix. If a radiolabelled material is being tested, similar considerations as those for the aqueous exposure method should be considered with feed analysis replacing water analysis (*cf.* paragraph 65).

Analysis of Fish

148. At each fish sampling event, 5-10 individuals will be sampled from exposure and control treatments (in some instances numbers of control fish can be reduced; *cf.* paragraph 134).

149. Sampling events should occur at the same time on each experimental day (relative to feeding time), and should be timed so that the likelihood of food remaining in the gut during the uptake phase and the early part of the depuration phase is minimised to prevent spurious contributions to total test substance concentrations (*i.e.* sampled fish should be removed at the end of an experimental day, keeping in mind that an experimental day starts at the time of feeding and ends at the time of the next feeding, approximately 24 hours later. Depuration begins with the first feeding of unspiked food; *cf.* paragraph 128). The first depuration phase sample (taken shortly before the second feeding with unspiked food) is important as extrapolation back one day from this measurement is used to estimate the time zero concentration ($C_{0,d}$, the concentration in the fish at the end of uptake/start of depuration). Optionally, the gastrointestinal tract of the fish can be removed and analysed separately at the end of uptake and at days 1 and 3 of depuration.

150. At each sampling event fish should be removed from both test vessels and treated in the same way as described in the aqueous method (*cf.* paragraphs 61-63).

151. Concentrations of test substance in whole fish (wet weight) are measured at least at the end of the uptake phase and during the depuration phase in both control and test groups. During the depuration phase, four to six sampling points are recommended (*e.g.* 1, 3, 7, 14 and 28 days). Optionally, an additional sampling point may be included after 1-3 days' uptake to estimate assimilation efficiency from the linear phase of uptake for the fish while still near the beginning of the exposure period. Two main deviations from the schedule exist: i) if an extended uptake phase is employed for the purposes of investigating uptake kinetics, there will be additional sampling points during the uptake phase and so additional fish will need to be included (*cf.* paragraph 126); ii) if the study has been terminated at the end of the uptake phase owning to no measurable uptake (*cf.* paragraph 131). Individual fish that are sampled should be weighed (and their total length measured) to allow growth rate constants to be determined. Concentrations of the substance in specific fish tissue (edible and non-edible portions) can also be measured at the end of uptake and selected depuration times. If a radiolabelled material is being tested, similar considerations as those for the aqueous exposure method should be considered with feed analysis replacing water analysis (*cf.* paragraph 65).

152. For the periodic use of a reference substance (*cf.* paragraph 25), it is preferable that concentrations are measured in the test group at the end of uptake and at all depuration times specified for the test substance (whole fish); concentrations need only be analysed in the control group at end of uptake (whole fish). In certain circumstances (for example if analysis techniques for test substance and reference substance are incompatible, such that additional fish would be needed to follow the sampling schedule) another approach may be used as follows to minimise the number of additional fish required. Concentrations of the reference substance are measured during depuration only on days 1, 3 and two further sampling points, selected such that reliable estimations of time zero concentration ($C_{0,d}$) and k_2 can be made for the reference substance.

153. If possible the lipid content of the individual fish should be determined on each sampling occasion, or at least at the start and end of the uptake phase and at the end of the depuration phase. (*cf.* paragraphs 56 and 67). Depending on the analytical method (refer to paragraph 67 and to Annex 4), it may be possible to use the same fish for both lipid content and test substance concentration determination. This is preferred on the grounds of minimising fish numbers. However, should this not be possible, the same approach as described in the aqueous exposure method can be used (see paragraph 56 for these alternative lipid measurement options). The method used to quantify the lipid content should be documented in the report.

Quality of the analytical method

154. Experimental checks should be conducted to ensure the specificity, accuracy, precision and reproducibility of the substance-specific analytical technique, as well as recoveries of the test substance from both food and fish.

Fish growth measurement

155. At the start of the test a sample of fish from the stock population need to be weighed (and their total length measured). These fish should be sampled shortly before the first spiked feeding (*e.g.* one hour), and assigned to experimental day 0. The number of fish for this sample should be at least the same as that for the samples during the test. Some of these can be the same fish used for lipid analysis before the start of the uptake phase (*cf.* paragraph 153). At each sampling interval fish are first weighed and their length measured. In each individual fish the measured weight (and length) should be linked to the analysed chemical concentration (and lipid content, if applicable), for example using a unique identifier code for each sampled fish. The measurements of these sampled fish can be used to estimate the weight (and length) of fish remaining in the test and control tanks.

Experimental Evaluation

156. Observations of mortality should be performed and recorded daily. Additional observations for adverse effects should be performed, for example for abnormal behaviour or pigmentation, and recorded. Fish are considered dead if there is no respiratory movement and no reaction to a slight mechanical stimulus can be detected. Any dead or clearly moribund fish should be removed.

DATA AND REPORTING

Treatment of results

157. Test results are used to derive the depuration rate constant (k_2) as a function of the total wet weight of the fish. Growth rate constant, k_g, based on mean increase in fish weight is calculated and used to produce the growth-corrected depuration rate constant, k_{2g}, if appropriate. In addition, the assimilation efficiency (α; absorption from the gut), the kinetic biomagnification factor (BMF_K) (if necessary growth corrected, BMF_{Kg}), its lipid-corrected value (BMF_{KL} or BMF_{KgL}, if corrected for growth dilution) and feeding rate should be reported. Also, if an estimate of the time to steady-state in the uptake phase can be made (*e.g.*, 95% of steady-state or $t_{95} = 3.0/k_2$), an estimate of the steady-state BMF (BMF_{SS}) can be included (*cf.* paragraphs 105 and 106, and Annex 5) if the t_{95} value indicates that steady-state conditions may have been reached. The same lipid correction should be applied to this BMF_{SS} as to the kinetically-derived BMF (BMF_K) to give a lipid-corrected value, BMF_{SSL} (note that no agreed procedure is available to correct a steady-state BMF for growth dilution). Formulae and example calculations are presented in Annex 7. Approaches are available that make it feasible to estimate a kinetic bioconcentration factor (BCF_K) from data generated in the dietary study. This is discussed in Annex 8.

Fish weight/length data

158. Individual fish wet weights and lengths for all time periods are tabulated separately for test and control groups for all sampling days during the uptake phase (stock population for start of uptake; control group and test group for end of uptake and, if conducted, the early phase (*e.g.* day 1-3 of uptake) and depuration phase (*e.g.* days 1, 2, 4, 7, 14, 28, for control and test group). Weight is the preferred measure of growth for growth dilution correction purposes. See below (paragraphs 162 and 163) and Annex 5 for the method(s) used to correct data for growth dilution.

Test substance concentration in fish data

159. Individual fish test substance residue measurements (or pooled fish samples if individual fish measurements are not possible), expressed in terms of wet weight concentration (w/w), are tabulated for test and control fish for individual sample times. If lipid analysis has been conducted on each sampled fish then individual lipid-corrected concentrations, in terms of lipid concentration (w/w lipid), can be derived and tabulated.

- Test substance residue measurements in individual fish (or pooled fish samples if individual fish measurements are not possible, *cf.* paragraph 66) for the depuration period are converted to their natural logarithms and plotted versus time (day). If a visual inspection of the plot shows obvious outliers, a statistically valid outlier test may be applied to remove spurious data points as well as documented justification for their omission.

- A linear least squares correlation is calculated for the ln(concentration) vs. depuration (day) data. The slope and intercept of the line are reported as the overall depuration rate constant (k_2) and natural logarithm of the derived time zero concentration ($C_{0,d}$) (*cf.* Annex 5 and Annex 7 for further details). Should this not be possible because concentrations fall below

the limit of quantification for the second depuration sample, a conservative estimate of k_2 can be made (*cf.* Annex 7).

- The variances in the slope and intercept of the line are calculated using standard statistical procedures and the 90% (or 95%) confidence intervals around these results evaluated and presented.

- The mean measured fish concentration for the final day of uptake (measured time zero concentration, $C_{0,m}$) is also calculated and compared with the derived value $C_{0,d}$. In case the derived value is lower than the measured value, the difference may suggest the presence of undigested spiked food in the gut. If the derived value is very much higher than the measured value, this may be an indication that the value derived from the depuration data linear regression is erroneous and should be re-evaluated (see Annex 7).

Depuration rate and biomagnification factor

160. To calculate the biomagnification factor from the data, first the assimilation efficiency (absorption of test substance across the gut, α) should be obtained. To do this, equation A7.1 in Annex 7 should be used, requiring the derived concentration in fish at time zero of the depuration phase ($C_{0,d}$), (overall) depuration rate constant (k_2), concentration in the food (C_{food}), food ingestion rate constant (I) and duration of the uptake period (t) to be known. The slope and intercept of the linear relationship between ln(concentration) and depuration time are reported as the overall depuration rate constant (k_2 = slope) and time zero concentration ($C_{0,d} = e^{intercept}$), as above. The derived values should be checked for biological plausibility (e.g. assimilation efficiency as a fraction is not greater than 1). *(I)* is calculated by dividing the mass of food by the mass of fish fed each day (if fed at 2% of body weight, (*I)* will be 0.02). However, the feeding rate used in the calculation may need to be adjusted for fish growth (this can be done using the known growth rate constant to estimate the fish weight at each time-point during the uptake phase; *cf.* Annex 7). In cases where k_2 and $C_{0,d}$ cannot be derived because, for example, concentrations fell below the limit of detection for the second depuration sample, a conservative estimate of k_2 and an "upper bound" BMF_k can be made (*cf.* Annex 7).

161. Once the assimilation efficiency (α) is obtained, the biomagnification factor can be calculated by multiplying α by the ingestion rate constant (I) and dividing by the (overall) depuration rate constant (k_2). The growth-corrected biomagnification factor is calculated in the same way but using the growth-corrected depuration rate constant (k_{2g}; *cf.* paragraphs 162 and 163. An alternative estimate of the assimilation efficiency can be derived if tissue analysis was performed on fish sampled in the early, linear phase of the uptake phase; *cf.* paragraph 151 and Annex 7. This value represents an independent estimate of assimilation efficiency for an essentially unexposed organism (*i.e.* the fish are near the beginning of the uptake phase). The assimilation efficiency estimated from depuration data is usually used to derive the BMF.

Lipid Correction and Growth-Dilution Correction

162. Fish growth during the depuration phase can lower measured chemical concentrations in the fish with the effect that the overall depuration rate constant, k_2, is greater than would arise from removal processes (*e.g.* metabolism, egestion) alone (*cf.* paragraph 72). Lipid contents of test fish (which are strongly associated with the bioaccumulation of hydrophobic chemicals) and lipid contents of food can vary enough in practice such that their correction is necessary to present biomagnification factors in a meaningful way. The biomagnification factor should be corrected for growth dilution (as is the kinetic BCF in the aqueous exposure method) and corrected for the lipid content of the food relative to that of the fish (the lipid-correction factor). Equations and examples for these calculations can be found in Annex 5 and Annex 7, respectively.

163. To correct for growth dilution, the growth-corrected depuration rate constant (k_{2g}) should be calculated (see Annex 5 for equations). This growth-corrected depuration rate constant (k_{2g}) is then used to calculate the growth-corrected biomagnification factor, as in paragraph 73. In some cases this approach is not possible. An alternative approach that circumvents the need for growth dilution correction involves using mass of test chemical per fish (whole fish basis) depuration data rather than the usual mass of test chemical per unit mass of fish (concentration) data. This can be easily achieved as tests according to this guideline should link recorded tissue concentrations to individual fish weights. The simple procedure for doing this is outlined in Annex 5. Note that k_2 should still be estimated and reported even if this alternative approach is used.

164. To correct for the lipid content of the food and fish when lipid analysis has not be conducted on all sampled fish, the mean lipid fractions (w/w) in the fish and in the food are derived(31). The lipid correction factor (L_c) is then calculated by dividing the fish mean lipid fraction by the mean food lipid fraction. The biomagnification factor, growth corrected or not as applicable, is divided by the lipid correction factor to calculate the lipid-corrected biomagnification factor.

165. If chemical and lipid analyses were conducted on the same fish at each sampling point, then the lipid-corrected tissue data for individual fish may be used to calculate a lipid-corrected BMF directly (*cf.* (37)). The plot of lipid-corrected concentration data gives $C_{0,d}$ on a lipid basis and k_2. Mathematical analysis can then proceed using the same equations in Annex 7, but assimilation efficiency (α) is calculated using the lipid-normalised food ingestion rate constant (I_{lipid}) and the dietary concentration on a lipid basis ($C_{food\text{-}lipid}$). Lipid corrected parameters are similarly then used to calculate BMF (note that growth rate constant correction should also be applied to the lipid fraction rather than the fish wet weight to calculated the lipid-corrected, growth corrected BMF_{KgL}).

Interpretation of results

166. Average growth in both test and control groups should in principle not be significantly different to exclude toxic effects. The growth rate constants or the growth curves of the two groups should be compared by an appropriate procedure (32)).

Test report

167. After termination of the study, a final report is prepared containing the information on *Test Substance*, *Test Species* and *Test Conditions* as listed in paragraph 81 (as for the aqueous exposure method). In addition, the following information is required:

Test Substance:

- Any information on stability of the test substance in prepared food;

Test Conditions:

- Substance nominal concentration in food, spiking technique, amount of (lipid) vehicle used in food spiking process (if used), test substance concentration measurements in spiked diet for each analysis (at least in triplicate before study start and at end of uptake) and mean values;

(31) This approach is specific to the dietary study, distinct from the procedure followed in the aqueous exposure, hence the word "correction" has been used rather than "normalisation" to prevent confusion – see also footnote (24).

(32) A *t*-test on growth rate constants can be performed , to test whether growth differs between control and test groups, or an *F*-test in case of analysis of variance. If needed, an *F*-test or likelihood ratio test can be used to assist in the choice of the appropriate growth model (OECD monograph 54, (32).

- If used, type and quality of carrier oil or solvent (grade, supplier, etc) used for food spiking;

- Food type employed (proximate analysis [(33)], grade or quality, supplier, etc.), feeding rate during uptake phase, amount of food administered and frequency (including any adjustments based on sampled fish weight);

- Time at which fish were collected and euthanized for chemical analysis for each sample point (e.g. one hour before the following day's feeding);

Results:

- Results from any preliminary study work;

- Information on any adverse effects observed;

- Complete description of all chemical analysis procedures employed including limits of detection and quantification, variability and recovery;

- Measured lipid concentrations in food (spiked and control diet), individual, mean values and standard deviations;

- Tabulated fish weight (and length) data linked to individual fish, both for control and exposure groups (for example using unique identifiers for each fish) and calculations, derived growth rate constant(s) and 95% confidence interval(s);

- Tabulated test substance concentration data in fish, mean measured concentration at end of uptake ($C_{0,m}$), and derived (overall) depuration rate constant (k_2) and concentration in fish at start of depuration phase ($C_{0,d}$) together with the variances in these values (slope and intercept);

- Tabulated fish lipid contents data (listed against specific chemical concentrations if applicable), mean values for test group and control at test start, end of uptake and end of depuration;

- Curves (including all measured data), showing the following (if applicable, concentrations may be expressed in relation to the whole body of the animal or specified tissues thereof):

    - growth (*i.e.* fish weight (and length) vs. time) or natural logarithm transformed weight vs. time;

    - the depuration of the test chemical in the fish; and

    - natural logarithm transformed concentration (ln concentration) vs. depuration time (including the derived depuration rate constant k_2, and natural logarithm derived concentration in fish at start of depuration phase, $C_{0,d}$).

- If a visual inspection of a plot shows obvious outliers, a statistically valid outlier test may be applied to remove spurious data points as well as documented justification for their omission.

- Calculated growth-corrected depuration rate constant and growth-corrected half-life.

(33) A foodstuff analysis technique for protein, lipid, crude fibre and ash content; this information is usually available from the feed supplier.

- Calculated assimilation efficiency (α).
- "Raw" dietary BMF, lipid and growth-dilution corrected kinetic BMF ("raw" and lipid-corrected based on whole fish wet weight), tissue-specific BMF if applicable.
- Any information concerning radiolabelled test chemical metabolites and their accumulation.
- Anything unusual about the test, any deviation from these procedures, and any other relevant information.
- A summary table of relevant measured and calculated data, as hereafter:

Substance Depuration Rate constants and Biomagnification Factors (BMF_K)	
k_g (growth rate constant; day^{-1}):	Insert Value (95% CI) (1)
k_2 (overall depuration rate constant, day^{-1}):	Insert Value (95% CI)
k_{2g} (growth-corrected depuration rate constant; day^{-1}):	Insert Value (95% CI) (1)
$C_{0,m}$ (measured time zero concentration, the concentration in fish at end of uptake) (μg/g):	Insert Value ± SD(2)
$C_{0,d}$ (derived time zero concentration of depuration phase; μg/g):	Insert Value ± SD(2)
I (set feed ingestion rate; g food/g fish/day):	Insert Value
I_g (effective feeding rate, adjusted for growth; g food/g fish/day)(2):	Insert Value ± SD(2)
C_{food} (chemical concentration in the food; μg/g):	Insert Value ± SD(2)
α (substance assimilation efficiency):	Insert Value ± SD(2)
BMF_K (kinetic dietary BMF):	Insert Value (95% CI) (1)
BMF_{Kg} (growth-corrected kinetic dietary BMF):	Insert Value (95% CI) (1)
$t_{1/2g}$ (growth-corrected half-life in days):	Insert Value ± SD(2)
Lc (lipid correction factor):	Insert Value
BMF_{KgL} (lipid-corrected growth-corrected kinetic BMF):	Insert Value
BMF_{SS-L} (indicative lipid-corrected steady-state BMF)(2):	Insert Value ± SD(2)

(1) CI: confidence interval (where possible to estimate)

(2) SD: Standard deviation (where possible to estimate)

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ANNEX 1

DEFINITIONS AND UNITS

The assimilation efficiency (α) is a measure of the relative amount of substance absorbed from the gut into the organism (α is unitless, but it is often expressed as a percentage rather than a fraction).

Bioaccumulation is generally referred to as a process in which the chemical concentration in an organism achieves a level that exceeds that in the respiratory medium (e.g., water for a fish or air for a mammal), the diet, or both (1).

Bioconcentration is the increase in concentration of the test substance in or on an organism (or specified tissues thereof) relative to the concentration of test substance in the surrounding medium.

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/kg) divided by the concentration of the chemical in the surrounding medium (C_w as mg/L). BCF is expressed in $L \cdot kg^{-1}$. Please note that corrections for growth and/or a standard lipid content are not accounted for.

Biomagnification is the increase in concentration of the test substance in or on an organism (or specified tissues thereof) relative to the concentration of test substance in the food.

The biomagnification factor (BMF) is the concentration of a substance in a predator relative to the concentration in the predator's prey (or food) at steady-state. In the method described in this Guideline, exposure via the aqueous phase is carefully avoided and thus a BMF value from this test method cannot directly be compared to a BMF value from a field study (in which both water and dietary exposure may be combined).

The dietary biomagnification factor (dietary BMF) is the term used in this guideline to describe the result of dietary exposure test, in which exposure via the aqueous phase is carefully avoided and thus the dietary BMF from this test method cannot directly be compared to a BMF value from a field study (in which both water and dietary exposure may be combined).

The depuration or post-exposure (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.

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

Dissolved organic carbon (DOC) is a measure of the concentration of carbon originating from dissolved organic sources in the test media.

The exposure or uptake phase is the time during which the fish are exposed to the test chemical.

The food ingestion rate (I) is the average amount of food eaten by each fish each day, relative to the estimated average fish whole body weight (expressed in terms of g food/g fish/day).

The kinetic bioconcentration factor (BCF_K) is the ratio of the uptake rate constant, k_1, to the depuration rate constant, k_2 (*i.e.* k_1/k_2 – see corresponding definitions in this annex). In principle the value should be comparable to the BCF_{SS} (see definition above), but deviations may occur if steady-state was uncertain or if corrections for growth have been applied to the kinetic BCF.

The lipid normalised kinetic bioconcentration factor (BCF_{KL}) is normalised to a fish with a 5% lipid content.

The lipid normalised, growth corrected kinetic bioconcentration factor (BCF_{KgL}) is normalised to a fish with a 5% lipid content and corrected for growth during the study period as described in Annex 5.

The lipid normalised steady-state bioconcentration factor (BCF_{SSL}) is normalised to a fish with 5% lipid content.

The octanol-water partition coefficient (K_{OW}) is the ratio of a chemical's solubility in *n*-octanol and water at equilibrium (OECD Guidelines 107 (2), 117 (3), 123 (4)); also expressed as P_{OW}. The logarithm of K_{OW} is used as an indication of a chemical's potential for bioconcentration by aquatic organisms.

Particulate organic carbon (POC) is a measure of the concentration of carbon originating from suspended organic sources in the test media.

Solid-phase microextraction (SPME) is a solvent-free analytical technique developed for dilute systems. In this method, a polymercoated fibre is exposed to the gas or liquid phase containing the analyte of interest. Generally, a minimum analysis time is imposed so that equilibrium conditions are established between the solid and fluid phases, with respect to the measured species. Subsequently the concentration of the analyte of interest can be determined directly from the fibre or after extracting it from the fibre into a solvent, depending on the determination technique.

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

The steady-state bioconcentration factor (BCF_{SS}) 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 (*cf.* Definition of steady-state).

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

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 L kg^{-1} day^{-1}).

Chemical substances of Unknown or Variable composition, Complex reaction products and Biological materials are known as UVCB

Litterature

(1) Gobas F.A.P.C., de Wolf W., Burkhard L.P., Verbruggen E. and Plotzke K. (2009), Revisiting bioaccumulation criteria for POPs and PBT assessments. Integr. Environ. Assess. Manag. 5: 624-637.

(2) OECD (1995), *Test No. 107: Partition Coefficient (n-octanol/water): Shake Flask Method*, OECD Guidelines for the Testing of Chemicals, Section 1, OECD Publishing. doi: 10.1787/9789264069626-en.

(3) OECD (2004),*Test No. 117: Partition Coefficient (n-octanol/water), HPLC Method*, OECD Guidelines for the Testing of Chemicals, Section 1, OECD Publishing. doi: 10.1787/9789264069824-en.

(4) OECD (2006), *Test No. 123: Partition Coefficient (1-Octanol/Water): Slow-Stirring Method*, OECD Guidelines for the Testing of Chemicals, Section 1, OECD Publishing. doi: 10.1787/9789264015845-en.

ANNEX 2

SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

Substance	Limit concentration
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	100 ng/L
Mercury	100 ng/L
Silver	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)[2]
Danio rerio[1] (Teleostei, Cyprinidae) (Hamilton-Buchanan) Zebra-fish	20 – 25	3.0 ± 0.5
Pimephales promelas (Teleostei, Cyprinidae) (Rafinesque) Fathead minnow	20 – 25	5.0 ± 2.0
Cyprinus carpio (Teleostei, Cyprinidae) (Linnaeus) Common carp	20 – 25	8.0 ± 4.0[3]
Oryzias latipes (Teleostei, Poecilliidae) (Temminck and Schlegel) Ricefish	20 – 25	4.0 ± 1.0
Poecilia reticulata (Teleostei, Poeciliidae) (Peters) Guppy	20 – 25	3.0 ± 1.0
Lepomis macrochirus (Teleostei Centrarchidae) (Rafinesque) Bluegill	20 – 25	5.0 ± 2.0
Oncorhynchus mykiss (Teleostei Salmonidae) (Walbaum) Rainbow trout	13 – 17	8.0 ± 4.0
Gasterosteus aculeatus (Teleostei, (Gasterosteidae) (Linnaeus) Three-spined stickleback	18 – 20	3.0 ± 1.0

(1) Meyer *et al.* (1)

(2) It should be noted that in the test itself weight is preferred as the measure for size and growth rate constant derivations. It is however recognised that length is a more practical measure if fish have to be selected by sight at the beginning of an experiment (*i.e.* from the stock population).

(3) This length range is indicated in the Testing Methods for New Chemical Substances etc. based on the Japan's Chemical Substances Control Law (CSCL).

Various estuarine and marine species have less widely been used, for example:

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

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.

Litterature

(1) Meyer A., Biermann C.H. and Orti G. (1993), The phylogenetic position of the zebrafish (*Danio rerio*), a model system in developmental biology: An invitation to the comparative method Proc. R. Soc. Lond. B. 252: 231-236.

ANNEX 4

SAMPLING SCHEDULES FOR AQUEOUS AND DIETARY EXPOSURE TESTS

1. Theoretical example of a sampling schedule for a full aqueous exposure bioconcentration test of a substance with log K_{OW} = 4.

Fish Sampling	Sample time schedule		No. of water samples[1]	No. of fish per sample[1]
	Minimal required frequency (days)[2]	Additional sampling (days)[2]		
Uptake phase				
1	-1 0		2[3] (2)	4[4] (3[6])
2	0.3	0.4	2 (2)	4 (4)
3	0.6	0.9	2 (2)	4 (4)
4	1.2	1.7	2 (2)	4 (4)
5	2.4	3.3	2 (2)	4 (4)
6	4.7		2	4 – 8[5] (3[6])
Depuration phase				Transfer fish to water free of test substance
7	5.0	5.3	2	4 (4)
8	5.9	7.0	2	4 (4)
9	9.3	11.2	2	4 (4)
10	14.0	17.5	2	4 – 8[5] (4+3[6])
TOTAL				40 – 72 (48 – 80)[5]

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

(2) Pre-test estimate of k_2 for log K_{OW} of 4.0 is 0.652 days^{-1}. The total duration of the experiment is set to $3 \times t_{SS} = 3 \times 4.6$ days, *i.e.* 14 days. For the estimation of t_{SS} refer to Annex 5.

(3) Sample water after a minimum of 3 "chamber-volumes" has been delivered.

(4) These fish are sampled from the stock population.

(5) If greater precision or metabolism studies are necessary that require more fish, these should be sampled particularly at the end of the uptake and depuration phases (*cf.* paragraph 40).

(6) At least 3 additional fish may be required for lipid content analysis if it is not possible to use the same fish sampled for substance concentrations at the start of the test, the end of the uptake phase and the end of the depuration phase. Note it should be possible in many cases to use the 3 control fish alone (*cf.* paragraph 56).

2. Theoretical example of sampling schedule for dietary bioaccumulation test of substance following 10 day uptake and 42 day depuration phases.

Sampling event	Sample time schedule		No. food samples	No. fish per sample	
	Day of phase	Additional fish samples?		Test Group	Control Group
Uptake phase					
1	0	Possible(1)(2)	3 – test group 3 – control group(1)	0	5 – 10 (8 – 13)(2)
1A(3)	1-3			5 – 10	5 – 10
2	10	Yes(4)	3 – test group 3 – control group(1)	10 – 15(4) (13 – 18)(5)	5 – 10 (8 – 13)(5)
Depuration phase					
3	1	Yes(4)		10 – 15(4)	5 – 10
4	2			5 – 10	5 – 10
5	4			5 – 10	5 – 10
6	7	Yes(4)		10 – 15(4)	5 – 10
7	14			5 – 10	5 – 10
8	28			5 – 10	5 – 10
9	42	Yes(4)		10 – 15(4) (13 – 18)(5)	5 – 10 (8 – 13)(5)
TOTAL				59 – 120 (63 – 126)(4,5)	50 – 110 (56 – 116)(4,5)

(1) 3 samples of feed from both control and test groups analysed for test substance concentrations and for lipid content.

(2) Fish are sampled from the stock population as near to the start of the study as possible; at least 3 fish from the stock population at test start should be sampled for lipid content.

(3) (Optional) sampling early in the uptake phase provides data to calculate dietary assimilation of test substance that can be compared with the assimilation efficiency calculated from the depuration phase data.

(4) 5 extra fish may be sampled for tissue-specific analysis.

(5) At least 3 additional fish may be required for lipid content analysis if it is not possible to use the same fish sampled for substance concentrations at the start of the test, the end of the uptake phase and the end of the depuration phase. Note it should be possible in many cases to use the 3 control fish alone (*cf.* paragraphs 56 and 153).

Note on phase and sampling timings: The uptake phase begins with the first feeding of spiked diet. An experimental day runs from one feeding until shortly before the next, 24 hours later. The first sampling event (1 in the table) should be taken shortly before the first feeding (*e.g.* one hour). Sampling during a study should ideally be carried out shortly before the following day's feeding (*i.e.* about 23 hours after the sample day's feeding). The uptake phase ends shortly before the first feeding with unspiked diet, when the depuration phase begins (test group fish are likely to be still digesting spiked feed in the intervening 24 hours after the last spiked diet feeding). This means that the end of uptake sample should be taken shortly before the first feeding with unspiked diet and the first depuration phase sample should be taken about 23 hours after the first feeding with unspiked feed.

ANNEX 5

GENERAL CALCULATIONS

1. 1. Introduction

2. Prediction of the duration of the uptake phase

3. Prediction of the duration of the depuration phase

4. Sequential method: determination of depuration (loss) rate constant k_2

5. Sequential method: determination of uptake rate *constant* k1 (aqueous exposure method only)

6. Simultaneous method for calculation of uptake and depuration (loss) rate constants (aqueous exposure method only)

7. Growth dilution correction for kinetic BCF and BMF

8. Lipid normalisation to 5% lipid content (aqueous exposure method only)

1. Introduction

The general fish aquatic bioaccumulation model can be described in terms of uptake and loss processes, ignoring uptake with food. The differential equation (dC_f/dt) describing the rate of change in fish concentration (mg·kg^{-1}·day^{-1}) is given by (1):

$$\frac{dC_{\mathrm{f}}}{dt} = k_1 \times C_{\mathrm{w}} - \left(k_2 + k_{\mathrm{g}} + k_{\mathrm{m}} + k_e\right) \times C_{\mathrm{f}} \qquad \text{[Equation A5.1]}$$

Where k_1= First order rate constant for uptake into fish (L·kg^{-1}·day^{-1}).

k_2 = First order rate constant for depuration from fish (day^{-1}).

k_g = First order rate constant for fish growth (‘growth dilution’) (day^{-1})

k_m = First order rate constant for metabolic transformation (day^{-1})

k_e = First order rate constant for faecal egestion (day^{-1})

C_{w} = Concentration in water (mg·L^{-1}).

C_f = Concentration in fish (mg·kg^{-1} wet weight).

For bioaccumulating substances, it can be expected that a time-weighted average (TWA) is the most relevant exposure concentration in water (C_w) within the allowed range of fluctuation (*cf.* paragraph 24). It is recommended to calculate a TWA water concentration, according to the procedure in Annex 6 of OECD TG 211 (2). It should be noted that the ln-transformation of the water concentration is suitable when exponential decay between renewal periods is expected, e.g. in a semi-static test design. In a flow through system, ln-transformation of exposure concentrations may not be needed. If TWA water concentrations are derived, they should be reported and used in subsequent calculations.

In a standard fish BCF test uptake and depuration can be described in terms of two first order kinetic processes.

$$\text{Rate of uptake} = k_1 \times C_{\text{w}} \qquad \text{[Equation A5.2]}$$

$$\text{Overall loss rate} = \left(k_2 + k_g + k_m + k_e\right) \times C_{\text{f}} \qquad \text{[Equation A5.3]}$$

At steady-state, assuming growth and metabolism are negligible (*i.e.* the values for k_{g} and k_{m} cannot be distinguished from zero), the rate of uptake equals the rate of depuration, and so combining Equation A5.2 and Equation A5.3 gives the following relationship:

$$\text{BCF} = \frac{C_{\text{f-SS}}}{C_{\text{w-SS}}} = \frac{k_1}{k_2} \qquad \text{[Equation A5.4]}$$

Where $C_{\text{f-SS}}$ = Concentration in fish at steady-state (mg kg^{-1} wet weight).

$C_{\text{w-SS}}$ = Concentration in water at steady-state (mg L^{-1}).

The ratio of k_1/k_2 is known as the kinetic BCF (BCF_K) and should be equal to the steady-state BCF (BCF_{SS}) obtained from the ratio of the steady-state concentration in fish to that in water, but deviations may occur if steady-state was uncertain or if corrections for growth have been applied to the kinetic BCF. However, as k_1 and k_2 are constants, steady-state does not need to be reached to derive a BCF_K.

Based on these first order equations, this Annex 5 includes the general calculations necessary for both aqueous and dietary exposure bioaccumulation methods. However, sections 5, 6 and 8 are only relevant for the aqueous exposure method but are included here as they are "general" techniques. The sequential (sections 4 and 5) and simultaneous (section 6) methods allow the calculation of uptake and depuration constants which are used to derive kinetic BCFs. The sequential method for determining k_2 (section 4) is important for the dietary method as it is needed to calculate both assimilation efficiency and BMF. Annex 7 details the calculations that are specific to the dietary method.

2. 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 (K_{OW}) or k_1 and BCF. It should be realised, however, that the equations in this section only apply when uptake and depuration follow first-order kinetics. If this is clearly not the case it is advised to seek advice from a biostatistician and/or pharmacokineticist, if predictions of the uptake phase are desirable.

An estimate of k_2 (day^{-1}) may be obtained by several methods. For example, the following empirical relationships could be used in the first instance [34]:

$$\log k_2 = 1.47 - 0.414 \log K_{OW} \qquad (r^2=0.95) \qquad \text{[(3); Equation A5.5]}$$

or

$$k_2 = \frac{k_1}{\text{BCF}} \qquad \text{[Equation A5.6]}$$

Where $k_1 = 520 \times W^{-0.32}$ (for substances with a log K_{OW} > 3) (r^2=0.85) [(4); Equation A5.7]

And $\text{BCF} = 10^{\left(0.910 \cdot \log K_{OW} - 1.975 \cdot \log\left(6.8 \cdot 10^{-7} K_{OW} + 1\right) - 0.786\right)}$ (r^2=0.90) [(5); Equation A5.8]

W = mean treated fish weight (grams wet weight) at the end of uptake/start of depuration [35]

For other related relationships *see* (6). It may be advantageous to employ more complicated models in the estimation of k_2 if, for example, it is likely that significant metabolism may occur (7) (8). However as the complexity of the model increases, greater care should be taken with the interpretation of the predictions. For example the presence of nitro groups might indicate fast metabolism, but this is not always the case. Therefore the user should weigh up the predictive method results against chemical structure and any other relevant information (for example preliminary studies) in the scheduling of a study.

The time to reach a certain 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), assuming growth and metabolism is negligible. If substantial growth occurs during the study, the estimations described below will not be reliable. In such cases, it is better to use the growth corrected k_{2g} as described later (see Section 7 of this Annex):

$$\frac{dC_f}{dt} = k_1 C_w - k_2 C_f \qquad \text{[Equation A5.9]}$$

or, if C_w is constant:

$$C_f = \frac{k_1}{k_2} \cdot C_w \left(1 - e^{-k_2 t}\right) \qquad \text{[Equation A5.10]}$$

When steady-state is approached ($t \rightarrow \infty$), Equation A5.10 may be reduced (*cf.* (9) (10)) to:

$$C_f = \frac{k_1}{k_2} \cdot C_w \qquad \text{[Equation A5.11]}$$

or

(34) As with every empirical relationship, it should be verified that the test substance falls within the applicability domain of the relationship

(35) The weight of fish at the end of the uptake phase can be estimated from previous study data or knowledge of the test species' likely increase in size from a typical test starting weight over a typical uptake duration (*e.g.* 28 days).

$$\frac{C_f}{C_w} = \frac{k_1}{k_2} = \text{BCF} \qquad \text{[Equation A5.12]}$$

Then BCF × C_w is an approximation to the concentration in the fish at steady-state ($C_{f\text{-}SS}$). [Note: the same approach can be used when estimating a steady-state BMF with the dietary test. In this case, BCF is replaced with BMF and C_w with C_{food}, concentration in the food, in the equations above]

Equation A5.10 may be transcribed to:

$$C_f = C_{f\text{-}SS}\left(1 - e^{-k_2 t}\right) \qquad \text{[Equation A5.13]}$$

or

$$\frac{C_f}{C_{f\text{-}SS}} = 1 - e^{-k_2 t} \qquad \text{[Equation A5.14]}$$

Applying Equation A5.14, the time to reach a certain percentage of steady-state may be predicted when k_2 is pre-estimated using Equation A5.5 or Equation A5.6.

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 at least 50% of steady-state (*i.e.* $0.69/k_2$), but not more than 95% of steady-state (*i.e.* $3.0/k_2$) (11). In case accumulation reaches beyond 95% of steady-state, calculation of a BCF_{SS} becomes feasible.

The time to reach 80 percent of steady-state is (using Equation A5.14):

$$0.80 = 1 - e^{-k_2 t} \qquad \text{[Equation A5.15]}$$

or

$$t_{80} = \frac{-\ln(0.20)}{k_2} = \frac{1.6}{k_2} \qquad \text{[Equation A5.16]}$$

Similarly the time to reach 95 percent of steady-state is:

$$t_{95} = \frac{-\ln(0.05)}{k_2} = \frac{3.0}{k_2} \qquad \text{[Equation A5.17]}$$

For example, the duration of the uptake phase (*i.e.* time to reach a certain percentage of steady-state, *e.g.* t_{80} or t_{95}) for a test substance with log K_{OW} = 4 would be (using Equation A5.5, Equation A5.16 and Equation A5.17):

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

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

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

or $$t_{95} = \frac{3.0}{0.652} = 4.60 \text{ days (110 hours)}$$

Alternatively, the expression:

$$t_{eSS} = 6.54 \cdot 10^{-3} \cdot K_{OW} + 55.31 \text{ (hours)} \qquad \text{[Equation A5.18]}$$

may be used to calculate the time for effective steady-state (t_{eSS}) to be reached (12). For a test substance with log K_{OW} = 4 this results in:

$$t_{eSS} = 6.54 \cdot 10^{-3} \cdot 10^{4} + 55.31 = 121 \text{ hours}$$

3. Prediction of the duration of the depuration phase

A prediction of the time needed to reduce the body burden to a certain percentage of the initial concentration may also be obtained from the general equation describing uptake and depuration (assuming first order kinetics, *cf.* Equation A5.9 (1) (13).

For the depuration phase, C_w (or C_{food} for the dietary test) is assumed to be zero. The equation may then be reduced to:

$$\frac{dC_f}{dt} = k_2 C_f \qquad \text{[Equation A5.19]}$$

or

$$C_f = C_{f,0} \cdot e^{-k_2 t} \qquad \text{[Equation A5.20]}$$

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

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

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

or

$$t_{50} = \frac{-\ln(0.50)}{k_2} = \frac{0.693}{k_2}$$

Similarly 95 percent depuration will be reached at:

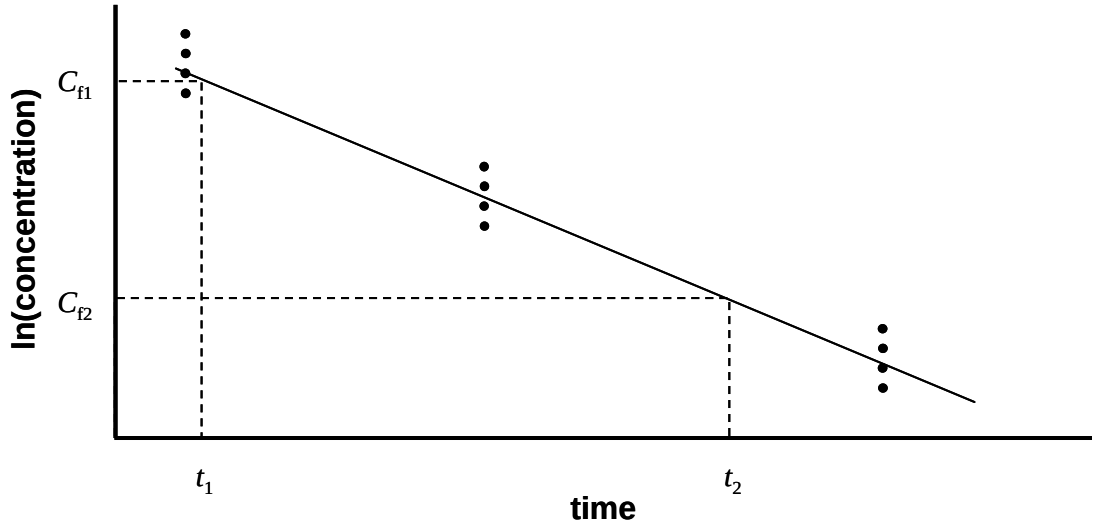
$$t_{95} = \frac{-\ln(0.05)}{k_2} = \frac{3.0}{k_2}$$

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.

Note that the estimations are based on the assumption that uptake and depuration patterns will follow first order kinetics. If first-order kinetics is obviously not obeyed, these estimations are not valid.

4. Sequential method: determination of depuration (loss) rate constant k_2

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 (on an ln scale), during the depuration phase.



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

To calculate k_2 for multiple time (sampling) points, perform a linear regression of ln(concentration) versus time. The slope of the regression line is an estimate of the depuration rate constant k_2 [(36)]. From the intercept the average concentration in the fish at the start of the depuration phase ($C_{0,d}$; which equals the average concentration in the fish at the end of the uptake phase) can easily be calculated (including error margins) [(36)]:

$$C_{0,d} = e^{\text{intercept}} \qquad \text{[Equation A5.21]}$$

To calculate k_2 when only two time (sampling) points are available (as in the minimised design), substitute the two average concentrations into the following equation

$$k_2 = \frac{\ln(C_{f1}) - \ln(C_{f2})}{t_2 - t_1} \qquad \text{[Equation A5.22]}$$

Where $\ln(C_{f1})$ and $\ln(C_{f2})$ are the natural logarithms of the concentrations at times t_1 and t_2, respectively, and t_2 and t_1 are the times when the two samples were collected relative to the start of depuration[(37)].

(36) In most programs that allow a linear regression, also standard errors and confidence interval (CI) of the estimates are given, *e.g.* in Microsoft Excel using the Data Analysis tool pack.

(37) In contrast with the linear regression method, using this formula will not yield a standard error for k_2.

5. Sequential method: determination of uptake rate constant k_1 (aqueous exposure method only)

To find a value for k_1 given a set of sequential time concentration data for the uptake phase, use a computer program to fit the following model:

$$C_f(t) = C_w(t) \cdot \frac{k_1}{k_2} \cdot \left(1 - e^{-k_2 t}\right) \qquad \text{[Equation A5.23]}$$

Where k_2 is given by the previous calculation, $C_f(t)$ and $C_w(t)$ are the concentrations in fish and water, respectively, at time t.

To calculate k_1 when only two time (sampling) points are available (as in the minimised design), use the following formula:

$$k_1 = \frac{C_f \cdot k_2}{C_w \left(1 - e^{-k_2 t}\right)} \qquad \text{[Equation A5.24]}$$

Where k_2 is given by the previous calculation, C_f is the concentration in fish at the start of the depuration phase, and C_w is the average concentration in the water during the uptake phase [(38)].

Visual inspection of the k_1 and k_2 slopes when plotted against the measured sample point data can be used to assess goodness of fit. If it turns out that the sequential method has given a poor estimate for k_1 then the simultaneous approach to calculate k_1 and k_2 should be applied (see next section 6). Again, the resulting slopes should be compared against the plotted measured data for visual inspection of goodness of fit. If the goodness of fit is still poor this may be an indication that first order kinetics do not apply and other more complex models should be employed.

6. Simultaneous method for calculation of uptake and depuration (loss) rate constants (aqueous exposure method only)

Computer programs can be used to find values for k_1 and k_2 given a set of sequential time concentration data and the model:

$$C_f = C_w \cdot \frac{k_1}{k_2} \cdot \left(1 - e^{-k_2 t}\right) \qquad 0 < t < t_c \qquad \text{[Equation A5.25]}$$

$$C_f = C_w \cdot \frac{k_1}{k_2} \cdot \left(e^{-k_2 (t - t_c)} - e^{-k_2 t}\right) \qquad t > t_c \qquad \text{[Equation A5.26]}$$

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

This approach directly provides standard errors for the estimates of k_1 and k_2. When k_1/k_2 is substituted by BCF (*cf.* Equation A5.4) in Equation A5.25 and Equation A5.26, the standard error and 95% CI of the BCF can be estimated as well. This is especially useful when comparing different estimates due to data transformation. The dependent variable (fish concentration) can be fitted with or without ln transformation, and the resulting BCF uncertainty can be evaluated.

As 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 (see above); k_2 in most cases can

(38) In contrast with a linear fitting procedure, this method will usually not yield a standard error or confidence interval for the estimated k_1.

be estimated from the depuration curve with relatively high precision. k_1 can be subsequently calculated from the uptake data using non-linear regression[39]. It is advised to use the same data transformation when fitting sequentially.

Visual inspection of the resulting slopes when plotted against the measured sample point data can be used to assess goodness of fit. If it turns out that this method has given a poor estimate for k_1 then the simultaneous approach to calculate k_1 and k_2 can be applied. Again, the fitted model should be compared against the plotted measured data for visual inspection of goodness of fit and the resulting parameter estimates for k_1, k_2 and resulting BCF and their standard errors and/or confidence intervals should be compared between different types of fit.

If the goodness of fit is poor this may be an indication that first order kinetics does not apply and other more complex models should be employed. One of the most common complications is fish growth during the test.

7. Growth dilution correction for kinetic BCF and BMF

This section describes a standard method for correction due to fish growth during the test (so called 'growth dilution') which is only valid when first order kinetics applies. In case there are indications that first order kinetics do not apply, it is advised to seek advice from a biostatistician for a proper correction of growth dilution or to use the mass based approach described below.

In some cases this method for correcting growth dilution is subject to a lack of precision or sometimes does not work (for example for very slowly depurating substances tested in fast growing fish the derived depuration rate constant corrected for growth dilution, k_{2g}, may be very small and so the error in the two rate constants used to derive it become critical, and in some cases k_g estimates may be larger than k_2). In such cases an alternative approach (*i.e.* mass approach), which also works when first order growth kinetics have not been obeyed, can be used which avoids the need for the correction. This approach is outlined at the end of this section.

Growth rate constant subtraction method for growth correction

For the standard method all individual weight and length data are converted to natural logarithms and ln(weight) or ln(1/weight) is plotted vs. time (day), separately for treatment and control groups. The same process is carried out for the data from the uptake and depuration phases separately. Generally for growth dilution correction it is more appropriate to use the weight data from the whole study to derive the growth rate constant (k_g), but statistically significant differences between the growth rate constants derived for the uptake phase and depuration phase may indicate that the depuration phase rate constant should be used. Overall growth rates from aqueous studies for test and control groups can be used to check for any treatment related effects.

A linear least squares correlation is calculated for the ln(fish weight) *vs.* day (and for ln(1/weight) *vs.* day) for each group (test(s) and control groups, individual data, not daily mean values) for the whole study, uptake and depuration phases using standard statistical procedures. The variances in the slopes of the lines are calculated and used to evaluate the statistical significance (p = 0.05) of the difference in the slopes (growth rate constants) using the student *t*-test (or ANOVA if more than one concentration is tested). Weight data are generally preferred for growth correction purposes. Length data, treated in the same way, may be useful to compare control and test groups for treatment related effects. If there is no statistically significant difference in the weight data analysis, the test and control

(39) It should be realised that the uncertainty in the k_2 estimate is not used properly in the bioaccumulation model when this is essentially regarded as constant when fitting k_1 in the sequential fit method. The resulting BCF uncertainty will therefore be different between the simultaneous and sequential fitting methods.

data may be pooled and an overall fish growth rate constant for the study (k_g) calculated as the overall slope of the linear correlation. If statistically significant differences are observed, growth rate constants for each fish group, and/or study phase, are reported separately. The rate constant from each treated group should then be used for growth dilution correction purposes of that group. If statistical differences between the uptake and depuration phase rate constants were noted, depuration phase derived rate constants should be used.

The calculated growth rate constant (k_g expressed as day^{-1}) can be subtracted from the overall depuration rate constant (k_2) to give the depuration rate constant, k_{2g}.

$$k_{2g} = k_2 - k_g \qquad \text{[Equation A5.27]}$$

The uptake rate constant is divided by the growth-corrected depuration rate constant to give the growth-corrected kinetic BCF, denoted BCF_{Kg} (or BMF_{Kg}).

$$BCF_{Kg} = \frac{k_1}{k_{2g}} \qquad \text{[Equation A5.28]}$$

The growth rate constant derived for a dietary study is used in Equation A7.5 to calculate the growth corrected BMF_{Kg} (*cf.* Annex 7).

Mass based method for growth correction

An alternative to the above "growth rate constant subtraction method" that avoids the need to correct for growth can be used as follows. The principle is to use depuration data on a mass basis per whole fish rather than on a concentration basis.

- Convert depuration phase tissue concentrations (mass of test chemical/unit mass of fish) into mass of test chemical/fish: match concentrations and individual fish weights in tabular form (*e.g.* using a computer spreadsheet) and multiply each concentration by the total fish weight for that measurement to give a set of mass test chemical/fish for all depuration phase samples.

- Plot the resulting natural logarithm of chemical mass data against time for the experiment (depuration phase) as would be done normally.

- For the aqueous exposure method, derive the uptake rate constant routinely (see sections 4 and 6) note that the "normal" k_2 value should be used in the curve fitting equations for k_1) and derive the depuration rate constant from the above data. Because the resulting value for the depuration rate constant is independent of growth as it has been derived on a mass basis per whole fish, it should be denoted as k_{2g} and not k_2.

8. Lipid normalisation to 5% lipid content (aqueous exposure method only)

BCF results (kinetic and steady-state) from aqueous exposure tests should also be reported relative to a default fish lipid content of 5% wet weight, unless it can be argued that the test substance does not primarily accumulate in lipid (*e.g.* some perfluorinated substances may bind to proteins). Fish concentration data, or the BCF, need to be converted to a 5% lipid content wet weight basis. If the same fish were used for measuring chemical concentrations and lipid contents at all sampling points, this requires each individual measured concentration in the fish to be corrected for that fish's lipid content.

$$C_{f,L} = \frac{0.05}{L} \cdot C_f$$ [Equation A5.29]

where $C_{f,L}$ = lipid-normalised concentration in fish (mg kg^{-1} wet weight)

L = lipid fraction (based on wet weight)

C_f = concentration of test substance in fish (mg kg^{-1} wet weight)

If lipid analysis was not conducted on all sampled fish, a mean lipid value is used to normalise the BCF. For the steady-state BCF, the mean value recorded at the end of the uptake phase in the treatment group should be used. For the normalisation of a kinetic BCF there may be some cases where a different approach is warranted, for example if the lipid content changed markedly during the uptake or depuration phase. However a feeding rate that minimises dramatic changes in lipid content should be used anyway routinely.

$$BCF_{KL} = \frac{0.05}{L_n} \cdot BCF_K$$ [Equation A5.30]

where BCF_{KL} = lipid-normalised kinetic BCF (L kg^{-1})

L_n = mean lipid fraction (based on wet weight)

BCF_K = kinetic BCF (L kg^{-1})

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ANNEX 6

EQUATION SECTION FOR AQUEOUS EXPOSURE TEST: MINIMISED TEST DESIGN

The rationale for this approach is that the bioconcentration factor in a full test can either be determined as a steady-state bioconcentration factor (BCF_{SS}) by calculating the ratio of the concentration of the test substance in the fish's tissue to the concentration of the test substance in the water, or by calculating the kinetic bioconcentration factor (BCF_K) as the ratio of the uptake rate constant k_1 to the depuration rate constant k_2. The BCF_K is valid even if a steady-state concentration of a chemical is not achieved during uptake, provided that uptake and depuration act approximately according to first order kinetic processes.

If a measurement of the concentration of the chemical in tissues (C_{f1}) is made at the time that exposure ends (t_1) and the concentration in tissue (C_{f2}) is measured again after a period of time has elapsed (t_2), the depuration rate constant (k_2) can be estimated using Equation A5.22 from Annex 5.

The uptake rate constant, k_1, can then be determined algebraically using Equation A5.23 from Annex 5 (where C_f equals C_{f1} and t equals t_1) (1). The kinetic bioconcentration factor for the minimised design (designated as BCF_{Km} to distinguish it from kinetic bioconcentration factors determined using other methods) is thus:

$$BCF_{Km} = \frac{k_1}{k_2} \qquad \text{[Equation A6.1]}$$

Concentrations or results should be corrected for growth dilution and normalised to a fish lipid content of 5%, as is described in Annex 5.

The minimised BCF_{SS} is the BCF calculated at the end of the uptake phase, assuming that steady-state has been reached. This can only be assumed, as the number of sampling points is not sufficient for proving this.

$$\text{minimised BCFss} = \frac{C_{f-minSS}}{C_{w-minSS}} \qquad \text{[Equation A6.2]}$$

Where $C_{f\text{-}minSS}$ = Concentration in fish at assumed steady-state at end of uptake (mg kg^{-1} wet weight).

$C_{w\text{-}minSS}$ = Concentration in water at assumed steady-state at end of uptake (mg L^{-1}).

Literature

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ANNEX 7

EQUATION SECTION FOR DIETARY EXPOSURE TEST

1. Example of constituent quantities of a suitable commercial fish food

2. Food spiking technique examples

3. Calculation of assimilation efficiency and biomagnification factor

4. Lipid correction

5. Evaluation of differences between measured time zero concentration (C0,m) and derived time zero concentration (*C0,d*)

6. Guidance for very fast depurating test substances

1. Example of constituent quantities of a suitable commercial fish food

Major constituent	fish meal
Crude Protein	≤ 55.0%
Crude fat	≤ 15.0%(1)
Crude Fibre	≥ 2.0%
Moisture	≥ 12%
Ash	≥ 8%

(1) In some regions it may only be possible to obtain fish food with a lipid concentration that falls far short of this upper limit. In such cases studies should be run with the lower lipid concentration in the food as supplied, and the feeding rate adjusted appropriately to maintain fish health. Diet lipids should not be artificially increased by the addition of excess oil.

2. Food spiking technique examples

General Points

- Control diets should be prepared in exactly the same way as the spiked diet, but with an absence of test substance.

- To check the concentration of the treated diet, triplicate samples of the dosed food should be extracted with a suitable extraction method and the test substance concentration or radioactivity in the extracts measured. High analytical recoveries (>85%) with low variation between samples (three sample concentrations for the substance taken at test start should not vary more than ± 15% from the mean) should be demonstrated.

- During the dietary test, three diet samples for analysis should be collected on day 0 and at the end of the uptake phase for the determination of the test substance content in the diet.

Fish food preparation with a liquid test material (neat)

A target, nominal test concentration in the treated fish food is set, for example 500 µg test substance/g food. The appropriate quantity (by molar mass or specific radioactivity) of neat test substance is added to a known mass of fish food in a glass jar or rotary evaporator bulb. The mass of fish food should be sufficient for the duration of the uptake phase (taking into account the need for increasing quantities at each feed owing to fish growth). The fish feed/test substance should be mixed overnight by slow tumbling (*e.g.* using a roto-rack mixer or by rotation if a rotary evaporator bulb is used). The spiked diet should be stored under conditions that maintain stability of the test chemical within the feed mix (*e.g.* refrigeration) until use.

Fish food preparation with a corn or fish oil vehicle

Solid test substances should be ground in a mortar to a fine powder. Liquid test substances can be added directly to the corn or fish oil. The test substance is dissolved in a known quantity of corn or fish oil (*e.g.* 5-15 mL). The dosed oil is quantitatively transferred into a rotary evaporation bulb of suitable size. The flask used to prepare the dosed oil should be flushed with two small aliquots of oil and these added to the bulb to make sure all dissolved test substance is transferred. To ensure complete dissolution/dispersion in the oil (or if more than one test substance is being used in the study), a micro-stirrer is added, the flask stoppered and the mixture stirred rapidly overnight. An appropriate quantity of fish diet (usually in pellet form) for the test is added to the bulb, and the bulb's contents are mixed homogeneously by continuously turning the glass bulb for at least 30 minutes, but preferably overnight. Thereafter, the spiked food is stored appropriately (*e.g.* refrigerated) to ensure test substance stability in the food until use.

Fish food preparation with an organic solvent

An appropriate quantity of test substance (by molar mass or specific radioactivity) sufficient to achieve the target dose is dissolved in a suitable organic solvent (*e.g.* cyclohexane or acetone; 10-40 mL, but a greater volume if necessary depending on the quantity of food to spike). Either an aliquot, or all (added portionwise), of this solution is mixed with the appropriate mass of fish food sufficient for the test to achieve the required nominal dose level. The food/test substance can be mixed in a stainless steel mixing bowl and the freshly-dosed fish food left in the bowl in a laboratory hood for two days (stirred occasionally) to allow the excess solvent to evaporate, or mixed in a rotary evaporator bulb with continuous rotation. The excess solvent can be "blown" off under a stream of air or nitrogen if necessary. Care should be taken to ensure that the test substance does not crystallise as the solvent is removed. The spiked diet should be stored under conditions (*e.g.* refrigeration) that maintain stability of the test chemical within the feed mix until use.

3. Calculation of assimilation efficiency and biomagnification factor

To calculate the assimilation efficiency, the overall depuration rate constant should first be estimated according to section 4 of Annex 5 (using the "sequential method", *i.e.* standard linear regression) using mean sample concentrations from the depuration phase. The feeding rate constant, *I*, and uptake duration, *t*, are known parameters of the study. C_{food}, the mean measured concentration of the test substance in the food is a measured variable in the study. $C_{0,d}$, the test substance concentration in the fish at the end of the uptake phase, is usually derived from the intercept of a plot of ln(concentration) *vs.* depuration day.

The chemical assimilation efficiency (α, absorption of test substance across the gut) is calculated as:

$$\alpha = \frac{C_{0,d} \cdot k_2}{I \cdot C_{food}} \cdot \frac{1}{1 - e^{-k_2 t}}$$ [Equation A7.1]

where: $C_{0,d}$ = derived concentration in fish at time zero of the depuration phase (mg kg^{-1});

k_2 = overall (not growth-corrected) depuration rate constant (day^{-1}), calculated according to equations in Annex 5, Section 3;

I = food ingestion rate constant (g food g^{-1} fish day^{-1});

C_{food} = concentration in food (mg kg^{-1} food);

t = duration of the feeding period (day)

However, the feeding rate, I, used in the calculation may need to be adjusted for fish growth to give an accurate assimilation efficiency, α. In a test where fish grow significantly during the uptake phase (in which no correction of feed quantities is made to maintain the set feeding rate), the effective feeding rate as the uptake phase progresses will be lower than that set, resulting in a higher 'real' assimilation efficiency. (Note this is not important for the overall calculation of BMF as the I terms effectively cancel out between Equation A7.1 and Equation A7.4). The mean feeding rate corrected for growth dilution, I_g, can be derived in several ways, but a straightforward and rigorous one is to use the known growth rate constant (k_g) to estimate the test fish weights at timepoints during the uptake phase, *i.e.*:

$$W_f(t) = W_{f,0} \times e^{k_g \cdot t}$$ [Equation A7.2]

in which $W_f(t)$ = mean fish weight at uptake day t

$W_{f,0}$ = mean fish weight at the start of the experiment

In this way (at least) the mean fish weight on the last day of exposure ($W_{f,end\text{-}of\text{-}uptake}$) can be estimated. As the feeding rate was set based on $W_{f,0}$, the effective feeding rate for each day of uptake can be calculated using these two weight values. The growth-corrected feeding rate, I_g (g food g^{-1} fish day^{-1}), to use instead of I in cases of rapid growth during the uptake phase, can then be calculated as

$$I_g = \frac{I \times W_{f,0}}{W_{f,end\text{-}of\text{-}uptake}}$$ [Equation A7.3]

Once the assimilation efficiency has been obtained, the BMF can be calculated by multiplying it with the feeding rate constant I (or I_g, if used to calculate α) and dividing the product by the overall depuration rate constant k_2:

$$BMF = \frac{I \times \alpha}{k_2}$$ [Equation A7.4]

The growth-corrected biomagnification factor should also be calculated in the same way, using the growth corrected depuration rate constant (as derived according to section 7 in Annex 5). Again, if I_g has been used to calculate α, it should also be used here instead of I:

$$BMF = \frac{I \times \alpha}{k_{2g}}$$ [Equation A7.5]

where: α = assimilation efficiency (absorption of test substance across the gut);

k_2 = overall (not growth-corrected) depuration rate constant (day^{-1}), calculated according to equations in Annex 5, Section 3;

k_{2g} = growth-corrected depuration rate constant (day^{-1});

I = food ingestion rate constant (g food g^{-1} fish day^{-1});

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

$$t_{1/2} = \frac{0.693}{k_{2g}} \qquad \text{[Equation A7.6]}$$

The chemical assimilation efficiency from the diet can also be estimated if tissue residues are determined during the linear phase of the uptake phase (between days 1 and 3). In this case the chemical assimilation efficiency (α) can be determined as follows

$$\alpha = \frac{C_{fish}(t)}{I \times C_{food} \times t} \qquad \text{[Equation A7.7]}$$

Where $C_{fish}(t)$ = the concentration of test substance in the fish at time t (mg kg^{-1} wet weight).

4. Lipid correction

If lipid content was measured on the same fish as chemical analysis for all sampling intervals, then individual concentrations should be corrected on a lipid basis and the ln(concentration, lipid corrected) plotted against depuration (day) to give $C_{0,d}$ and k_2. Assimilation efficiency (Equation A7.1) can then be calculated on a lipid basis, using C_{food} on a lipid basis (*i.e.* C_{food} is multiplied by the mean lipid fraction of the food). Subsequent calculation using Equation A7.4 and Equation A7.5 will give the lipid-corrected (and growth-dilution corrected) BMF directly.

Otherwise, the mean lipid fraction (w/w) in the fish and in the food are derived for both treatment and control groups (for food and control group fish this is usually from data measured at exposure start and end; for treatment group fish this is usually from data measured at end of exposure only). In some studies, fish lipid content may increase markedly; in such cases it is more appropriate to use a mean test fish lipid concentration calculated from the measured values at the end of exposure and end of depuration. In general, data from the treatment group only should be used to derive both of the lipid fractions.

The lipid-correction factor (L_c) is calculated as:

$$L_c = \frac{L_{fish}}{L_{food}} \qquad \text{[Equation A7.8]}$$

where L_{fish} and L_{food} are the mean lipid fractions in fish and food, respectively.

The lipid-correction factor is used to calculate the lipid-corrected biomagnification factor (BMF_L):

$$BMF_L = \frac{BMF}{L_c} \qquad \text{[Equation A7.9]}$$

5. Evaluation of differences between measured time zero concentration ($C_{0,m}$) and derived time zero concentration ($C_{0,d}$)

The measured time zero concentration ($C_{0,m}$) and derived time zero concentration ($C_{0,d}$) should be compared. If they are very similar, then this supports the first order model used to derive the depuration parameters.

In some studies there may be a marked difference between the derived time zero value, $C_{0,d}$, and the mean measured time zero concentration. $C_{0,m}$ (see last bullet point of paragraph 159 of this Guideline). If $C_{0,d}$ is very much lower than $C_{0,m}$ ($C_{0,d} << C_{0,m}$), the difference may suggest the presence of undigested spiked food in the gut. This may be tested experimentally by conducting separate analysis on the excised gut if additional (whole fish) samples were taken and stored at the end of the uptake phase. Otherwise, if a statistically valid outlier test applied to the depuration phase linear regression indicates that the first sample point of depuration is erroneously elevated, carrying out the linear regression to derive k_2 but omitting the first depuration concentration point may be appropriate. In such cases, if the uncertainty in the linear regression is greatly decreased, and it is clear that approximately first order depuration kinetics were obeyed, it may be appropriate to use the resulting $C_{0,d}$ and k_2 values in the assimilation efficiency calculation. This should be fully justified in the report. It is also possible that non-first order kinetics were operating in the depuration phase. If this is likely (*i.e.* the natural logarithm transformed data appear to follow a curve compared with the straight-line linear regression plot), then the calculations of k_2 and $C_{0,d}$ are unlikely to be valid and the advice of a biostatician should be sought.

If $C_{0,d}$ is very much higher than the measured value ($C_{0,d} >> C_{0,m}$) this may indicate: that the substance was depurated very fast (*i.e.* sampling points approached the limit of quantification of the analytical method very early in the depuration phase, *cf.* Section 6 below); that there was a deviation from first order depuration kinetics; that the linear regression to derive k_2 and $C_{0,d}$ is flawed; or that a problem with the measured concentrations in the study occurred at some sampling time points. In such cases the linear regression plot should be scrutinised for evidence of samples at or near the limit of quantification, for outliers and for obvious curvature (suggestive of non-first order kinetics), and highlighted in the report. Any subsequent re-evaluation of the linear regression to improve estimated values should be described and justified. If marked deviation from first order kinetics is observed, then the calculations of k_2 and $C_{0,d}$ are unlikely to be valid and the advice of a biostatician should be sought.

6. Guidance for very fast depurating test substances

As discussed in paragraph 129 of the Guideline, some substances may depurate so fast that a reliable time zero concentration, $C_{0,d}$, and k_2 cannot be derived because in samples very early in the depuration phase (*i.e.* from the second depuration sample onwards) the substance is effectively no longer measured (concentrations reported at the limit of quantification). This situation was observed in the ring test carried out in support of this test method with benzo[a]pyrene, and has been documented in the validation report for the method. In such cases linear regression cannot be carried out reliably, and is likely to give an unrealistically high estimate of $C_{0,d}$, resulting in an apparent assimilation efficiency much greater than 1. It is possible to calculate a conservative estimate of k_2 and an "upper bound" BMF in these instances.

Using those data points of the depuration phase where a concentration was measured, up to and including the first “non-detect” concentration (concentration set at limit of quantification), a linear regression (using natural logarithm transformed concentration data against time) will give an estimate of k_2. For these sorts of cases this is likely only to involve two data points (*e.g.* sample days 1 and 2 of depuration) and then k_2 can be estimated using Equation A5.22 in Annex 5. This k_2 estimate can be used to estimate an assimilation efficiency according to equation A7.1, substituting the $C_{0,d}$ value in the equation with the measured time zero concentration ($C_{0,m}$) in cases where $C_{0,d}$ is clearly estimated to be much higher than could have been achievable in the test. If $C_{0,m}$ was not measureable, then the

limit of detection in fish tissue should be used. If, in some cases, this gives a value of $\alpha > 1$, then the assimilation efficiency is assumed to 1 as a “worst case”.

The maximum BMF_K can then be estimated using Equation A7.4, and should be quoted as a "much less than" (<<) value. For example, for a study carried out with a feeding rate of 3% and a depuration half-life less than 3 days, and a “worst case” α of 1, the BMF_K is likely to be below about 0.13. Given the purpose of this estimation and the fact that values will be conservative in nature, it is not necessary to correct them for growth dilution or fish and food lipid content.

ANNEX 8

APPROACHES TO ESTIMATE TENTATIVE BCFs FROM DATA COLLECTED IN THE DIETARY EXPOSURE STUDY

The dietary method is included in this guideline for the bioaccumulation testing of substances that cannot in practice be tested using the aqueous exposure method. The aqueous exposure method gives a bioconcentration factor, whereas the dietary method leads directly to information on feeding biomagnification potential. In many chemical safety regimes information on aquatic bioconcentration is required (for example in risk assessment and the Globally Harmonization System of Classification). Hence there is a need to use the data generated in a dietary study to estimate a bioconcentration factor that is comparable to tests conducted according to the OECD 305 aqueous exposure method[(40)]. This section explores approaches that may be followed to do this, while recognising the shortcomings that are inherent in the estimations.

The dietary study measures depuration to give a depuration rate constant, k_2. If an uptake rate constant can be estimated with the available data for the situation where the fish had been exposed to the test substance via the water, then a kinetic BCF could be estimated.

The estimation of an uptake rate constant for water exposure of a test substance is reliant on many assumptions, all of which will contribute to the estimate's uncertainty. Furthermore, this approach to estimating a BCF assumes that the overall rate of depuration (including contributory factors like distribution in the body and individual depuration processes) is independent of the exposure technique used to produce a test substance body burden.

The main assumptions inherent in the estimation approach can be summarised as follows.

- Depuration following dietary uptake is the same as depuration following aqueous exposure for a given substance
- Uptake from water would follow first order kinetics
- Depending on the method used to estimate uptake:
    - uptake can be correlated with fish weight alone
    - uptake can be correlated with the substance's octanol-water partition coefficient alone
    - uptake can be correlated with a combination of fish weight and the substance's octanol-water partition coefficient

(40) In the wild the route leading to greatest exposure in aqueous environments is likely to be through ingestion for very hydrophobic substances and so an estimated BCF is not strictly representative of such a substance's bioaccumulation potential.

- factors that can affect uptake in an aqueous exposure study in practice such as substance bioavailability, adsorption to apparatus, molecular size etc. have little effect

and, crucially:

- The database ("training set") used to develop the uptake estimation method is representative of the substance under consideration

Several publications in the open literature have derived equations relating uptake from water in fish via the gills to a substance's octanol-water partition coefficient, fish weight (1) (2) (3) (4), volume and/or lipid content, membrane permeation/diffusion (5) (6), fish ventilation volume (7) and by a fugacity/mass balance approach (8) (9) (10). A detailed appraisal of such methods in this context is given in Crookes & Brooke (11). A publication by Barber (12) focussed on modelling bioaccumulation through dietary uptake is also useful in this context as it includes contributions from gill uptake rate models. A section of the background document to the 2004 dietary protocol (13) was also devoted to this aspect.

Most of these models seem to have been derived using limited databases. For models where details of the database used to build the model are available, it appears that the types of substances used are often of a similar structure or class (in terms of functionality, *e.g.* organochlorines). This adds to the uncertainty in using a model to predict an uptake rate constant for a different type of substance, in addition to test-specific considerations like species, temperature, etc.

A review of available techniques (11) highlighted that no one method is "more correct" than the others. Therefore a clear justification should be given for the model used. Where several methods are available for which the use can be justified, it may be prudent to present several estimates of k_1 (and so BCF) or a range of k_1 values (and BCF) according to several uptake estimation methods. However, given the differences in model types and datasets used to develop them, taking a mean value from estimates derived in different ways would not be appropriate.

Some researchers have postulated that BCF estimates of this sort require a bioavailability correction to account for a chemical's adsorption to dissolved organic carbon (DOC) under aqueous exposure conditions, to bring the estimate in line with results from aqueous exposure studies (*e.g.* (13) (14)). However this correction may not be appropriate given the low levels of DOC required in an aqueous exposure study for a 'worst case' estimate (*i.e.* ratio of bioavailable substance to substance as measured in solution). For highly hydrophobic substances uptake at the gill may become limited by the rate of passive diffusion near the gill surface; in this case it is possible that the correction may be accounting for this effect rather than what it was designed for.

It is advised to focus on methods that require inputs for which data will be readily available for substances tested according to the dietary study described here (*i.e.* log K_{OW}, fish weight). Other methods that require more complex inputs may be applied, but may need additional measurements in the test or detailed knowledge on the test substance or fish species that may not be widely available. In addition, choice of model may be influenced by the level of validation and applicability domain (see (11)for a review and comparison of different methods).

It should be borne in mind that the resulting k_1 estimate, and estimated BCF, are uncertain and may need to be treated in a weight-of-evidence approach along with the derived BMF and substance parameters (*e.g.* molecular size) for an overall picture of a substance's bioaccumulation potential. Interpretation and use of these parameters may depend on the regulatory framework.

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