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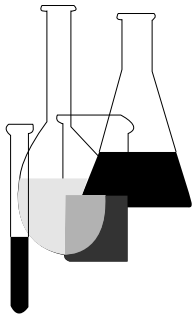
Ecological Effects Test Guidelines

OPPTS 850.1730
Fish BCF



“Public Draft”

INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Public Draft Access Information. This draft guideline is part of a series of related harmonized guidelines that need to be considered as a unit. *For copies.* These guidelines are available electronically from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines’’ or in paper by contacting the OPP Public Docket at (703) 305–5805 or by e-mail. guidelines@epamail.epa.gov.

To Submit Comments. Interested persons are invited to submit comments. By mail. Public Docket and Freedom of Information Section, Office of Pesticide Programs, Field Operations Division (7506C), Environmental Protection Agency, 401 M St. SW., Washington, DC 20460. In person. bring to. Rm. 1132, Crystal Mall #2, 1921 Jefferson Davis Highway, Arlington, VA. Comments may also be submitted electronically by sending electronic mail (e-mail) to. guidelines@epamail.epa.gov.

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OPPTS 850.1730 Fish BCF

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*).

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline is 40 CFR 797.1520 Fish Bioconcentration Test; OPP guideline 72–6 Aquatic Organism Bioavailability/ Biomagnification/Toxicity Tests (Pesticide Assessment Guidelines, Subdivision E—Hazard Evaluation; Wildlife and Aquatic Organisms) EPA report 540/09–82–024, 1982 and OPP 165–4 Laboratory Studies of Pesticide Accumulation in Fish (Pesticide Assessment Guidelines, Subdivision N—Environmental Fate) EPA report 540/09–82–031, 1982; and OECD 305E Bioaccumulation: Flow-Through Fish Test.

(b) **Introduction**—(1) **Purpose.** The purpose of the study is to determine uptake and depuration rate constants and bioconcentration factors (BCFs) for fish exposed to a test chemical in aqueous solution. Another purpose is to identify and quantify major degradates at steady state. BCF values for the test chemical should always be based on concentrations of the chemical in fish tissue and exposure water, and not on total radiolabeled residues. BCFs may be used to help assess risks to the fish and to nontarget organisms (including humans) above them in the food chain.

(2) **Criteria for performing test.** The test is most commonly required for chemicals that are relatively persistent (stable) in water and have a relatively high potential for bioaccumulation as indicated by log P_{ow} (log of the octanol/water partition coefficient) values less than or equal to 1.0.

(3) **Criteria for degradate characterization.** BCFs based on total radiolabeled residues in fish tissue and exposure water can be used to help determine whether major degradates should be identified and quantified. If the BCF in terms of total radiolabeled residues is greater than or equal to 1,000, OPP requires that an attempt be made to identify and quantify pesticide degradates representing greater than or equal to 10 percent of total residues in fish tissues at steady state. If degradates representing greater than or equal to 10 percent of total radiolabeled residues in the fish tissue are identified and quantified, then degradates in the test water should also be identified and quantified.

(4) **Desired information on the test chemical.** To determine whether a BCF test is warranted (see paragraph (b)(2) of this guideline), it is necessary to know aqueous fate characteristics of the test chemical that determine its persistence in water and its octanol/water partition coefficient. Aqueous fate characteristics include rates of abiotic hydrolysis, biodegradation, direct photolysis in natural sunlight, and volatilization from water. Henry's law constant (approximated by the ratio of the chemi-

cal's vapor pressure to its solubility in water) is a good indicator of volatilization potential. It is necessary to know the test chemical's solubility in water to ensure that exposure concentrations do not exceed it. It is also necessary to know the toxicity of the chemical to test fish to ensure exposure concentrations do not adversely effect them (see paragraph (g)(2) of this guideline). The purity of the test chemical should be known as well as its radiopurity if radiolabeled. The structure and radiolabeled positions should be known. An appropriate analytical method, of known accuracy, precision, and sensitivity, for the quantification of the substance in the test solutions and in biological material must be available, together with details of sample preparation and storage. Analytical detection limit of test substance in both water and fish tissues should also be known.

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline. The following definitions also apply to this test guideline.

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

The bioconcentration factor (BCF or KB) at any time during the uptake phase of this accumulation test is the concentration of test substance (expressed in milligrams per gram or parts per million) in/on the fish or specified tissues thereof, divided by the concentration of the chemical in the surrounding medium ($BCF = C_f/C_w$).

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}).

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

Kinetic concentration factors (BCFK) are bioconcentration factors calculated directly from kinetic rate constants (k_1/k_2).

The *octanol-water partition coefficient* (P_{ow}) is the ratio of the solubility of a chemical in *n*-octanol and water at equilibrium and can also be expressed as K_{ow}. Log P_{ow} is used as an indication of a chemical's potential for bioconcentration by aquatic organisms.

A *plateau or steady-state* is reached when the the plot of yhe concentration of test substance in fish (C_f) against time becomes parallel to the time axis and three successive analyses of C_f made on samples taken at intervals of at least 2 days are within ± 20 percent of each other, and there are no significant differences among the three sampling periods. At

least four successive analyses are required when pooled samples are analyzed. For test substances which are taken up slowly, the intervals would more appropriately be 7 days.

The *postexposure* or *depuration (loss)* phase is the time, following the transfer of the test fish from a medium containing test substance to a medium free of that substance, during which the depuration (or the net loss) of the substance from the test fish (or specified tissue thereof) is studied.

The *steady state bioconcentration factor* is found when the BCF does not change significantly over a prolonged period of time, the concentration of the test substance in the surrounding medium being constant during this period of time.

The *uptake rate constant (k_1)* is the numerical value defining the rate of increase in the concentration of test substance in/on test fish (or specified tissues thereof) when the fish are exposed to that chemical (k_1 is expressed in day^{-1}).

(d) **Principle of test**—(1) **Uptake and depuration phase.** The test consists of two phases—the exposure (uptake) and postexposure (depuration) phases. During the uptake phase, separate groups of fish of one species are exposed to at least two concentrations of the test substance until steady state is achieved or to a maximum of 28–60 days (see paragraph (g)(3) of this guideline). They are then transferred to a medium free of the test substance for a depuration phase of adequate duration (see paragraph (g)(4) of this guideline). The concentration of the test substance in/on the fish (or specified tissue thereof) and in water is followed through both phases of the test.

(2) **Determination of rate constants and BCFs.** (i) Concentrations of the test chemical in fish tissue and water as a function of time throughout the uptake and depuration phases are used to determine the uptake (k_1) and depuration (k_2) rate constants (see paragraph (i)(1) of this guideline.

(ii) Both the steady state and kinetic bioconcentration factors should be calculated (see paragraph (i)(2) of this guideline). The steady state bioconcentration factor (BCFs) is calculated as the ratio of the concentration in the fish (C_f) and to that in the water (C_w) at apparent steady-state. The kinetic bioconcentration factor (BCFK) is calculated as the ratio of the uptake rate constant (k_1) to the depuration rate constant (k_2) assuming first-order kinetics

(iii) At a minimum, BCFs should be computed for the whole fish. Whenever possible, they should also be calculated for edible and nonedible tissue. BCFs should be related to both the weight and lipid content of

the fish. If first-order kinetics are obviously not obeyed, more complex models should be employed under paragraph (d)(2)(iv) of this guideline.

(iv) **Model discrimination.** Most bioconcentration data have been assumed to be reasonably well described by a simple two-compartment/two-parameter model, as indicated by the rectilinear curve which approximates to the points for concentrations in fish, during the depuration phase, when these are plotted on semilog paper. (Where these points cannot be described by a rectilinear curve then more complex models should be employed, see paragraph (k)(20) of this guideline.)

(A) Graphical method for determination of depuration (loss) rate constant k_2.

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

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

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

(B) Graphical method for determination of uptake rate constant k_1.

Given k_2, calculate k_1 as follows:

Equation 1

$$k_1 = C_f k_2/C_w \; T1\times (1 - e^{-k2t})$$

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

(C) Computer method for calculation of uptake and depuration (loss) rate constants.

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

Equation 2

$$C_f = C_w \times k_1/k_2 \times (1 - e^{-k2t}) \qquad 0 < t < t_c$$

Equation 3

$$C_f = C_w \times k_1/k_2 \times (e^{-k2(t - tc)} - e^{k-2t} \qquad t > t_c$$

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

This approach provides standard deviation estimates of k_1 and k_2.

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

(e) **Materials**—(1) **Exposure tanks and tubes.** Care should be taken to avoid the use of materials, for all parts of the equipment, that can dissolve, sorb or leach and have an adverse effect on the fish. Standard rectangular or cylindrical tanks, made of chemically inert material and of a suitable capacity in compliance with loading rate (see paragraph (e)(7) of this guideline), can be used. The use of soft plastic tubing should be minimized. Use Teflon, stainless steel and/or glass tubing. Experience has shown that for substances with high adsorption coefficients, such as the synthetic pyrethroids, silanized glass may be required. In these situations the equipment will have to be discarded after use.

(2) **Diluter.** For flow-through tests, a system which continuously dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) is required to deliver the test concentrations to the test chambers. Preferably allow at least five volume replacements through each test chamber per day. The flow 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 percent either within or between chambers. The flow-through mode is to be preferred, but where this is not possible (e.g. when the test organisms are adversely affected) a semi-static technique may be used provided that the validity criteria are satisfied (see paragraph (g)(11) of this guideline).

(3) **Dilution water.** (i) Natural water is generally used in the test and should be obtained from uncontaminated and uniform quality source. The dilution water must be of a quality that will allow the survival of the chosen fish species for the duration of the acclimation and test periods without them showing any abnormal appearance or behavior. Ideally, it should be demonstrated that the test species can survive, grow and reproduce in the dilution water (e.g. in laboratory culture or a life-cycle toxicity test). The water should be characterized at least by pH, hardness, total solids, total organic carbon and, preferably also ammonium, nitrite and alkalinity and, for marine species, salinity. Although the parameters which

are important for optimal fish well-being are not fully known, the following Table 1. gives recommended maximum concentrations of a number of parameters for fresh and marine test waters.

Table 1.—Some chemical characteristics of an acceptable dilution water

Substance	Limit concentration
Particulate matter	5 mg/L
Total organic carbon	2 mg/L
Un-ionized ammonia	1 mg/L
Residual chlorine	10 mg/L
Total organophosphorus pesticides	50 ng/L
Total organochlorine pesticides	50 ng/L
plus polychlorinated biphenyls	25 ng/L
Total organic chlorine	1 µg/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

(ii) The water should be of constant quality during the period of a test. The pH value should be within the range 6.0 to 8.5, but during a given test it should be within a range of ± 0.5 pH units. In order to ensure that the dilution water will not unduly influence the test result (for example, by complexation of the test substance) or adversely affect the performance of the stock of fish, samples should be taken at intervals for analysis. Determination of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca, Mg, Na, K, C1, SO_4), pesticides (e.g. total organophosphorus and total organochlorine pesticides), total organic carbon and suspended solids should be made, for example, every 3 months where a dilution water is known to be relatively constant in quality. If water quality has been demonstrated to be constant over at least 1 year, determinations can be less frequent and intervals extended (e.g. every 6 months).

(iii) The natural particle content as well as the total organic carbon (TOC) of the dilution water should be as low as possible to avoid adsorption of the test substance to organic matter which may reduce its bioavailability. The maximum acceptable value is 5 mg/L for particulate matter (dry matter, not passing a 0.45 µm filter) and 2 mg/L for total

organic carbon. If necessary, the water should be filtered before use. The contribution to the organic carbon content in water from the test fish (excreta) and from the food residues should be as low as possible. Throughout the test, the concentration of organic carbon in the test vessels should not exceed the concentration of organic carbon originating from the test substance and, if used, the solubilizing agent by more than 10 mg/L (±20 percent).

(4) **Test chemical.** Whether radiolabeled or not, the chemical purity of the test chemical should be as high as practical (preferably greater than or equal to 98 percent). If radiolabeled, the radiopurity should be greater than or equal to 95 percent.

(5) **Test chemical stock solutions.** Prepare a stock solution of the test substance at a suitable concentration. The stock solution should preferably be prepared by simply mixing or agitating the test substance in the dilution water. The use of solvents or dispersant (solubilizing agents) is not recommended; however this may occur in some cases in order to produce a suitably concentrated stock solution. Solvents which may be used are, ethanol, methanol, ethylene glycol monomethyl ether, ethylene glycol dimethyl ether, dimethylformamide and triethylene glycol. Dispersant which may be used are Cremophor RH40, Tween 80, methylcellulose 0.01 percent and HCO-40. Care should be taken when using readily biodegradable agents as these can cause problems with bacterial growth in flow-through tests.

(6) **Test species.** (i) 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 and test conditions are given in the following Table 2. Other species may be used but the test procedure may have to be adapted to provide suitable test conditions. The rationale for the selection of the species and the experimental method should be reported in this case.

Table 2.—Fish species recommended for testing

Species	Test temperature (°C)	Total length of test animal (cm)
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	5.0±3.0
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 A. and G. Orti. *Proceedings of the Royal Society of London* 252 (Series B):231 (1993).

(ii) Various estuarine and marine species have been used in different countries, for example: Spot (*Leiostomus xanthurus*); Sheepshead minnow (*Cyprinodon variegatus*); Silverside (*Menidia beryllina*); Shiner perch (C*ymatogaster aggregata*); English sole (*Parophrys vetulus*); Staghorn sculpin (*Leptocottus armatus*); Three-spined stickleback (*Gasterosteus aculeatus*); Sea bass (*Dicentracus labrax*); Bleak (*Alburnus alburnus*)

(iii) The fresh water fish listed 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.

(7) **Reference chemicals.** The use of reference compounds of known bioconcentration potential would be useful in checking the experimental procedure, when required. However, specific substances cannot yet be recommended.

(f) **Fish care and health**—(1) **Acclimation.** Acclimate the stock population of fish for at least 2 weeks in water at the test temperature and

feed throughout on a sufficient diet (see paragraph (f)(3) of this guideline) and of the same type to be used during the test.

(2) **Pretest mortality and health.** (i) Following a 48–h settling-in period (during acclimation), mortalities are recorded and the following criteria applied:

(A) Mortalities of greater than 10 percent of population in 7 days, reject the entire batch.

(B) Mortalities of between 5 and 10 percent of population in 7 days, acclimate for 7 additional days.

(C) Mortalities of less than 5 percent of population in 7 days, accept the batch. If more than 5 percent mortality during the second 7 days, reject the entire batch.

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

(3) **Feeding.** (i) During the acclimation and test periods, feed an appropriate diet of known lipid and total protein content to the fish in an amount sufficient to keep them in a healthy condition and to maintain body weight. Feed daily throughout the acclimation and test periods at a level of approximately 1 to 2 percent of body weight per day; this keeps the lipid concentration in most species of fish at a relatively constant level during the test. The amount of feed should be recalculated, for example, once per week, in order to maintain consistent body weight and lipid content. For this calculation, the weight of the fish in each test chamber can be estimated from the weight of the fish sampled most recently in that chamber. Do not weigh the fish remaining in the chamber.

(ii) Siphon uneaten food and faeces daily from the test chambers shortly after feeding (30 min to 1 h). Keep the chambers as clean as possible throughout the test so that the concentration of organic matter is kept as low as possible (see paragraph (e)(3), since the presence of organic carbon may limit the bioavailability of the test substance under paragraph (k)(6) of this guideline.

(iii) Since many feeds are derived from fishmeal, the feed should be analyzed for the test substance. It is also desirable to analyze the feed for pesticides and heavy metals.

(g) **Exposure conditions during test**—(1) **Optional preliminary test to determine optimal conditions.** It may be useful to conduct a preliminary experiment in order to optimize the test conditions of the definitive test, e.g. selection of test substance concentrations, duration of the uptake and depuration phases.

(2) **Exposure concentrations of test chemical.** (i) During the uptake phase, expose fish under flow-through conditions to at least two concentrations of the test substance in water. Normally, select the higher (or highest) concentration of the test substance to be about 1 percent of its acute asymptotic LC50, and to be at least tenfold higher than its detection limit in water by the analytical method used. The highest test concentration can also be determined by dividing the acute 96–h LC50 by an appropriate acute/chronic ratio (e.g. appropriate ratios for some chemicals are about 3, but a few are above 100). If possible, choose the other concentrations such that it differs from the one above by a factor of 10. If this is not possible because of the 1 percent of LC50 criterion and the analytical limit, a lower factor than 10 can be used or the use of ^{14}C labeled test substance should be considered.

(ii) No exposure concentration used should be above the solubility in water of the test substance.

(iii) Where a solubilizing agent is used in the stock solution, its diluted concentration in the exposure water should not be greater than 0.1 mL/L and should be the same in all test vessels. Its contribution (together with the test substance) to the overall content of organic carbon in the test water should be known. However, every effort should be made to avoid the use of such materials.

(iv) Minimize results reported as ‘‘not detected at the limit of detection’’ by pretest method development and experimental design, since such results cannot be used for rate constant calculations. Pretest results can be used to determine the exposure concentrations necessary to ensure that concentrations in fish tissue are generally above method detection limits.

(3) **Duration of uptake phase.** (i) A prediction of the duration of the uptake phase and time required to reach steady state 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 utilizing knowledge of either the solubility in water or the octanol/water partition coefficient of the test substance (see paragraph (g)(5) of this guideline).

(ii) The uptake phase should be run for 28 days unless it can be demonstrated that equilibrium has been reached earlier. If the steady-state has not been reached by 28 days, the uptake phase should be extended, taking further measurements, until steady-state is reached or 60 days, whichever is shorter. The depuration phase is then begun.

(4) **Duration of depuration phase.** (i) The depuration period is begun by transferring the fish to the same medium but without the test substance in another clean vessel. A depuration phase is always necessary unless uptake of the substance during the uptake phase has been insignificant (e.g. the BCF is less than 10).

(ii) A period of half the duration of the uptake phase is usually sufficient for an appropriate (e.g. 95 percent) reduction in the body burden of the substance to occur (see paragraph (g)(5) of this guideline for an explanation of the estimation). If the time required to reach 95 percent 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 percent of steady-state concentration). However, for substances having more complex patterns of uptake and depuration than are represented by a one-compartment fish model, yielding first order kinetics, allow longer depuration phases for determination of loss rate constants. The period may, however, be governed by the period over which the concentration of test substance in the fish remains above the analytical detection limit.

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

(B) (*1*) An estimate of k_2 (day–1) may be obtained from the following empirical relationship (see paragraph (k)(20) of this guideline):

Equation 1

$$\log k_2 = -0.414 \log P_{ow} + 1.47 (r^2 = 0.95)$$

For other relationships see see paragraph (k)(14) of this guideline.

(*2*) If the partition coefficient (P_{ow}) is not known, an estimate can be made (see paragraph (k)(4) of this guideline) from a knowledge of the aqueous solubility (s) of the substance using:

Equation 2

$$\log P_{ow} = 0.862 \log(s) + 0.710 (r^2 = 0.994)$$

where s = solubility expressed as moles per liter: (n=36)

(*3*) These relationships apply only to chemicals with log P_{ow} values between 2 and 6.5 (see paragraph (k)(12) of this guideline).

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

$$dC_f/dt = k_1 C_w - k_2 C_f$$

or, if C_w is constant:

Equation 3

$$C_f = k_1/k_2 \cdot C_w(1 - (\exp)^{-k2t}$$

When steady-state is approached (as t approaches infinity), equation 3 may be reduced (see paragraphs (k)(3) and (k)(9) of this guideline) to:

$$C_f = k_1/k_2 C_w$$

or

$$C_f/C_w = k_1/k_2 = BCF$$

Then $k_1/k_2 \cdot Cw$ is an approach to the concentration in the fish at steady-state ($C_{f,s}$). Equation 3 may be transcribed to:

$$C_f = C_{f,s}(1 - (\exp)^{-k2t}$$

or

Equation 4

$$C_f/C_{f,s} = 1 - e^{-k2t}$$

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

As a guideline, the statistically optimal duration of the uptake phase for the production of statistically acceptable data (BCFK) is that period which is required for the curve of the logarithm of the concentration of the test substance in fish plotted against linear time to reach its midpoint, or $1.6/k_2$, or 80 percent of steady-state but not more than $3.0/k_2$ or 95 percent of steady-state (see paragraph (k)(19) of this guideline).

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

$$0.8 = 1 - e^{-k2t}$$

or

Equation 5

$$t_{80} = 1.6/k_2$$

Similarly 95 percent of steady-state is:

Equation 6

$$t_{95} - 3.0/k_2$$

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

$$\log k_2 = -0.414.(4) + 1.47$$

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

up (80 pct)= 1.6/0.652 i.e. 2.45 days (59 h)

or

up (95 pct)= 3.0/0.652 i.e. 4.60 days (110 h)

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

$$\log (P_{ow}) = -0.862 (-5.0) + 0.710 = 5.02$$

$$\log k_2 = -0.414 (5.02) + 1.47$$

$$k2 = 0.246 \text{ days}^{-1}$$

up (80 pct) = 1.6/0.246, i.e. 6.5 days (156 hours)

or

*u*p (95 pct) = 3.0/0.246, i.e. 12.2 days (293 hours)

Alternatively, the expression:

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

may be used to calculate the time for effective steady-state to be reached (see paragraph (k)(12) of this guideline).

(ii) **Prediction of the duration of the depuration phase.** (A) A prediction of the time needed to reduce the body burden to some percentage of the initial concentration may also be obtained from the general equation describing uptake and depuration (first order kinetics) (see paragraphs (k)(13) and (k)(20) of this guideline.

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

$$dC_f/dt = -k_2 C_f$$

or

$$C_f = C_{f,0}(\exp)^{-k2t}$$

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}):

or

Similarly 95 percent depuration will be reached at:

$$t_{95} = 3.0/k_2$$

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

It is important to note, however, that the estimations are based on the assumption that uptake and depuration patterns will follow first order kinetics. If first order kinetics are obviously not obeyed, more complex models should be employed (e.g. paragraph (k)(16) of this guideline).

(6) **Numbers and characteristics of test fish.** (i) Select the numbers of fish per test concentration such that a minimum of four fish per sample are available at each sampling. If greater statistical power is required, more fish per sample will be necessary.

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

(iii) In any one test, select fish of similar weight such that the smallest are no smaller than two-thirds of the weight of the largest. All should be of the same year-class and come from the same source. Since weight and age of a fish appear sometimes to have a significant effect on BCF values (see paragraph (k)(6) of this guideline) record these details accurately. It is recommended that a sub-sample of the stock of fish is weighed before the test in order to estimate the mean weight (see paragraph (h)(2) of this guideline).

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

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

(8) **Light and temperature.** The photoperiod is usually 12 to 16 h and the temperature (±2 °C) should be appropriate for the test species (see Table 3. under paragraph (e)(6)(i) of this guideline). 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.

(9) **Water quality measurements.** During the test, dissolved oxygen, TOC, pH and temperature should be measured in all vessels. Total hardness and salinity (if relevant) should be measured in the controls and one vessel at the higher (or highest) concentration. As a minimum, dissolved oxygen and salinity (if relevant) should be measured 3 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 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.

(10) **Controls.** In addition to the two test concentrations, a control group of fish is held under identical conditions except for the absence of the test substance, to relate possible adverse effects observed in the bioconcentration test to a matching control group and to obtain background concentrations of test substance. One dilution water control and if relevant, one control containing the solubilizing agent should be run.

(11) **Validity of test.** For a test to be valid the following conditions apply:

(i) The temperature variation is less than ± 2 °C.

(ii) The concentration of dissolved oxygen does not fall below 60 percent saturation.

(iii) The concentration of the test substance in the chambers is maintained within ± 20 percent of the mean of the measured values during the uptake phase.

(iv) The mortality or other adverse effects/disease in both control and treated fish is less than 10 percent 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 percent per month and not exceed 30 percent in all.

(h) **Sampling and analysis of fish and water**—(1) **Fish and water sampling schedule.** (i) Sample water from the test chambers for the determination of test substance concentration before addition of the fish and during both uptake and depuration phases. As a minimum, sample the

water at the same time as the fish and before feeding. During the uptake phase, the concentrations of test substance are determined in order to check compliance with the validity criteria (see paragraph (g)(11) of this guideline).

(ii) Sample fish on at least five occasions during the uptake phase and at least on four occasions during the depuration phase. Since on some occasions it will be difficult to calculate a reasonably precise estimate of the BCF value based on this number of samples (especially when other than simple first-order depuration kinetics are indicated), it may be advisable to take samples at a higher frequency in both periods (see the following Table 3.) Store the extra samples as described in paragraph (h)(3) and analyze them only if the results of the first round of analyses prove inadequate for the calculation of the BCF with the desired precision.

(iii) An example of an acceptable sampling schedule is given in the following Table 3.

Table 3.—Theoretical example of sampling schedule for bioconcentration tests of substances with log P_{ow} = 4

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

*Sample water after minimum of 3 ‘‘chamber-volumes’’ have been delivered.

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

Note: Pretest estimate of k_2 for log P_{ow} of 4.0 is 0.652 days^{-1}. The total duration of the experiment is set to $3 \times up = 3 \times 4.6$ days = 14 days. For the estimation of *up* see paragraph (g)(5) of this guideline.

Other schedules can readily be calculated using other assumed values of P_{ow} to calculate the exposure time for 95 percent uptake.

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

(2) **Sampling methodology.** (i) Obtain water samples for analysis by siphoning through inert tubing from a central point in the test chamber. Since neither filtration nor centrifuging appears always to separate the nonbioavailable fraction of the test substance from that which is bioavailable (especially for superlipophilic chemicals, those chemicals with a log P_{ow} greater than or equal to 5) (see paragraphs (k)(6) and (k)(8) of this guideline), samples may not be subjected to those treatments. Instead, measures should be taken to keep the tanks as clean as possible and the content of total organic carbon should be monitored during both the uptake and depuration phases (see paragraph (g)(9) of this guideline).

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

(3) **Sample storage.** (i) It is preferable to analyze fish and water immediately after sampling in order to prevent degradation or other losses and to calculate approximate uptake and depuration rates as the test proceeds. Immediate analysis also avoids delay in determining when a plateau has been reached.

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

(4) **Analysis of fish samples.** (i) Radiolabeled test substances can facilitate the analysis of water and fish samples, and may be used to determine whether degradate identification and quantification should be made. BFCs based on total radiolabeled residues (e.g. by combustion or tissue solubilization) can serve as one of the criteria for determining if degrades identification and quantification is necessary. However, BCF determinations for the parent compound should be based upon the concentration of the parent compound in fish and water, not upon total radiolabeled residues.

(ii) If the BCF in terms of total radiolabeled residues is greater than or equal to 1,000, it may be advisable, and for certain categories of chemicals such as pesticides strongly recommended, to identify and quantify degradates representing greater than or equal to 10 percent of total residues in fish tissues at steady state. If degradates representing greater than or equal to 10 percent of total radiolabeled residues in the fish tissue are identified and quantified, then it is also recommended to identify and quantify degradates in the test water. The major metabolites may be characterized at steady-state or at the end of the uptake phase, whichever is the sooner. It is possible to combine a fish metabolism study with a bioconcentration study to identify and quantify residues in tissues.

(iii) The concentration of the test substance should usually be determined for each weighed individual fish. If this is not possible, pooling of the samples on each sampling occasion may be done but pooling does restrict the statistical procedures which can be applied to the data. If a specific statistical procedure and power are important considerations, then an adequate number of fish to accommodate the desired pooling, procedure and power, should be included in the test. See paragraphs (k)(7) and (k)(10) of this guideline for an introduction to relevant pooling procedures.

(5) **Determination of lipid content.** BCF should be expressed both as a function of total wet weight and, for high lipophilic substances, as a function of the lipid content. Determine the lipid content of the fish on each sampling occasion if possible. Suitable methods should be used for determination of lipid content (see paragraphs (k)(5) and (k)(15) of this guideline). Chloroform/methanol extraction technique may be recommended as standard method (see paragraph (k)(11) of this guideline). The various methods do not give identical values (see paragraph (k)(18) of this guideline), so it is important to give details of the method used. When possible, the analysis for lipid should be made on the same extract as that produced for analysis for the test substance, since the lipids often have to be removed from the extract before it can be analyzed chromatographically. The lipid content of the fish (as mg/kg wet weight) at the end of the experiment should not differ from that at the start by more ± 25 percent. The tissue percent solids should also be reported to allow conversion of lipid concentration from a wet to a dry basis.

(6) **Quality of analytical method.** Since the whole procedure is governed essentially by the accuracy, precision, and sensitivity of the analytical method used for the test substance, check the precision and reproducibility of the chemical analysis experimentally, as well as recovery of the test substance from both water and fish to ensure that they are satisfactory for the particular method. Also, check that the test substance is not detectable in the dilution water used. If necessary, correct the values of C_w and C_f obtained from the test for the recoveries and background values of controls. Handle the fish and water samples throughout in such a manner as

to minimize contamination and loss (e.g. resulting from adsorption by the sampling device).

(i) **Data analysis**—(1) **Determination of uptake and depuration rate constants.** (i) Obtain the uptake and depuration curves of the test substance by plotting its concentration in/on fish (or specified tissues) in the uptake and in the depuration phase against time on arithmetic scales. The depuration rate constant (k_2) is usually determined from the depuration curve (i.e. a plot of the decrease in test substance concentration in the fish with time). The uptake rate constant (k_1) is then calculated given k_2 and a value of C_f which is derived from the uptake curve. See paragraph (d)(2)(iv) of this guideline for a description of these methods. The preferred method for obtaining BCFK and the rate constants, k_1 and k_2, is to use nonlinear parameter estimation methods on a computer (see paragraph (k)(15) of this guideline). Otherwise, graphical methods may be used to calculate k_1 and k_2. If the depuration curve is obviously not first-order, then more complex models should be employed (see paragraphs (k)(3), (k)(4), (k)(9), (k)(12), (k)(13), (k)(14), (k)(19, and (k)(20) of this guideline) and advice sought from a biostatistician.

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

(iii) The results should be interpreted with caution where measured concentrations of test solutions occur at levels near the detection limit of the analytical method. Clearly defined uptake and loss curves are an indication of good quality bioconcentration data. The variation in uptake/depuration constants between the two test concentrations should be less than 20 percent. Observed significant differences in uptake/depuration rates between the two applied test concentrations should be recorded and possible explanations given. Generally the confidence limit of BCFs from well-designed studies approach ± 20 percent.

(2) **Determination of the steady state and kinetic BCFs.** (i) Obtain the uptake curve of the test substance by plotting its concentration in/on fish (or specified tissues) in the uptake phase against time on arithmetic scales. If the curve has reached a plateau, that is, become approximately asymptotic to the time axis, calculate the steady state BCFs from the following relationship:

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

(ii) When no steady state is reached, it may be possible to calculate a BCFs of sufficient precision for hazard assessment from a steady-state at 80 percent (1.6/k2) or 95 percent (3.0/k2) of equilibrium.

(iii) Determine the concentration factor (BCFK) as the ratio k_1/k_2, the two first-order kinetic constants.

(iv) The BCF is expressed as a function of the total wet weight of the fish. However, for special purposes, specified tissues or organs (e.g. muscle, liver), may be used if the fish are sufficiently large or the fish may be divided into edible (fillet) and nonedible (viscera) fractions. Since, for many organic substances, there is a clear relationship between the potential for bioconcentration and lipophilicity, there is also a corresponding relationship between the lipid content of the test fish and the observed bioconcentration of such substances. Thus, to reduce this source of variability in test results for those substances with high lipophilicity (i.e. with log P_{ow} greater than or equal to 3), bioconcentration should be expressed in relation to lipid content in addition to whole body weight. The lipid content should be determined on the same biological material as is used to determine the concentration of the test substance, when feasible.

(j) **Test report.** The test report must include the following information.

(1) **Summary.** Test chemical and test species, uptake and depuration rate constants, and steady state and kinetic BCFs

(2) **Materials.** (i) Exposure tanks and tubes--material and size of tanks.

(ii) Diluter-type and description.

(iii) Dilution water. Source, description of any pretreatment, and water characteristics including 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.

(iv) Test substance. Physical nature and, where relevant, physicochemical properties; chemical identification data (including the organic carbon content, if appropriate); if radio-labeled, the precise position of the labeled atoms and the percentage of radioactivity associated with impurities.

(v) Stock solutions. Method of preparation of stock solutions and frequency of renewal (the solubilizing agent, its concentration and its contribution to the organic carbon content of test water must be given, when used).

(vi) Test species. Scientific name, strain, source, any pretreatment, age, size-range, etc.

(vii) Care of fish. Acclimation, pretest mortality and health, feeding (e.g. type of foods, source, composition—at least lipid and protein content if possible, amount given and frequency).

(3) **Test conditions**— (i) **Test design.** Number and size of test chambers, water volume replacement rate, number of replicates, number of fish per replicate tank, number of test concentrations, controls.

(ii) **Exposure concentrations.** The nominal concentrations, the means of the measured values and their standard deviations in the test vessels.

(iii) **Length of uptake and depuration phases.** Give the lengths of the uptake and depuration phases and the rationale behind them

(iv) **Light.** Type and characteristics of illumination used and photoperiods.

(v) **Water quality within test vessels.** pH, hardness, TOC, temperature and dissolved oxygen concentration.

(4) **Sampling and analysis.** (i) Sampling frequency for fish and water samples.

(ii) Sample storage.

(iii) Sample extraction and analysis.

(iv) Detection and quantification limits.

(v) Accuracy and precision—results of spike and replicate analyses

(5) **Results.** (i) Data obtained in any preliminary test.

(ii) Validity of the test. Fish mortality and/or abnormal behavior for exposed and control, variations in exposure concentrations, variations in temperature, and minimum dissolved oxygen with respect to test validity criteria.

(iii) Lipid content of the test fish.

(iv) Uptake and depuration curves of the test chemical in fish; graphical representation of data.

(v) Concentrations of parent in fish tissue and exposure water. Tabular representation of data; C_f and C_w (with standard deviation and range, if appropriate) for all sampling times (C_f expressed in milligrams per gam of wet weight (parts per million) of whole body or specified tissues thereof e.g. lipid, and C_w expressed in milligrams per gam of wet weight (parts per million). C_w values for the control series (background should also be reported).

(vi) Uptake and depuration rate constants. Give values and 95 percent confidence limits for the uptake and depuration (loss) rate constants, describe the computation.

(vii) Steady state and kinetic BCFs. The BCFs and the BCFK (both expressed in relation to the whole body and the total lipid content, if measured, of the animal or specified tissues thereof), confidence limits and standard deviation (as available).

(viii) Degradate concentrations. Where radiolabeled substances are used, and when required, the accumulation of any major metabolites at steady state or at the end of the uptake phase.

(ix) Deviations and/or unusual observations. Report anything unusual about the test, any deviation from these procedures, and any other relevant information.

(k) **References.** The following references should be consulted for additional background material on this test guideline.

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