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# Guidance Document on Aspects of OECD TG 305 on Fish Bioaccumulation

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# GUIDANCE DOCUMENT ON ASPECTS OF OECD TG 305 ON FISH BIOACCUMULATION

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## FOREWORD

The project to develop this Guidance Document on Aspects of OECD Test Guideline 305 (TG 305) on Fish Bioaccumulation was co-led by Germany, the Netherlands and the United Kingdom. Following major revisions to TG 305 in 2012, it became clear that separate and detailed guidance was necessary to address complex areas of fish bioaccumulation testing, data treatment and interpretation.

Additionally, a User Guide for the R-Package software supporting the treatment of data generated in studies following TG 305 has been developed and is available on the OECD public site (http://www.oecd.org/env/ehs/testing), together with the downloadable software elements.

The Guidance Document was approved by the Working Group of the National Co-ordinators of the Test Guidelines Programme (WNT) at its 29th meeting in April 2017. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 10th July, 2017. This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

# GUIDANCE DOCUMENT ON ASPECTS OF OECD TG 305 ON FISH BIOACCUMULATION

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# 1 GENERAL INTRODUCTION

1. OECD Test Guideline 305 (OECD TG 305 (1)) was revised in 2012 with the following main topics:

- The testing of only one test concentration can be considered, when it is likely that the bioconcentration factor (BCF[1]) is independent of the test concentration.
- A minimised aqueous exposure test design with a reduced number of sample points is possible, if specific criteria are met.
- Measurement of fish lipid content to enable BCF to be expressed on a 5% lipid content basis.
- Measurement of fish weight to enable the (kinetic) BCF to be corrected for growth dilution.
- Greater emphasis on kinetic BCF estimation.
- Addition of a dietary exposure test for substances where aqueous exposure testing is technically unfeasible, or for cases where the objective is specifically to generate information on exposure via the dietary route.

2. On several of these issues, additional information has been generated that has an impact on the use of the bioaccumulation test. The aim of this document is to give guidance to the experimenter and user of the bioconcentration or bioaccumulation data on how to perform the test, calculate the results and interpret them. This guidance document should be seen as an explanation to the revised OECD TG 305, not as a substitute for it.

3. Chapter 2 focuses on some important practical issues of performing the aqueous test. These include avoiding the use of solvents and dispersants by using column generated stock solutions for fish BCF studies with highly hydrophobic test substances (Section 2.1), the influence of total organic carbon (TOC) and dissolved organic carbon (DOC) on BCF values (Section 2.2), the use of solid-phase microextraction (SPME) as an alternative analytical method for the determination of aqueous test substance concentrations within aqueous exposure studies (Section 2.3), and some considerations on ionisable chemicals (Section 2.4). Finally, it gives some guidance on the use of the minimised test design (Section 2.5.1).

4. Chapter 3 introduces the general mathematical models for uptake and elimination of chemicals, where these apply both to the aqueous exposure and to the dietary exposure test (Section 3.2). It also introduces the general procedure to calculate the kinetic BCF (Sections 3.3 and 3.4), how to take account of growth during the experiment when determining the kinetic BCF (Section 3.5), and how to calculate the uncertainty of the kinetic BCF, including growth (Sections 3.4 and 3.5).

5. Chapter 4 focuses on the dietary exposure test. It contains sections on: steps to take in deciding when to run a dietary study (Section 4.1); test conduct (Sections 4.2 and 4.3); the effects of varying study parameters on results (Section 4.4); uncertainty in dietary biomagnification parameters (Section 4.5, this parallels that in chapter 3 for the aqueous method); and a section on how to use the results of a dietary study, including BCF estimation (Section 4.6).

[1] Where BCF is used further in the guidance document, it is intended to cover both the kinetic BCF ($BCF_K$) and the steady-state BCF ($BCF_{SS}$). Where necessary, either $BCF_K$ or $BCF_{SS}$ is specified.

6. The main mathematical models and statistical methods to fit these models to either aqueous or dietary exposure test data are made available as an R-package that accompanies this guidance document. The R-package, named '*bcmfR*', is currently a developmental version (0.3-2) that can be used for evaluation purposes, and to apply most of the statistical methods in this guidance document. To run the package, the statistical software environment called 'R' needs to be installed. The additional installation of the 'RStudio' development environment facilitates running the models and statistical methods. A short User Guide on how to install and use '*bcmfR*' accompanies this guidance document. All supporting elements of the Guidance Document will be made available on a dedicated page on the OECD public site and the URL address inserted here at the time of publication. The R-package was developed to facilitate the complex mathematics and statistics needed to interpret the data. Nevertheless, the mathematics and statistics can be done in other statistical packages as well and the R-package that is provided is not seen as mandatory.

# 2 GENERAL GUIDANCE FOR 305-I: AQUEOUS EXPOSURE BIOCONCENTRATION FISH TEST

7. This chapter focuses on practical issues to consider when conducting the aqueous esposure bioconcentration fish test and should be read together with the OECD Test Guideline 305 (1). As stated in paragraph 30 of that OECD TG 305, stock solutions for fish BCF studies should preferably be prepared by simply mixing or agitating the test substance in the dilution water. However, for highly hydrophobic test substances this may prove a challenge. The use of solvents and dispersants (solubilising agents) is not generally recommended but may be acceptable in order to produce a suitably concentrated stock solution. OECD Guidance Document 23 on Aquatic Toxicity Testing of Difficult Substances and Mixtures (2) focuses on the issues of testing hydrophobic and volatile substances and provide guidance on alternatives to the use of solvents. In cases where it is difficult to achieve a stable and fully solved concentration of the test chemical, either a dietary fish test can be chosen or further work done to conduct an aqueous exposure test. Further guidance is given in paragraph 7 of OECD TG 305

## 2.1 An alternative method to achieve constant concentrations in BCF testing

8. An alternative method to achieve constant $C_{free}$ conditions in BCF testing has been explored by Adolfsson-Erici et al. (3). Here, a polymer phase (silicone rubber) with fast diffusion kinetics was used to maintain the freely dissolved concentrations ($C_{free}$) of a mixture of hydrophobic substances in a bioconcentration test. The advantage of this approach is that any desired concentration can be maintained by changing the concentration in the polymer and the water flow across its surface. When testing a more biodegradable substance, source water may need to be treated to minimise dissolved organic carbon (DOC) and bacterial load. By matching the volume of the polymer phase to the physicochemical characteristics of the chemical of interest and the total volume of water generated, $C_{free}$ concentrations can be maintained. However, to reach steady state concentrations of highly hydrophobic substances extended exposure periods up to 60 days are required which may be difficult to maintain by the polymer phase system. An alternative that may be appropriate under such conditions is the use of a solid phase desorption dosing system (4). Also, the use of column generated test concentrations allows the preparation of test solutions without using solubilizing agents in those test solutions (2).

### *2.1.1 Use of column generated stock solutions*

#### *2.1.1.1 Spiking of carrier matrix with the test items*

9. A solution of the highly hydrophobic test item is prepared using an organic solvent. The solution is then mixed with a carrier matrix with a sufficiently high surface area and a sufficient affinity for the test item. The carrier matrix is a suitable adsorbing matrix, for example silica gel, glass beads, or commercially available optimised specific matrix. Testing is required to choose the right matrix, which should be selected to guarantee a stable loading of the solid phase allowing a constant release of the test item over extended periods of up to 60 days. To reach a suitable eluate concentration a loading of up to 5 mg $g^{-1}$ is recommended (4). The method is not suitable for volatile compounds (due to expected high losses during column preparation and desorption) or surface active compounds (which may form micelles or emulsions, and tend to adsorb at water-solid interfaces.

*Preparing the glass columns*

10. The solvent is then evaporated to dryness. The dry carrier material of each test item is then mixed with water and filled into a glass column or a column from another sufficiently inert material. The top and the bottom of the fillings are covered with glass fibre filters to avoid the loss of matrix material. A constant flow of water (membrane pump) through the column from bottom to top needs to be maintained at a level to allow sufficient time for the test item to desorb from the matrix material into the water column (approximately 5–30 mL $min^{-1}$). Careful investigations are necessary prior to the onset of a flow-through study to estimate the right settings for the optimal dosing procedure. Flow rates (membrane pump) to the mixing chamber may need to be adjusted in response to the trajectory of the column generated concentrations. The pathway of the water through the solid phase desorption dosing system is presented in Figure 2-1. A second test concentration may be generated by further diluting in a second mixing chamber. Further details are provided by Schlechtriem et al. (4).

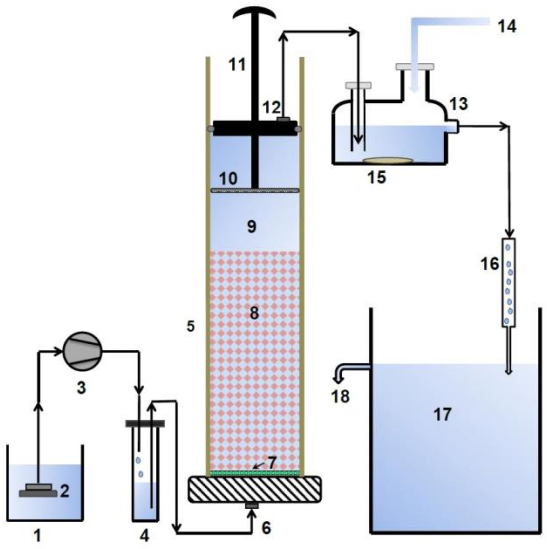


**Figure 2-1: Example set-up of a solid phase desorption dosing system for the generation of column generated test concentrations for fish BCF studies.**

**1: fresh water reservoir; 2: filter unit with glass fibre filter; 3: peristaltic pump; 4: damper; 5: glass column; 6: column inlet; 7: glass fibre filter; 8: test item on a carrier matrix; 9: clearing zone of the water phase; 10: perforated stainless steel screen plate; 11: variable column head gasket; 12: column outlet; 13: mixing vessel; 14: fresh water supply; 15: magnetic stirrer; 16: glass inlet tube; 17: flow-through fish tank; 18: water outlet (4).**

*Pros*

11. OECD TG 305 (1) demands the verification that the aqueous exposure concentration(s) to be applied in flow-through tests are within the aqueous solubility in the test media (cf. para 24). Column-generated test concentrations prevent that test chemicals exceed their water solubility under the given test concentrations. The solid phase desorption dosing system has been successfully applied in fish BCF studies with different highly hydrophobic test items characterised by a high hydrophobicityup to log $K_{OW}$ 7.8 (4). With all substances tested (e.g. PCB 153, hexachlorobenzene, *o*-terphenyl, dibenz[*a*,*h*]anthracene) stable average concentrations (± 20%) could be maintained over a period of 8 weeks ensuring that steady-state concentrations in fish could be reached.

*Cons*

12. Test set-up, including pre-exposure choice of the most appropriate adsorbing matrix and setting flow rates to ensure useable and consistent test concentrations, is more time consuming and difficult than more conventional dosing systems. In some cases flow rates to the mixing chamber need to be adjusted in response to the trajectory of the column generated concentrations. The preparation of eluates is not possible for quickly hydrolysable substances. The growth of bacteria in the columns as well as destruction of the test substances by photolysis may be a problem and must be avoided as far as possible.

## 2.2 Influence of total organic carbon (TOC) and dissolved organic carbon (DOC) on the determination of BCF values

### *2.2.1 General information*

13. As stated in OECD TG 305 (1) in paragraphs 30-31, organic matter content, quantified as total organic carbon (TOC) and dissolved organic carbon (DOC) can have a significant effect on the amount of freely dissolved test substance during flow-through fish tests, especially for highly hydrophobic substances. Sorption of the test substance to organic matter may reduce its bioavailability and therewith result in an underestimation of the BCF (5) (6).

14. Depending on origin, organic matter can be highly varying regarding its qualitative composition. This can result in consequences for the organic carbon concentration in the test system. Organic matter most relevant for flow-through fish tests is fish feed and fish faeces, which differ in their qualitative composition regarding e.g., TOC and DOC content, the fractions of protein, fat, fibre, and ash, and molecular structure and size. Those characteristics cause differences in sorption of the test substances, i.e. at a given TOC concentration, a different quality of organic matter can have a different impact on the reduction of freely dissolved/bioavailable substance concentrations by sorption processes (5) (7).

15. Throughout the test, the concentration of TOC in the test vessels should not exceed the concentration of organic carbon originating from the test substance (and solubilising agents, if used) by more than 10 mg $L^{-1}$ according to OECD TG 305 (1). The results of bioconcentration studies on highly hydrophobic compounds show that TOC concentrations of the water in the test chambers during the flow-through fish test can be maintained below this threshold concentration (4) (5).

16. Solid-phase microextraction (SPME, cf. Section 2.3) is suitable to distinguish between freely dissolved and total test substance concentrations. This can help to elucidate the influence of organic matter on the reduction of the test substance's bioavailability (cf. Section 2.3.2).

### *2.2.2 Handling*

17. According to OECD TG 305 (1), a concentration of up to 10 mg $L^{-1}$ TOC is acceptable. Cleaning of the test system is highly recommended to avoid artefacts. However, an impact of TOC on the results can hardly be eliminated, because 10 mg $L^{-1}$ TOC is a realistic value to reach even in thoroughly cleaned systems. Sorption to organic matter may occur far below a TOC content of 10 mg $L^{-1}$, especially for highly hydrophobic test substances (5) (7). To minimise adsorption of the test substance to organic matter, the guideline recommends keeping the natural particle content as well as the total organic carbon of the dilution water as low as possible. Further, the contribution to the organic carbon content in test water from the test fish (excreta) and from the food residues should be kept as low as possible. Uneaten food and faeces should be siphoned daily from the test chambers shortly after feeding (30 minutes to one hour), to keep the concentration of organic matter as low as possible throughout the test (cf. paragraphs 12, 29, 30, 46 in (1)).

## 2.3 Solid-Phase Microextraction (SPME) as alternative analytical method for the determination of aqueous test substance concentrations within aqueous exposure studies

### *2.3.1 General remarks*

18. OECD TG 305 (1) does not provide defined methods for the extraction of the aqueous phase as this may to some extent depend on the test chemical. However, a commonly used method is liquid-liquid extraction (LLE). The guideline mentions the use of solid-phase microextraction (SPME) to get information on the ratio between bound and freely dissolved analyte specifically when testing highly hydrophobic compounds (*cf.* paragraphs 30 and 60). SPME allows for the determination of freely dissolved substance concentrations. Furthermore, SPME can further be used instead of LLE to determine total aqueous concentrations of the test substances (5) (9). BCF values can be calculated based on total and freely dissolved test substance concentrations, respectively. However, the determination of freely dissolved substance concentrations and the calculation of the BCF value based on freely dissolved substance concentrations are not mandatory.

19. With LLE an exhaustive extraction resulting in total analyte concentrations is assumed, provided that a suitable solvent system is used. Extraction with LLE is an equilibrium-based process with most of the analytes getting dissolved within the solvent. By repeating the extraction process, an exhaustive extraction is approached.

20. If LLE is used to measure aqueous concentrations of the test substance, extraction efficiency can be influenced by both the solvent used for extraction and by the total organic carbon (TOC) content in the sample (7). The impact of TOC depends on both its concentration and its qualitative composition. Therefore, it is recommended to check for an appropriate solvent and to use internal standards for the extraction ($^{13}C$ or $^{2}H$-labelled analogues of the test substance) (12) (cf. 2.3.2.2).

### *2.3.2 Solid-phase microextraction (SPME)*

#### *2.3.2.1 General information*

*Principle of SPME*

21. Solid-phase microextraction (SPME) is a solvent-free analytical technique developed for dilute systems. It combines selective extraction and enrichment of analytes from the sample. In this method, a polymer coated fibre is exposed to the gas or liquid phase containing the analyte of interest. Analytes partition from the sample to the fibre coating in the course of an equilibration. This process is highly dependent on the characteristics of the analyte, the sample matrix, the ambient conditions, as well as the composition of the fibre coating. Generally, a minimum analysis time is imposed so that equilibrium conditions are established between the solid and fluid phases, with respect to the measured analyte. Subsequently the concentration of the analyte of interest can be determined directly from the fibre after thermal desorption or after extracting it from the fibre into a solvent, depending on the determination technique.

*Instrumentation*

22. Extraction by SPME can be processed manually or automatically by an autosampler. The use of automated SPME is recommended because it guarantees equal conditions during the extraction process for all samples (cf. Figure 2-2).

23. SPME can be coupled to gas chromatography (GC), with a direct thermo-desorption of the analytes in the injection system. Alternatively, fibres can be extracted by solvents and measured by GC or high performance liquid chromatography (HPLC).

24. If coupled to GC, SPME generally has a high sensitivity for hydrophobic organic compounds (HOCs) and quantification is possible at trace levels, allowing for studies with low concentrations of test substances.

25. Small sample volumes of 5 to 20 mL can be handled. Due to the small sample volumes, depletion during extraction can be a relevant issue, if freely dissolved concentrations are determined (i.e. absorption or adsorption of the analyte to the fibre in sufficient mass for detection, without significantly disturbing the equilibrium between dissolved and total analyte, cf. 2.3.2.4). The quantification of multiple analytes in water is possible. Preliminary studies should always be carried out to assess extraction temperature, kinetics, and time, to optimise extraction conditions for the analytes.

*SPME mode*

26. Two modes of SPME are mainly used: immersed extraction and headspace extraction (HS-SPME). During immersed extraction, the SPME fibre remains in the liquid sample and the analytes partition from the sample matrix to the fibre coating. For the determination of freely dissolved analyte concentrations, diffusion layer effects have to be considered as an issue if extraction is stopped before equilibrium of fibre and sample (cf. 2.3.2.4).

27. In the headspace mode, the analytes migrate from the aqueous to the gaseous phase and sorb to the fibre. In headspace mode, the fibre coating is protected from interfering matrix influences such as organic matter, proteins or strong acidic/alkaline conditions. For extraction in headspace mode, sufficient volatility of analytes has to be ensured. Partition to the gaseous phase can be enhanced by higher extraction temperatures.. If headspace mode is used, the influence of diffusion layer effects (cf. 2.3.2.4) can be prevented by extraction under non-equilibrium conditions.

*Fibre coating*

28. Different fibre coatings are commercially available and their selection depends on the required sensitivity and on the polarity and volatility of the analytes. Selection of an appropriate coating of the fibre is crucial for extraction efficiency and selectivity. For highly hydrophobic compounds, the use of polydimethylsiloxane (PDMS) coated fibres is recommended. For such compounds, the use of fibres with reduced coating thickness (e.g. PDMS 7 µm) should be considered to reduce a potential carry-over caused by an incomplete thermodesorption. Smaller fibre coatings can as well help to avoid significant depletion and help to reduce potential diffusion layer effects (cf. 2.3.2.4).

*Extraction conditions*

29. Within the extraction process, the parameters extraction time, extraction temperature, agitation, and sample composition influence the mass of extracted analytes. Prior to extraction, it has to be ensured that each sample is equilibrated according to extraction parameters.

30. To maintain reproducibility, extraction parameters have to be consistent during a series of analyses.

*Extraction in dynamic range vs. equilibrium*

31. Sample extraction by SPME is a non-exhaustive, equilibrium-based process. However, in small sample volumes a large fraction of the total mass can be extracted. To save time, and to prevent significant sample depletion (i.e. disturbing the original equilibrium), extraction of samples can be stopped in the dynamic range of the equilibration process, i.e. before the equilibrium between sample and fibre is reached. Since the equilibration of the analyte between sample and fibre can take more than a day, it is recommended to perform the extraction under non-equilibrium conditions (e.g., for 20−60 min), which is possible if ambient conditions are held constant. Here it is essential that temperature, extraction time and stirring are absolutely identical amongst all samples and standard solutions, which makes an autosampler with SPME device and agitator for well-defined shaking and heating indispensable. However, when freely dissolved analyte concentrations are extracted under non-equilibrium conditions with immersed SPME, diffusion layer effects have to be considered (cf. 2.3.2.4).

*Calibration*

32. As all these factors (cf. paragraphs 22–31) influence the outcome of the SPME method, calibration of the method is essential. Calibration methods such as external calibration and internal calibration are the methods most frequently used. An excellent overview of the various calibration methodologies that are available for SPME is given in (11).

33. The external standard calibration compares the detector response from the sample to the response from the target compound in the calibration standard. Different standard solutions must be prepared over the range of concentrations expected in the sample. The external standard calibration is well-suited for homogeneous aqueous samples with minor interference. However, care must be taken in ensuring that the calibration standards are freely dissolved and not a mixture of freely dissolved and precipitated/ undissolved substance (e.g. it has to be ensured, that the calibration concentrations do not exceed water solubility of the analytes). Generally, the freely dissolved concentration is measured using external calibration.

34. Internal standard calibration requires the addition of a known amount of a known compound into the calibration standards and samples. Internal standards must be similar in analytical behaviour to the target analytes but not found in the sample. Ideal internal standards are analogues of the analytes which are

labelled with stable isotopes ($^{2}H$ or $^{13}C$) (12) (13). Minor errors in process, the continuous decrease of fibre extraction efficiency, as well as potential variation in instrument sensitivity can be eliminated by the use of internal standards. Accordingly, sample to sample variations in extraction and desorption efficiency caused by the sample matrix, i.e. due to the presence of organic matter, can be corrected. Generally, the total concentration is measured using an internal calibration.

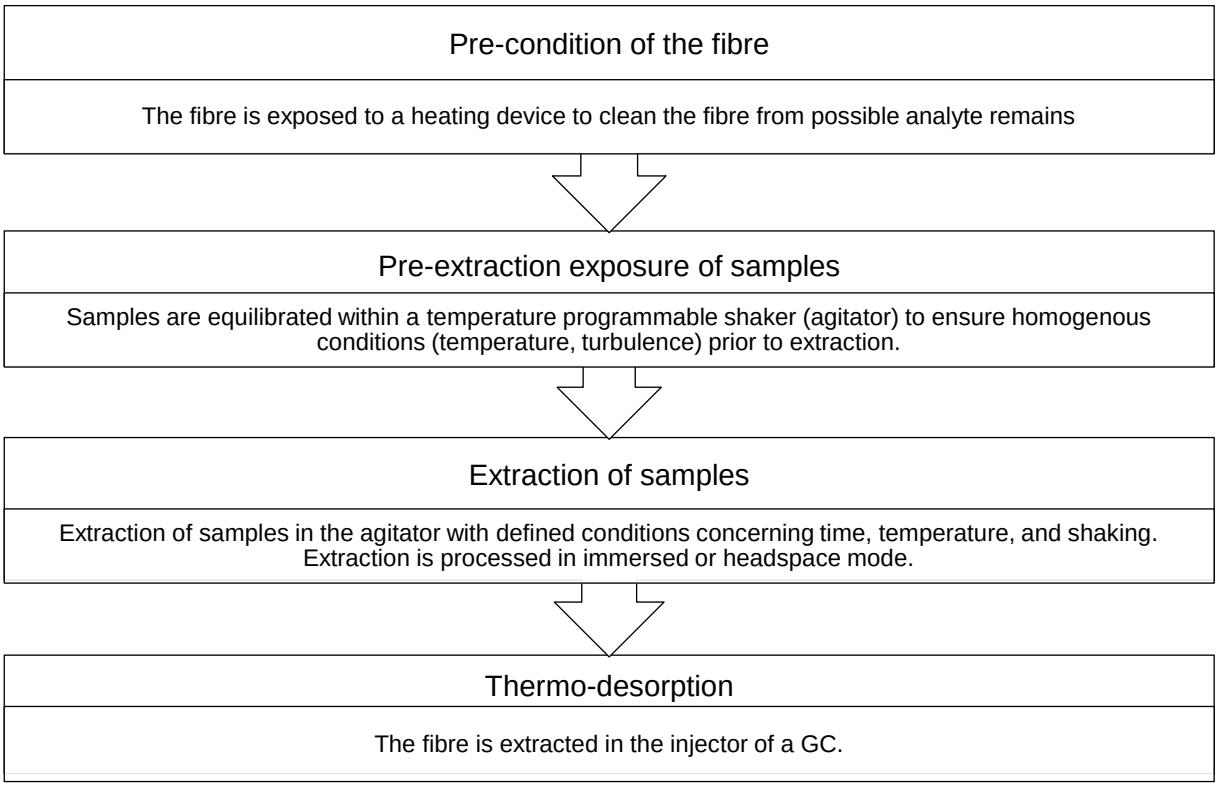


**Figure 2-2: Automated SPME analysis – general process**

*2.3.2.2 Total concentrations*

35. SPME can yield total analyte concentrations when an internal standard is added (5). Results are comparable to LLE results, and processing of the extraction can be automated. In addition, as SPME is a solvent-free procedure, costs of solvents are saved.

36. Using SPME, the total concentrations are determined indirectly, due to the extraction of only freely dissolved analyte concentrations. If an internal standard is added and equilibrated with the sample, the internal standard can be assumed to bind to the organic matter in an equal amount as the test substance, if an analogue of the analyte labelled with stable isotopes ($^{2}H$ or $^{13}C$) is used.

37. Within the extraction step, only the freely dissolved amounts of test substance and internal standard partition to the fibre. Concentrations of the analyte and the internal standard are then compared to references of the internal standard in samples without organic matter (determined as part of the calibration procedure, cf. paragraphs 32–34).

38. A factor can be calculated for the reduction of the internal standard in the samples compared to the internal standard in the references. If the amount of test substance extracted from the sample is divided by this factor, results correspond to the total concentrations. Within this step, variability of fibre and instrument (GC/MS) is eliminated as well.

39. It is highly recommended to use analogues of the test substance labelled by stable isotopes as internal standards, because this ensures an equal behaviour of internal standard and test substance. This is crucial for the sorption process of the internal standard when equilibrated with the sample prior to extraction, as well as to eliminate variance in fibre sensitivity and variances during GC/MS analysis.

*2.3.2.3 Free concentrations*

40. Several properties and effects of dissolved organic chemicals such as transport behaviour, bioavailability and toxicity are heavily dependent on the freely available concentration (14) (15) which can be determined by solid-phase microextraction (SPME). In contrast to the estimation of total concentrations, no correction using internal standards is applied to reach the freely dissolved analyte concentrations. However, the resulting values for free concentrations are to be considered as an assessment rather than a determination of exact concentrations and provide evidence of reduced bioavailability due to sorption processes within the test system (5). The use of this method is a relevant option when the test system is prone to accumulation of organic matter in water, but is not the recommended default procedure. Although no internal standards are used, precise data on freely dissolved analyte concentrations can still be obtained using the following approaches:

- Use a high number of replicates: Due to efficiency of the method, a higher number of replicates can be processed. Several replicates can be measured to reduce variability. With statistical methods, outliers can be eliminated. For example, outliers may be identified in box-and-whisker plots as values outside the range of Q1 – 1.5 × Interquartile range (IQR) and Q3 + 1.5 × IQR or with other methods.
- Use more robust detectors: Detectors such as flame ionization detector (FID) or electron capture detector (ECD) could help to reduce uncertainty. However, linear range and sensitivity could be relevant constraints for these detectors. Here, the variability of the fibre (e.g. by a changing sensitivity) remains.
- Use disposable fibres with solvent extraction: Disposable SPME-fibres can be extracted by solvents after their equilibration within the aqueous sample. Here, the variability between the different fibres remains. However, variability of the instrument (GC/MS) can be eliminated by the addition of an internal standard to the obtained solvent extracts prior to measurement. In case disposable fibres are used for extraction under equilibrium conditions, the amount of depletion has to be considered (cf. paragraphs 42−45 and Annex 1).

*2.3.2.4 Limitations of SPME*

*Diffusion layer effects*

41. When freely dissolved test substance concentrations of highly hydrophobic substances are measured with immersed SPME in the dynamic range (cf. paragraph 31), the occurrence of diffusion layer effects has to be avoided by choosing a sufficient extraction time. Diffusion layer effects or matrix accelerated transport can occur when desorption of the test substance from the matrix is faster than its diffusion in the stagnant water layer around the fibre. This can lead to an increased uptake rate of the test substance in the presence of matrix (e.g. dissolved organic matter, DOM) and further to an overestimation of freely dissolved test substance concentrations (16). For the extraction of substances affected by diffusion layer effects, the sampling time has to be sufficiently enhanced. However, at the same time care should be taken that significant depletion during extraction is prevented.

*Depletion*

42. A further aspect that may limit the applicability of SPME for the measurement of freely dissolved test substance concentrations in BCF studies is the issue of depletive extraction that may be encountered when highly hydrophobic substances are analysed in small volume samples obtained from the system (17).

43. The degree of depletion that is desired is defined by the critical ratio $r_C$ which is the ratio of the concentration in water after SPME to concentration in water prior to SPME ($r_C = C_W/C_W^0$). Generally the degree of depletion should be minimised to less than 10% ($r_C = 0.9$) of the mass of material in the system, ideally less than 5% ($r_C = 0.95$). This is necessary if one wants to measure the $C_{free}$ that the organisms were exposed to during the study, rather than an erroneous measurement due to the shift in the equilibrium between $C_{total}$ and $C_{free}$.

44. An exemplary calculation on the critical sample volume ($V_C$) that is needed to avoid depletion, as well as related partitioning equations are given in Annex 1. Those calculations show that for highly hydrophobic substances, the critical sample volume needed under equilibrium conditions mostly exceeds the volumes used in automated SPME procedures. However, because the equilibration process between fibre and sample can last up to more than a day, for those substances automated SPME is not recommended for equilibrium extraction anyway. Instead, the use of automated SPME is recommended under non-equilibrium conditions (cf. paragraphs 31 and 45).

45. Depletion could be prevented using automated SPME under non-equilibrium conditions (cf. Sections 2.3.2.1 and 2.3.2.3). If extraction should be performed under equilibrium conditions, significant depletion could be prevented by choosing smaller fibre coatings (e.g. 7 µm). Alternatively, (disposable) SPME fibres could be left in situ during the BCF test, and analysed after reaching equilibrium. During flow-through conditions where the freely dissolved concentration is continuously replenished, issues of depletion due to the partitioning to the fibre-phase should not occur.

## 2.4 Ionisable chemicals

46. It has been estimated that about 40% of chemicals on the market could be present in the environment in an ionised form, including weak and strong acids and bases. Many of these compounds are relatively hydrophilic when present either in the ionized or in the neutral form. However, some ionisable substances may tend to accumulate in fish through mechanisms not related to storage in lipids, e.g. certain perfluoroalkyl acids (PFAs).

47. Several empirical and mechanistic models are described in the literature, as cited by Nichols et al. (18), that take into account the prediction of bioaccumulation as a function of pH and a chemical's p$K_a$ value. It has been suggested that accumulation is predominantly driven by the concentration of the neutral form in water, because this is the form that diffuses easily across the water-gill interface (19).

48. The fraction that is dissociated (and thus the neutral fraction as well) can be easily estimated using the pH of the medium and the p$K_a$ of the chemical. When organic acids are added to water, they partially dissociate to yield an equilibrium mixture of the original undissociated neutral acid and its dissociated anionic form (the conjugate base):

$$HA \leftrightarrow H^+ + A^- \;,\; K_a = \frac{[H^+]\cdot[A^-]}{[HA]} \;;\; [Acid] = [HA] + [A^-] \;;\; \phi_{anion} = \frac{1}{1 + 10^{pK_a - pH}} \qquad \text{Equation 2-1}$$

Similarly, organic bases associate with protons in water to yield their cationic acid form (the conjugate acid):

$$HB^{+} \leftrightarrow H^{+} + B \;,\quad K_a = \frac{[H^{+}] \cdot [B]}{[HB^{+}]} \;;\; [Base] = [HB^{+}] + [B] \;;\; \phi_{cation} = \frac{1}{1 + 10^{pH - pK_a}} \qquad \text{Equation 2-2}$$

49. Recent studies show that several factors influence the transport of the ionised form into the fish, such as acidification of the gill surface caused by elimination of metabolically produced acid. In addition to lipid partitioning, other factors such as specific binding to proteins also contribute to bioaccumulation in fish (18). This means that bioaccumulation predictions based on models driven by lipid partitioning may underestimate bioaccumulation potential for certain substances (e.g. some perfluorinated compounds).

50. For the purpose of comparison with empirical bioconcentration data generated with OECD TG 305 (1), or development of the relevant OECD TG 305 experimental conditions, bioaccumulation of ionisable substances can be predicted using the model developed by Armitage et al. (20), although it cannot be used for zwitterions and multiprotic acids and bases. This model accounts for speciation of ionisable compounds in respired water and possible uptake of ionised species across the gills. In general, model performance was good for weak ($pK_a > 6$) acids and weak ($pK_a < 8$) bases. Somewhat poorer performance was obtained for stronger ($pK_a \leq 6$) acids and stronger ($pK_a \geq 8$) bases (19).

51. OECD TG 305 states that aqueous exposure tests should be conducted at a pH that ensures the test substance is in its neutral form and within the pH range appropriate for the test species, which ensures testing at a physiologically and environmentally relevant pH. In almost all cases this should be achievable since only weakly acidic or basic test substances would be considered for testing. As stated the presumption is that the neutral form will be better taken up by the test organisms and have the greater potential for accumulation through lipid storage. In cases where comparison of a BCF prediction based on log $K_{OW}$ or the Armitage model (20) and the measured BCF show that the measured value is significantly higher than the predicted value, this may indicate that accumulation mechanisms other than lipid partitioning are dominant (e.g. protein binding).

52. For further guidance on testing ionisable substances, please refer to OECD Guidance Document No. 23 on Difficult to Test Substances (2).

## 2.5 Number of test concentrations in a fish BCF Test

53. There have recently been several publications on evidence (21) (22) that demonstrates that for the vast majority of plant protection products and general chemicals, BCF values tend to be independent of the test concentration.

54. Thus, testing at a single concentration is permissible (cf. paragraph 49 of (1))and recommended if

- Testing at two concentrations is not a requirement under the regulatory regime of concern.
- The test substance is a moderately hydrophobic organic compound. Driven by diffusion, hydrophobic organic compounds would generally accumulate in lipids. For other groups of substances with other possible ways of uptake, e.g. ionisable substances or metals, not enough may be known to exclude a potential of concentration dependence.

- Testing is done clearly within the water solubility of the test substance, since testing at concentrations near or above the solubility limit of the test substance in the test water will lead to an underestimation of the BCF due to reduced bioavailability of the test substance.
- TOC and DOC in the test chamber is measured regularly and fully reported, and remain clearly within the permissible limits (cf. (1), paragraph 30), since adsorption of part of the test substance to organic matter in the test chamber will lead to an underestimation of the BCF due to reduced bioavailability of the test substance (cf. Section 2.2),
- The freely available concentration ($C_{free}$) of the test substance in the test chamber is measured and reported, e.g. by using SPME (cf. Section 2.2) or any other suitable method.

It may be useful to contact the relevant regulatory authority and to discuss the testing strategy in advance.

55. If, on the other hand, there is any evidence of possible concentration dependence, one of the following paths may be followed:

- Conduct of a full study at two concentrations, or
- Conduct of a minimised test at two concentrations as a pilot study for determining the need for testing at two test concentrations in a subsequent definitive test (cf. OECD TG 305 (1), paragraph 91). In Annex 2 of this guidance document, this option is further explored to define further criteria for when this will be a valid option or not. It is suggested to use a maximum permissible percent difference (MPD) of 50 % with no offset for cases where the results of a minimised test with two concentrations are not far from a regulatory level of concern. To define the term "not far from a regulatory level of concern", the analysis of Hashizume et al. (23) is useful (cf. paragraph 58). In this analysis, margins for $BCF_{Km}$ were estimated that correspond to regulatory values of concern. Should the result of one concentration of a two (or more) concentration minimized BCF test where the MPD is ≥ 50% fall into the relevant margin (depending on specific criteria in the different jurisdictions, e.g. 1,400 to 2,700 for the 2,000 criterion (23)), a full bioconcentration test with two or more concentrations should be performed. For minimised tests with two concentrations that demonstrate a concentration dependence (i.e. where the MPD is ≥ 50%) but both $BCF_{Km}$ are very low (e.g. below any values of concern as set out in regulation(s)), then conduct of a definitive test should not normally be necessary, depending on the requirements of the relevant regulatory authority (cf. (1), paragraphs 94 and 95).

### *2.5.1 Use of the minimised test design*

56. The minimised test is in principle the same as the main aqueous BCF (305-I) test but with reduced fish sampling, and the possibility of one or two test concentrations (cf. (1)). The minimised test is best used when integrated into an overall strategy for assessing bioaccumulation. At the outset, the assessor should consider the purpose and certainty required from the result of the bioaccumulation testing.

57. This will help ensure adequacy and acceptability of testing, thereby avoiding waste of animals and resources if tests have to be repeated. If the BCF estimate from the minimised BCF test is "far away" from values that are of regulatory concern, then performance of a definitive (full design) test might not be required (recognising that each regulatory agency will have its own policy regarding acceptance).

58. The work of Hashizume et al. (23) provides a means of defining what "far away" means. They collected BCF data of full BCF tests according to OECD TG 305 ($BCF_{full}$) of 298 curves from 155

chemicals from the Japanese Chemical Substances Control Law (CSCL) database[2] and resampled to simulate a minimised test and determination of a BCF from a minimised test ($BCF_{Km}$). In this analysis, the 5th and 95th percentile of the ratio of $BCF_{full}$:$BCF_{Km}$ were estimated to be 0.74 and 1.45, respectively. With these values, it is possible to identify a margin around the respective regulatory values of concern. The Japanese analysis suggests that a $BCF_{full}$ of 2,000 corresponds to a $BCF_{Km}$ of 1,400 to 2,700, and a $BCF_{full}$ of 5,000 corresponds to a $BCF_{Km}$ of 3,400 to 6,800 (23).

59. The minimised test design can also be used as a framework for a pilot study to provide information that allows optimisation of the design of a subsequent definitive test, should one be required. The minimised test design can address questions such as the following:

- Will BCF estimates for the test chemical depend on exposure concentration in test solutions? If not, it may be permissible to run a definitive test using a single test concentration (depending on regulatory authorities' policies). Performing the minimised test at two concentrations can provide information to make this decision.
- Do metabolites occur at levels that will necessitate fraction collection and/or metabolite analysis? If so, knowing the level of metabolite and metabolite profile to expect will allow optimisation of sampling design in a definitive test. The minimised design can provide samples for assessment of metabolite levels and profile.
- What is the likely length of depuration period that will be required? The minimised test provides a dependable estimate of depuration rate constant that allows efficient allocation of samples over time, whereas estimates of depuration rate constants based on relationships with $K_{OW}$ are unable to account for metabolism and other mechanisms of accumulation than hydrophobic partitioning.
- Are problems likely to occur with maintaining test substance concentrations in the test solutions during a definitive test? Analysis of test solutions during the minimised test will readily reveal problems, and additional preliminary work can be performed to ensure that methods are adequate.
- Are analytical methods adequate to support a definitive test? The minimised design can provide samples that will help determine requirements in terms of limit of quantification, and to allow analytical recovery of test substance to be assessed at appropriate concentrations.

60. For each of these questions, the assessor should consider whether a minimised test is necessary. In some instances other preliminary experimental work may address the query.

61. A further use of the test could be to re-confirm old tests where validity cannot be confirmed due to the absence of particular information, for example growth dilution or a depuration period.

## 3 ESTIMATING THE BIOCONCENTRATION FACTOR IN THE AQUEOUS EXPOSURE TEST

62. In OECD TG 305 (1) it is indicated that the bioconcentration factor (BCF) can be calculated as the ratio of concentration in the fish ($C_f$) and in the water ($C_w$) at steady-state ($BCF_{SS}$) and as a kinetic

[2] Available at: http://www.safe.nite.go.jp/jcheck/top.action?request_locale=en

bioconcentration factor ($BCF_K$), which is estimated as the ratio of the rate constants of uptake ($k_1$) and depuration ($k_2$) assuming first order kinetics. In this chapter of the Guidance further statistical background is given on estimating these different parameters, including influences of factors like hydrophobicityand growth. After a general introduction of the issues (Section 3.1), the basic parameters and their relationships are introduced (Section 3.2). The major part of this chapter focuses on estimation of kinetic BCF (Section 3.3), for which a stepwise approach is introduced (Section 3.4) that includes a check on model assumptions and influences of data transformations. To facilitate the calculations for this stepwise approach, a so-called package for the freely available statistical software *R* is made available. The final part of this chapter (Section 3.5) discusses ways to correct for growth of the fish during the test and how to correct for differences in lipid content. The chapter ends with a short overview of outcomes to report for the approach that is described (Section 3.6). For most users of OECD TG 305 Sections 3.4, 3.5, and 3.6 will be most relevant, but to fully understand these sections, the background in the previous sections is essential.

## 3.1 Main BCF estimation issues

63. As indicated in OECD TG 305 (1), the bioconcentration factor (BCF) can be calculated as the ratio of concentration in the fish ($C_f$) and in the water ($C_w$) at steady-state ($BCF_{SS}$) and as a kinetic bioconcentration factor ($BCF_K$), which is estimated as the ratio of the rate constants of uptake ($k_1$) and depuration ($k_2$) assuming first order kinetics.

64. Estimation $BCF_{SS}$ is relatively straight-forward, but it also has some disadvantages. It does not use all data generated in the experiment, growth dilution and loss processes may not have been taken into account, and steady-state may not be reached within the standard duration of the experiment. For this and other reasons, it is desirable to always report the $BCF_K$ as well as $BCF_{SS}$.

65. Because $k_1$ and $k_2$ are constants estimated from the experiment, the kinetic BCF can be calculated in the absence of steady-state. Statistical methods such as non-linear regression can be used to report the confidence limits of the kinetic BCF. This allows the assessor to explore the fit of the model to the experimental data as discussed later on. The rest of this chapter further explores these issues. The relatively straight-forward estimation of $BCF_{SS}$ is considered sufficiently discussed in OECD TG 305 (1), and thus not further discussed in this Guidance document.

66. In order to estimate $k_1$, $k_2$ and the $BCF_K$, a general differential equation describing the rate of change of the concentration in a fish is shown in Section 3.2. This also shows how this relates to the estimation of the dietary biomagnification factor (BMF). Therefore, Section 3.2 is relevant for the dietary BMF estimation as well.

67. Different estimation techniques can be used to estimate the $BCF_K$ (OECD TG 305 (1), Annex 5). Each of these techniques has its pros and cons. The main issue addressed here is that the $BCF_K$ estimate is co-determined by the statistical fitting procedure used, as witnessed by its mean value and its confidence interval. General guidance is given in Section 3.3 on statistical procedures to estimate the $BCF_K$. To accompany this guidance, a package for the freely available R software has been developed to automate these various statistical procedures, called '*bcmfR*'[3].

[3] The current version of the *R*-package (*bcmfR*, version 0.3-2) has been verified to work with *R Studio* release 1.0.44 and *R* release 3.3.2. That does not mean that it will not work in newer versions of *R* or its packages, but as the *R* software is open source and continuously under development, some functionalities may disappear in newer versions, in particular in certain packages (further details on necessary packages and their version release numbers can be found in the accompanying "OECD-TG305 R-Package bcmfR

68. Several biological variables also influence the $BCF_K$ estimation. For reasons of availability, cost and ease of experimentation, young fish are often used. Depending on age, feeding regime and species, rapid growth may occur. Growth may obscure the $BCF_K$ estimation if not taken into account properly. This is further explained in Section 3.5.

69. On a concentration basis, growth is seen as a 'biomass dilution' or pseudo-elimination effect in the fish. Simple fish growth models can be used to estimate the growth rate constant and its uncertainty, which can be used to correct kinetic BCF values but not steady state BCF values for growth. This in turn influences the estimation of the kinetic BCF, further discussed in Section 3.5. Next to growth during the experiments, the lipid content may change as well. Different fish lipid contents may influence the rates of depuration for hydrophobic substances that partition into fish lipids, which is described in Section 4.2.3.

70. OECD TG 305 (1) prescribes that the BCF is corrected for a 5% lipid content (both $BCF_{SS}$ and $BCF_K$ and corrected for growth during the study period (only applicable to $BCF_K$) as described in Annex 5 of OECD TG 305. Both of these additional calculations are included in the calculation of the final lipid normalised growth corrected $BCF_{KgL}$ and the corresponding confidence interval.

## 3.2 Basic parameter estimation for the BCF and the BMF

71. In a natural situation, fish can take up chemicals from water and from food (e.g. (24)). A simplified general equation shows the relationship between these two processes. To keep track of which type of mass is referred to in the units, this guidance proposes adding an identifier to the weights: *W* for 'wet weight' of the fish, and *X* for the amount of test chemical under study.

72. The basic equation to describe the rate of change of the concentration in a fish (with first order rate constants and constant exposure concentration) exposed by water and diet[4] simultaneously is given by:

$$\frac{dC_{\text{fish}}}{dt} = k_1 \cdot C_{\text{water}} + k_f \cdot C_{\text{food}} - k_t \cdot C_{\text{fish}}(t) \qquad \text{Equation 3-1}$$

Where $\frac{dC_{\text{fish}}}{dt}$: rate of change of fish chemical concentration (mgX $kgW^{-1}$ $d^{-1}$),
$k_1$: uptake rate constant from water (L $kgW^{-1}$ $d^{-1}$),
$k_f$: uptake rate constant from food ($d^{-1}$)
$C_{\text{water}}$: exposure concentration (mgX $L^{-1}$),
$C_{\text{food}}$: food concentration (mgX $kgW^{-1}$),
$k_t$: total depuration rate constant ($d^{-1}$),
$C_{\text{fish}}(t)$: chemical concentration in fish over time (mgX $kgW^{-1}$).

73. The uptake from food ($k_f$) is determined by the feeding rate ($I$) and the absorption efficiency[5] ($\alpha$), i.e. the absorption of a chemical from food across the gut

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User Guide (v0.3-2)". Potential future updates of the R-package bcmfR will be made available via the OECD public website (www.oecd.org/env/ehs/testing).

4 This situation is not included in the experimental set up of OECD Test Guideline 305 (1) but is shown here to introduce the general model.

5 In OECD TG305 the term "assimilation efficiency" is used. It was pointed out, however, that assimilation is not the correct term, since it refers to uptake and subsequent incorporation into tissue, i.e. it refers to uptake and transformation.

$$k_f = I \cdot \alpha \qquad \text{Equation 3-2}$$

Where *I:* food ingestion rate constant (kg food $kgW^{-1}$ $d^{-1}$)
*α:* assimilation efficiency (mgX $mgX^{-1}$)

74. The total depuration ($k_t$) is the sum of all loss processes acting on the fish, when expressed on a concentration basis

$$k_t = k_2 + k_g + k_m + k_e \qquad \text{Equation 3-3}$$

Where $k_{2:}$ first order rate constant for depuration from fish ($d^{-1}$)
$k_{g:}$ first order rate constant for fish growth ('growth dilution') ($d^{-1}$)
$k_{m:}$ first order rate constant for metabolic transformation ($d^{-1}$)
$k_{e:}$ first order rate constant for faecal egestion ($d^{-1}$)

75. The basic equation to describe the rate of change of the concentration in a test fish (with first order rate constants and constant exposure concentration) exposed via food only is defined as

$$\frac{dC_{fish}}{dt} = k_f \cdot C_{food} - k_t \cdot C_{fish} \qquad \text{Equation 3-4}$$

76. This equation, together with paragraph 83 and 84 form the basis for the calculations to derive the dietary BMF from the dietary bioaccumulation study data as discussed in Chapter 4.

77. The basic equation to describe the rate of change of the concentration in a test fish (with first order rate constants and constant exposure concentration) exposed via water only, and assuming that fish are not growing during the test and no metabolism occurs (i.e. assuming that $k_g$, $k_m$ and $k_e$ can be ignored) is:

$$\frac{dC_{fish}}{dt} = k_1 \cdot C_{water} - k_2 \cdot C_{fish} \qquad \text{Equation 3-5}$$

78. The concentration in fish as a function of time then becomes the familiar equation

$$C_{fish}(t) = C_{water} \cdot \frac{k_1}{k_2}(1 - e^{(-k_2 \cdot t)}) \qquad \text{Equation 3-6}$$

79. At steady state, which may not be reached in typical BCF experiments, the steady state fish concentration can now be calculated, indicated by the asterisk *:

$$C^*_{fish} = \frac{k_1}{k_2} \cdot C_{water} \qquad (mgX \cdot kgW{-}1) \qquad \text{Equation 3-7}$$

and so the steady state $BCF^*$ can be seen to be theoretically equivalent to the kinetic BCF in the absence of fish growth:

$$\mathrm{BCF}^{*} = \frac{C^{*}_{\mathrm{fish}}}{C_{\mathrm{water}}} = \frac{k_1}{k_2} \qquad (\mathrm{L \cdot kgW{-}1}) \qquad \text{Equation 3-8}$$

80. In many cases, the kinetic BCF will be the preferred measure of bioconcentration in the aqueous exposure test, but whenever possible, both $BCF_K$ and $BCF_{SS}$ should be determined and reported. Because it can be estimated in several ways, the statistical estimation technique should be reported and parameter estimates for $k_1$ and $k_2$, including their standard error and covariance matrix (if available) should also be reported. From this, the confidence limits and correlation between $k_1$ and $k_2$ can be estimated and from that the $BCF_K$ and its confidence limits. Standard errors and confidence limits of the expressions for $BCF_K$ and BMF can be estimated through the delta method (cf. Annex 3, A3.1.2). This is an analytical approximation to the error propagation through these expressions. The covariance between regression coefficients is taken into account. Details are given in Fox and Weisberg ((25), p. 200) and in the R-package 'car' supporting the book (26). Procedures and methodology of nonlinear regression are described in Bates and Watts (27), Seber and Wild (28), and Draper and Smith (29).

81. Traditionally, the $BCF_K$ was estimated in a sequential way (see OECD TG 305 (1), Annex 5). First $k_2$ is estimated from the depuration phase. To estimate $k_2$, a linear regression is usually done of *ln*(concentration in fish) versus time. Subsequently, the estimated average concentration in fish at the start of the depuration phase (when no samples have yet been taken) can be determined by extrapolation at the end of the uptake phase. Finally, $k_1$ can be estimated based on these estimates. Annex 3 (A3.1) describes the sequential procedure and explores its pros and cons. Although this is a robust procedure, it ignores the fact that $k_1$ and $k_2$ are correlated. In addition, $k_2$ co-determines the uptake phase (as seen in Equation 3-6). This means that the estimate of the $BCF_K$ uncertainty will be different between these two regression procedures with no obvious way of combining the two measures of uncertainty.

82. Nowadays, non-linear regression techniques make it relatively easy to perform a simultaneous fit of both the uptake and the depuration phase and this is the preferred method to provide $BCF_K$ estimates[6], including a single direct measure of uncertainty (confidence limits for the model's fit). Section 3.4 introduces a procedure to find an appropriate $BCF_K$ estimate.

83. In BCF experiments according to OECD TG 305 (1), there is an uptake phase during chemical exposure, and a depuration phase (starting at $t_{\mathrm{dep}}$) in which the exposure concentration is put to zero. This makes $C_{\mathrm{water}}$ time-dependent:

$$C_{\mathrm{water}}(t) = \begin{cases} C_{\mathrm{w}}, & t < t_{\mathrm{dep}} \\ 0, & t \geq t_{\mathrm{dep}} \end{cases} \qquad \text{Equation 3-9}$$

at constant $C_{\mathrm{water}}$ up to the depuration phase (i.e. up to $t_{\mathrm{dep}}$), and 0 otherwise.

The solution of Equation 3-5 is:

[6] In some cases with noisy data, e.g. when elimination is very slow, a simultaneous non-linear fit may not converge (i.e. an optimal fit through the data will not be reached) and sequential fitting may appear to give a better estimate (mainly based on visual inspection of the plot). It may also be more (visually) obvious from the sequential fit whether data are not exhibiting first order kinetics. Also in these cases, however, the pros and cons of the sequential fitting procedure should be considered (cf. Annex 3, A3.1).

$$C_{\text{fish}}(t) = \begin{cases} C_{\text{w}} \cdot \dfrac{k_1}{k_2} \cdot \left(1 - \text{e}^{-k_2 \cdot t}\right), & t < t_{\text{dep}} \\ C_{\text{fish}}\left(t_{\text{dep}}\right) \cdot \text{e}^{-k_2 \cdot \left(t - t_{\text{dep}}\right)}, & t \geq t_{\text{dep}} \end{cases} \qquad \text{Equation 3-10}$$

which reduces to:

$$C_{\text{fish}}(t) = \begin{cases} C_{\text{w}} \cdot \dfrac{k_1}{k_2} \cdot \left(1 - \text{e}^{-k_2 \cdot t}\right), & t < t_{\text{dep}} \\ C_{\text{w}} \cdot \dfrac{k_1}{k_2} \cdot \left(\text{e}^{k_2 \cdot t_{\text{dep}}} - 1\right) \cdot \text{e}^{-k_2 \cdot t}, & t \geq t_{\text{dep}} \end{cases} \qquad \text{Equation 3-11}$$

84. In both phases, growth can be taken into account as detailed in Section 3.5.2, but is ignored just for now for simplicity.

85. In order to estimate the $BCF_K$ and its uncertainty directly, the accumulation function can be re-parameterised in the following way by taking Equation 3-11 and substituting

$$\text{BCF} = \frac{k_1}{k_2}, \quad \text{i.e.} \quad k_1 = \text{BCF} \cdot k_2 \qquad \text{Equation 3-12}$$

to obtain a re-parameterisation of the accumulation function with parameters $BCF_K$ and $k_2$, as follows:

$$C_{\text{fish}}(t) = \begin{cases} C_{\text{water}} \cdot \text{BCF} \cdot \left(1 - \text{e}^{-k_2 \cdot t}\right), & t < t_{\text{dep}} \\ C_{\text{water}} \cdot \text{BCF} \cdot \left(\text{e}^{k_2 \cdot t_{\text{dep}}} - 1\right) \cdot \text{e}^{-k_2 \cdot t}, & t \geq t_{\text{dep}} \end{cases} \qquad \text{Equation 3-13}$$

By using nonlinear regression as detailed in Section 3.4, this yields a direct estimate of the $BCF_K$, its standard error, and its confidence limits.

## 3.3 $BCF_K$ estimation

86. The preferential statistical estimation of the kinetic bioconcentration factor in aqueous exposure tests involves the application of nonlinear regression techniques to fit the parameters in $BCF_K$ models. Simultaneous fitting of the uptake and depuration phase is recommended to find the $BCF_K$ and $k_1$ and $k_2$ estimates (cf. Annex 3, A3.1).

87. The null-hypothesis is that the general $BCF_K$ model (Equation 3-13) is an appropriate description of bioaccumulation in fish. It is also assumed that the error structure comes from a Normal (Gaussian) distribution, with constant, but often unknown, standard deviation. Moreover, the errors are considered to be uncorrelated. However there may be various reasons why this may not be the case. For instance, the simplification that first order kinetics apply may not be appropriate (e.g. due to various sorbing phases in the organism with different sorption constants or induction of metabolism of the test compound during the

experiment). Furthermore, model diagnostics might point out that data transformation is needed to accomplish that the measurement errors follow a Normal (Gaussian) distribution afterwards.

88. It is therefore important to check the assumptions that underlie a non-linear regression model to ensure that Equation 3-13 is indeed an appropriate description of bioaccumulation and that the $BCF_K$ that we derive from it is an appropriate metric for the process.

89. A special role in the assessment of $BCF_K$ model fits will be reserved for possible transformations of the data, when the analysis of the model results indicates e.g. that the variation in fish concentrations increases as a function of the mean (variance heterogeneity). This is often observed when dealing with concentrations of chemicals in field populations (e.g. (30) (31)).

90. As the depuration phase in a standard fish test is always modelled through a first-order clearance rate, whatever the nature of it, a log-transformation, e.g. natural logarithm with base *e* (ln), seems reasonable. Exponential decay, when log-transformed, becomes a straight-line model. Moreover, accumulated fish concentrations are positive values, once more making a log-transformation an interesting option. Theoretically, however, the doubly infinite tails of the Normal distribution cannot hold strictly for positive data. Furthermore, the log-transformation may have an undesirable effect on the fit. This may even happen in the simple exponential decay model for the depuration phase.

91. This guidance recommends analysing the bioaccumulation model (Equation 3-13) in a stepwise fashion:

1. Fit the model to the data, without data transformation, using a set of model diagnostics.
2. Fit the model to the data with ln-transformed response variable $C_{fish}(t)$ using a set of model diagnostics.
3. Find an optimum data transformation using the Box-Cox optimisation procedure.
4. Decide on the appropriateness of the model and data transformation used in steps 1, 2 and 3. Provide justification (based on model diagnostics and graphical plots) for the preferred data transformation used to estimate the kinetic BCF (and underlying $k_1$ and $k_2$ values).
5. Correct for growth and lipid content.

92. Section 3.4 describes this stepwise progression using a relevant example drawn from real data (Annex 5, A5.1, Table A – 6). Rather than postulating the optimal transformation, this guidance encourages the user to find a suitable model fit and data transformation with the *R*-package that is made available, following the steps that are outlined in Section 3.4. It should be noted that all of the above steps and the growth and lipid correction that is discussed in Section 3.5 in principle is done in one go, and reported as output of the *R*-package that accompanies this guidance.

93. The following model diagnostics can be used as discussed in Annex 3 (A3.1):

- Correct mean function for bioaccumulation $C_{fish}(t)$
- Checking (for heteroscedasticity) of measurement errors
- Normal distribution of measurement errors
- Mutually independent measurement errors

94. Making graphical plots is the first and main approach to check model assumptions. It would be unwise to put too much weight on statistical tests alone to judge these assumptions. It is therefore

recommended to use graphical procedures in conjunction with statistical tests, especially for deciding on variance homogeneity and normality assumptions (the output of the *R*-package includes such graphical plots).

## 3.4 $BCF_K$ estimation by non-linear regression

### *3.4.1 Step 1: Fit the model to the data, no data transformation*

95. Non-linear regression of bioaccumulation data is done according to the steps of paragraph 91. In order to explore if the model (Equation 3-13) fits the experimental data in a satisfactory way, a set of model diagnostics is recommended. We will closely follow some of the practical guidance discussed in Ritz and Streibig (32), with the advantage that the relevant methods are made available in the *R*-based software package '*bcmfR*' that accompanies this guidance. To facilitate the fitting procedures, the *R*-package is made available via OECD including instructions on how to use it, but it is not a mandatory element of the test conduct and reporting.

96. Equation 3-13 is used to fit the bioaccumulation model to data from bioaccumulation experiments[7] (*Example 1*, values are given in Annex 5, A5.1, Table A – 6) to illustrate the stepwise approach of paragraph 91. First of all, the data on the original concentration scale of the experiment will be shown and analysed using model diagnostics that will be generated using the dedicated *R*-package.

97. For *Example 1* (Annex 5, A5.1, Table A – 6) fitting $C_{fish}(t)$ on the untransformed scale gives a visually good fit (Figure 3-1). The parameter estimates are shown in Table 3-1.

**Table 3-1: Parameter estimates for *Example 1* (Annex 5, A5.1, Table A – 6), untransformed $C_{fish}$ data (for fitting).**

| | Estimate | Std. Error | 2.5% | 97.5% |
|---|---|---|---|---|
| $k_1$ | 451.3 | 79.0 | 317.2 | 653.9 |
| $k_2$ | 0.1784 | 0.0379 | 0.1123 | 0.2731 |
| $BCF_K$ | 2529 | 180 | 2189 | 2982 |

98. It is important to realise that the confidence interval is always relative to the model complexity and the model evaluation method. It is not the confidence that we have in a specific parameter, nor the confidence we have in the resulting $BCF_K$. These are estimates depending on the model structure, data structure, and fitting method. This also stresses the importance of model diagnostics and data transformation as shown later. The plot on the ln-scale (Figure 3-1, bottom panel) also seems to show an acceptable fit to the data, with the $BCF_K$ estimated at 2529 L/kg and the 95% confidence interval between 2189 and 2982 L/kg.

[7] In many cases chemical concentrations in fish at the end of the depuration phase in BCF studies will be very low and may fall below the limit of detection (l.o.d.). For these concentrations it will be difficult to decide on their true value, in particular when the l.o.d. is relatively high. For this reason it may be advisable to not use the time-points showing chemical concentrations in fish below the l.o.d. in data analysis. However, in some cases (e.g. when depuration is fast and many of the chemical concentrations in the fish fall below the l.o.d.), it may be advisable to allocating a specific value to those values below l.o.d. (e.g., 0.5 × l.o.d.). This would then allow a sensitivity analysis to be performed for the influence of these values below l.o.d. on the outcome of the test. An example where such a consideration may be needed is the situation where both values below and above l.o.d. are observed at the same time point.

99. Model diagnostics are used to further evaluate model fit (cf. paragraph 93 and Annex 3,A3.2), starting with a check on the correct mean function for bioaccumulation $C_{fish}(t)$. It is generally easier to check if the data are spread in a random manner around the fitted model of Figure 3-1 by using residuals plots. The residuals are defined as the difference between the response values $C_{fish}(t)$ and the corresponding fitted values (Figure 3-2, top left). They help to answer the question if a systematic pattern is visible in the plot, e.g. some form of curvature or asymmetry around the x-axis. If this is the case, the mean function may not be appropriate to capture the average trend in the data. However, other diagnostics can be used as well since the answer is not always that straightforward.

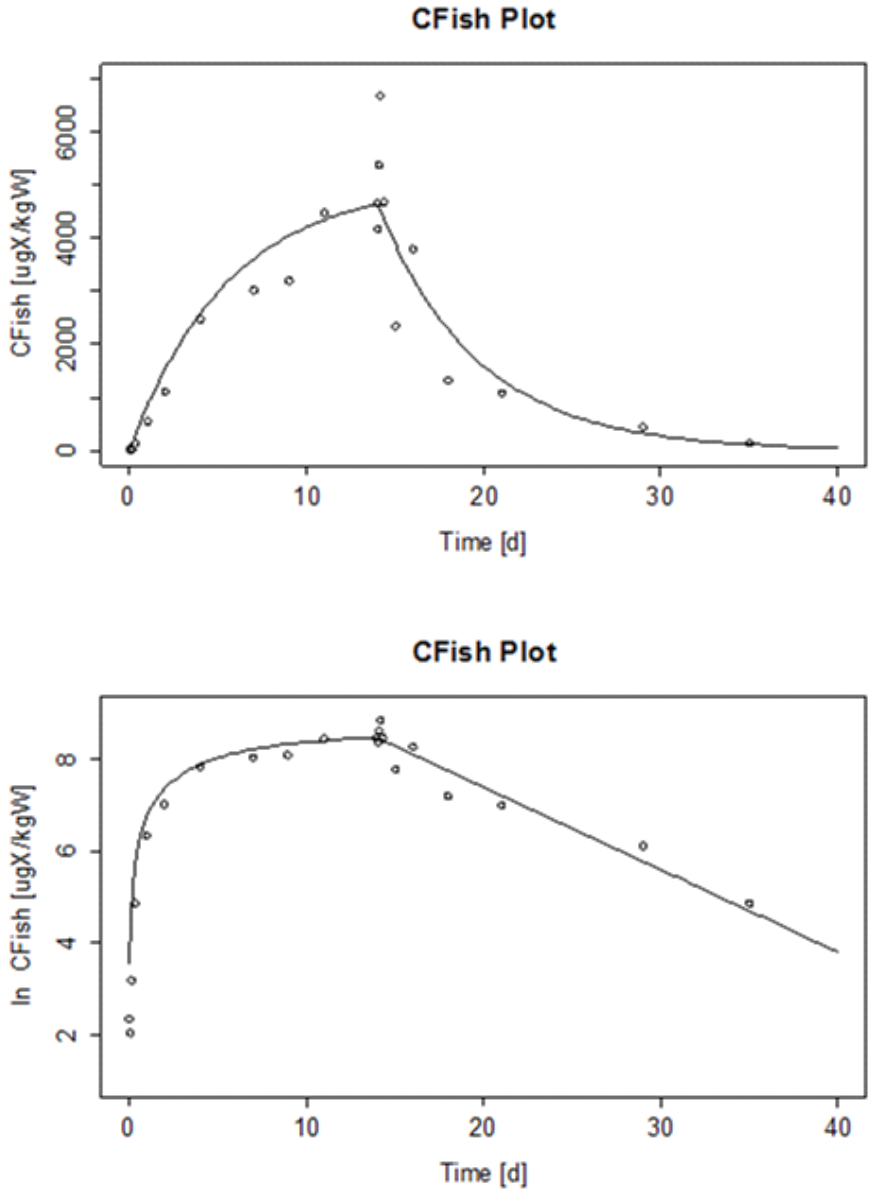


**Figure 3-1: Untransformed fit (*Example 1,* Annex 5, A5.1, Table A – 6) of the joint $k_1$-$k_2$ model to estimate $BCF_K$ parameters (top panel shows original curve fit), plotted on normal scale (top panel) and ln-scale (bottom panel).**

100. The residuals plot for Figure 3-2 (top left) shows that the values of the residuals increase (plus or minus) with the magnitude of the fitted values, indicating that the current model may be improved to describe the data in a better way. If we look at the standardised residuals (Figure 3-2, top right) the assumption is that they should approximately follow a standard normal distribution (having expectation 0 and deviation of 1). The y-axis is now based on plus or minus 2 standard deviations of the errors to allow for a quick check on outliers. The same conclusion can be drawn that the now standardised residuals increase with the magnitude of the fitted values, with one outlier at high values of $C_{fish}$, however no strong deviation from the normality assumption seems indicated. These plots show what is called "heteroscedasticity" (variability of a parameter is unequal across the range of fitted values in this case) something that often can be improved by transforming the dependent variable.

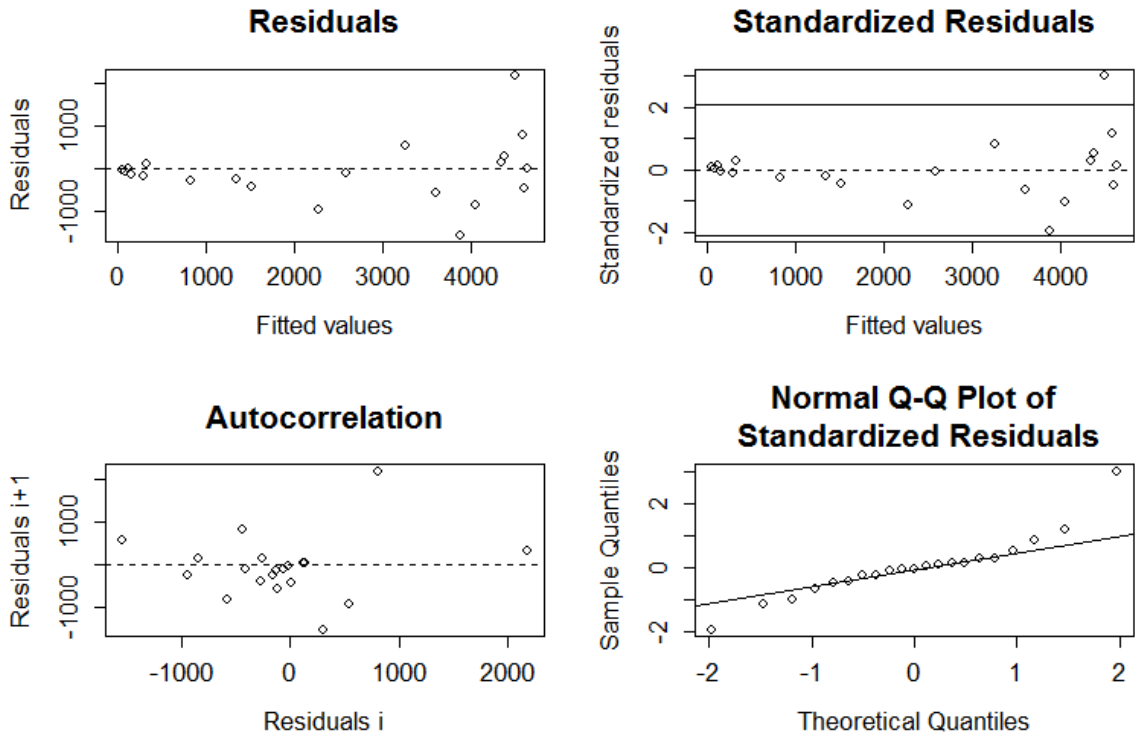


**Figure 3-2: Model diagnostic plots for the bioaccumulation model (Equation 3-13) in Figure 3-1 (*Example 1*).**

101. The residuals should not show any specific pattern over time. If this would be the case it would indicate a systematic error. An additional diagnostic tool is that of autocorrelation, which detects similarity between residuals as a function of the time lag between them. It is a tool for finding repeating patterns or time trends that otherwise might go undetected. This can be visually inspected by looking at the autocorrelation diagnostic plot (Figure 3-2, bottom left). In this case, the autocorrelation plot seems to confirm that the model seems generally adequate. In other cases a clear trend in subsequent values of the residuals can be shown (with a trend from negative to positive consecutive residuals over the experiment), indicating that the bioaccumulation model or experiment is flawed somewhere (e.g. Figure 3-3).

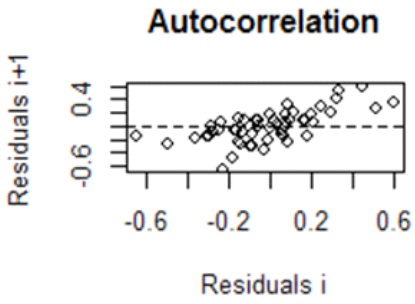


**Figure 3-3: Autocorrelation plot for residuals of the bioaccumulation model for *Example 2* (Annex 5, A5.1, Table A – 7; ln-transformed), indicating correlation between residuals over the course of the experiment.**

102. In support of the autocorrelation plot, the runs test (33) can be used to indicate if the residuals are independent (random) or not. The null-hypothesis is that they are, and at low *p*-values this hypothesis is rejected. For Figure 3-2 (bottom left panel), the runs test indicates a $p = 0.2664$ confirming the interpretation of the autocorrelation plot. For Figure 3-3 (*Example 2*), the runs test indicates $p = 5.791 \cdot 10^{-5}$, casting strong doubts on the independence of the data.

103. The normal probability plot or normal Q-Q plot (Figure 3-2, bottom right) allows inspection of whether the residuals approximately follow a normal distribution. The measurement errors are plotted against a straight line that indicates the standardised normal distribution. Departures from normality are indicated by deviations of the residuals from the straight line. In Figure 3-2, the data are approximately normal, but with clear deviations near the tails (left and right ends of the plot).

104. In addition to graphical inspection, a statistical test can be used for confirmation. The Shapiro-Wilk test for residuals assumes that they are normally distributed. In case the *p*-value is less than the alpha (significance) level (e.g. at 5%), the residuals do not follow a normal distribution. If the *p*-value exceeds the alpha level, then the null hypothesis that the data came from a normally distributed population cannot be rejected. In case of Figure 3-2, the deviations in the tails as seen in the Q-Q plot seem to indicate non-normality of the model error which is confirmed by the outcome of the Shapiro-Wilk normality test:

```
Shapiro-Wilk normality test

data: stdres
W = 0.8917, p-value = 0.02419
```

### *3.4.2 Step 2: Fit the model to the data, ln-transformed*

105. Equation 3-13 is now used to fit the bioaccumulation model where the model is transformed by taking the natural logarithm (Annex 3). Again *Example 1* (Annex 5, A5.1, Table A – 6) is used. The parameter estimates are shown in Table 3-2, with remarkably different $BCF_K$ estimates (1882, 95% CI 1408–2548) that are much lower than in the untransformed case (Table 3-1).

**Table 3-2: Parameter estimates for *Example 1* (Annex 5, A5.1, Table A – 6), ln-transformed $C_{fish}$ data (for fitting)**

| | Estimate | Std. Error | 2.5% | 97.5% |
|---|---|---|---|---|
| $k_1$ | 240.4 | 42.9 | 164.7 | 349.5 |
| $k_2$ | 0.1277 | 0.0207 | 0.0843 | 0.1723 |
| $BCF_K$ | 1882 | 264 | 1408 | 2548 |

106. The two plots of Figure 3-4 show that a systematic underestimation occurs in the uptake phase, from the 5$^{th}$ data point onwards, and at the start of the depuration phase. This is obviously important for the estimation of the BCF. So, one may have doubts as to the estimated value of the $BCF_K$ (i.e. $BCF_K$ = 1882). Especially for the untransformed result, but also for the parameter estimates of ln-transformed $C_{fish}(t)$, Figure 3-4 displays a dramatic underfitting of most of the uptake phase.

107. The model clearly did not improve by the ln-transformation, even though this seemed to be indicated by the results in Step 1 (Section 3.4.1), mostly because of the poor fit in the uptake phase. The diagnostic plots can be used to further detail the problems of the specific fit. The residuals plot (Figure 3-5, top left) shows that especially at lower values, only underfitting occurs (values below the zero line on the y-axis). This is confirmed by the standardised residuals, indicating again outliers but in contrast to Step 1 results (Figure 3-2), the outliers are now in the low range of fish concentrations instead of in the high range.

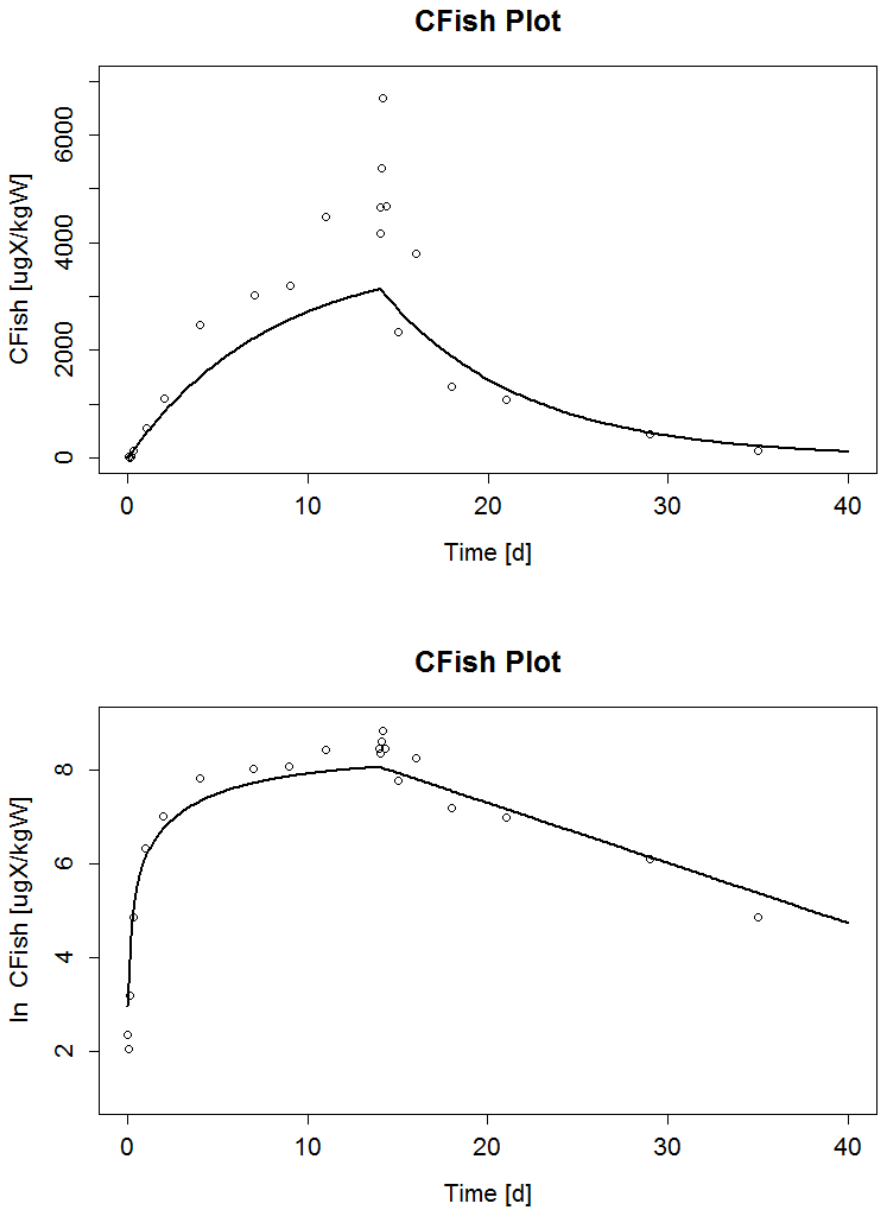


**Figure 3-4: Transformed fit (*Example 1,* Annex 5, A5.1, Table A – 6) of the joint $k_1$-$k_2$ model to estimate $BCF_K$ parameters (bottom panel shows original curve fit), plotted on (back-transformed) normal scale (top panel) and ln-scale (bottom panel).**

108. The autocorrelation plot also indicates the same trend where first underestimation occurs, and later on overestimation. The outcome of the runs test (34) is p = 0.004023, which clearly supports the conclusion from the graphical diagnostic plot: strong doubts on the independence of errors.

109. The normal probability plot or normal Q-Q plot (Figure 3-5, bottom right) shows even stronger departures from normality in the tails (deviations from the normal distribution straight line) than in Step 1 example results (Figure 3-2), also indicating problems with the underlying assumption on the normal error distribution. The Shapiro-Wilk test for residuals in case of Figure 3-4 also indicates non-normality of the model error which is confirmed by the outcome of the Shapiro-Wilk normality test:

```
Shapiro-Wilk normality test

data: stdres
W = 0.8784, p-value = 0.01364
```

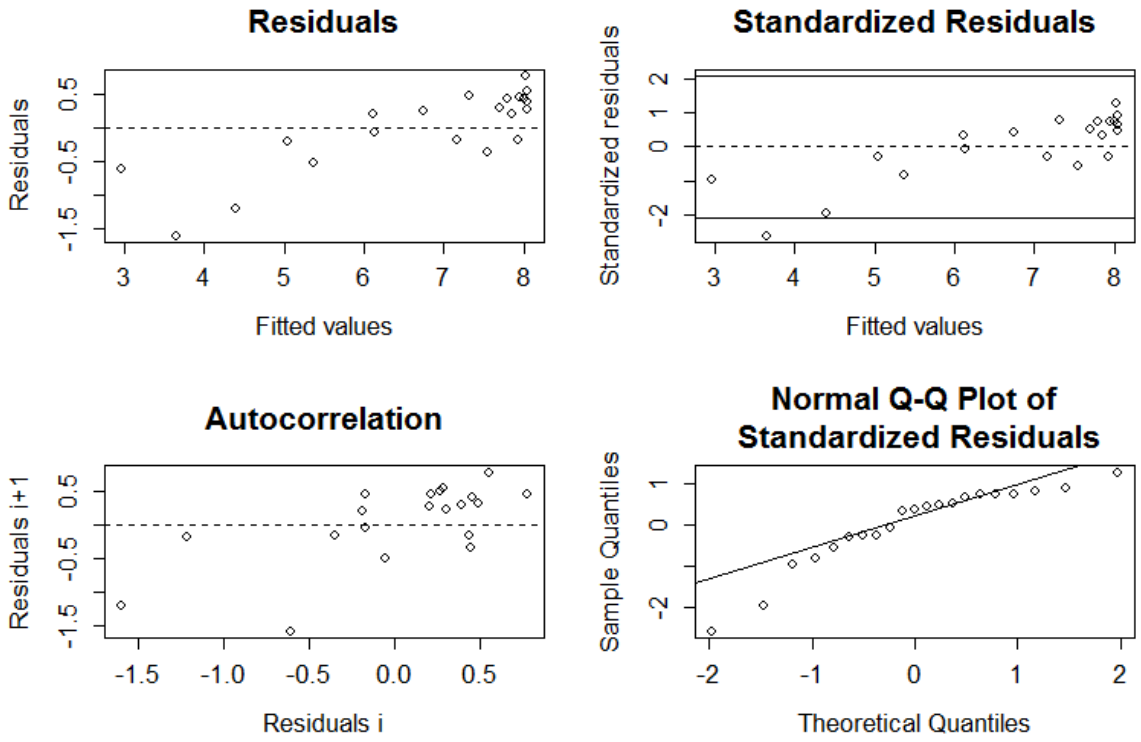


**Figure 3-5: Model diagnostic plots for the bioaccumulation model (Equation 3-13) in Figure 3-4 (*Example 1*).**

### *3.4.3 Step 3: Find an optimum data transformation using the Box-Cox optimisation procedure*

110. It is common practice that the chemical concentrations in a fish during uptake and depuration phase of a bioaccumulation test are untransformed when fitting the model to the data. It has often been observed that concentrations measured in biota vary between normally and log-normally distributed (30) (31). When steps 1-3 indicate that data transformation is still needed, other types of transformation can be explored. To find an appropriate data transformation, the Box-Cox power transformation can be used. The procedure is discussed briefly in the next paragraphs. The method is implemented as part of the *R*-package '*bcmfR*', using standardised *R* procedures and further explained in Ritz and Streibig (32). Both model predictions and response data are transformed the same way (transform-both-sides approach; (32), p. 81). To take the process-oriented nature of the nonlinear $BCF_K$ models seriously, transformation of both data and model need to be done by the same power coefficient.

111. The Box-Cox power transformation is defined as ((35) (32), p. 81):

$$y^{(\lambda)} = \begin{cases} \dfrac{x^{\lambda} - 1}{\lambda} & \text{when } \lambda \neq 0 \\ \ln(y) & \text{when } \lambda = 0 \end{cases} \qquad \text{Equation 3-14}$$

For zero or very small power coefficients λ, the transformation is (approximately) logarithmic. Other powers are 1.0 (corresponding to untransformed data), while 0.5 corresponds to the square root transformation, 1/3 is the cube root transformation, –1 is the reciprocal transformation, and so on. Note that the same transformation is applied to both the fish concentration data and model predictions. A very effective way is to fit the $BCF_K$ model parameters and transformation parameters jointly using a profile likelihood approach. The log-likelihood is the result of a function that is maximised, which corresponds to finding the best possible fit of the model to the data over a range of λ values.

112. The log-likelihood values of the fit are plotted over a range of λ values (Figure 3-6). This greatly extends the possibilities to obtain acceptable error distributions. For our *Example 1* data set, this is the Box-Cox transformation plot (Figure 3-6).

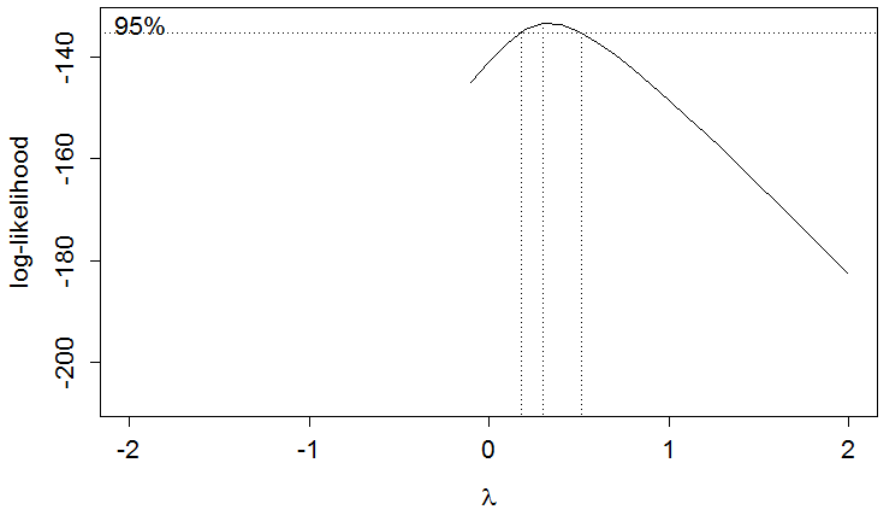


**Figure 3-6: Plot of the log likelihood function for the Box-Cox parameter λ applied to both $C_{fish}$ data and model prediction for *Example 1***

113. The estimated optimal λ value is 0.3 and its confidence interval is 0.18 up to 0.51. The interpretation is that the Box-Cox-transformation parameter λ is at most the square root (0.5), which is half-way between not transforming (1.0) and 0 (log-transformation). The most likely value (0.3) is near the *square root of the square root* (0.25). It may be difficult in this example to accept the square root of concentration values, let alone the square root of the square root, as log-values, such as pH, p*K*, and so on, seem more typical for data transformations of this kind. The Box-Cox transformation has been an important breakthrough in statistics to correct for asymmetry in either, or both, explanatory and response variables, and for non-constant standard deviations. It is a numerical device, as well as a graphical device, just like the logarithm of the $H^+$ concentration. In this way, lack of Normality, often accompanied by a non-constant standard deviation, can often be corrected. The model is still the same. The optimal Box-Cox transformation is *not a guarantee* for establishing Normally distributed errors with constant standard deviation, although improvements are likely (32).

114. Equation 3-13 is now used to fit the bioaccumulation model with the optimal λ value of 0.3, The parameter estimates are shown in Table 3-3, with a different $BCF_K$ estimate of 2351 (95% CI 1993 – 2709).

**Table 3-3: Parameter estimates and $BCF_K$ uncertainty from optimal Box-Cox fit to *Example 1*.**

| | Estimate | Std. Error | 2.5%[1] | 97.5%[1)] |
|---|---|---|---|---|
| $k_1$ | 367.8 | 45.3 | 273.0 | 462.6 |
| $k_2$ | 0.1565 | 0.0194 | 0.1158 | 0.1971 |
| $BCF_K$ | 2351 | 183 | 1993 | 2709 |

[1)] These *t*-based confidence intervals come from nlstools, and may slightly differ from previous confidence intervals.

115. The Box-Cox optimally transformed $C_{fish}$ fit and plot (with $\lambda = 0.3$) is shown in Figure 3-7, indicating a good fit to the data.

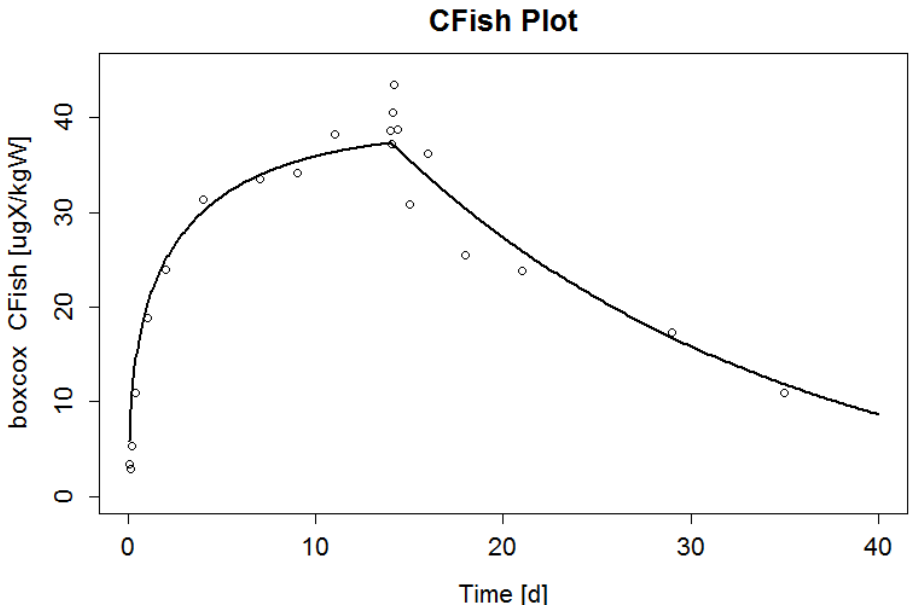


**Figure 3-7: Box-Cox (0.3)-transformed fit of $C_{fish}$ and plot (*Example 1*).**

116. The diagnostic plots are used once again to see if the optimal value of $\lambda = 0.3$ did improve on the distribution of the errors and variance homogeneity. The residuals plot (Figure 3-8, top left) shows that especially at lower values, some underfittting (values below the zero line on the y-axis) still occurs. The standardised residuals plot shows that with a single exception, model errors fall within the expected range of normal distributed values and no clear heteroscedasticity is observed anymore.

117. The autocorrelation plot does not indicate a trend anymore as in the ln-transformed cases in Step 2 (cf. Section 3.4.2). The outcome of the runs test is $p = 0.2664$ which supports the conclusion from the graphical diagnostic plot that the errors seem independent.

118. The normal probability Q-Q plot (Figure 3-8, bottom right) shows that the optimal λ value did improve on the departure from normality in the tails for the untransformed and the ln-transformed cases in Steps 1 and 2 (cf. Sections 3.4.1 and 3.4.2 respectively). The Shapiro-Wilk test for residuals in case of Figure 3-8 also indicates normality of the model error with a high *p*-value:

```
Shapiro-Wilk normality test

data: stdres
W = 0.9709, p-value = 0.7527
```

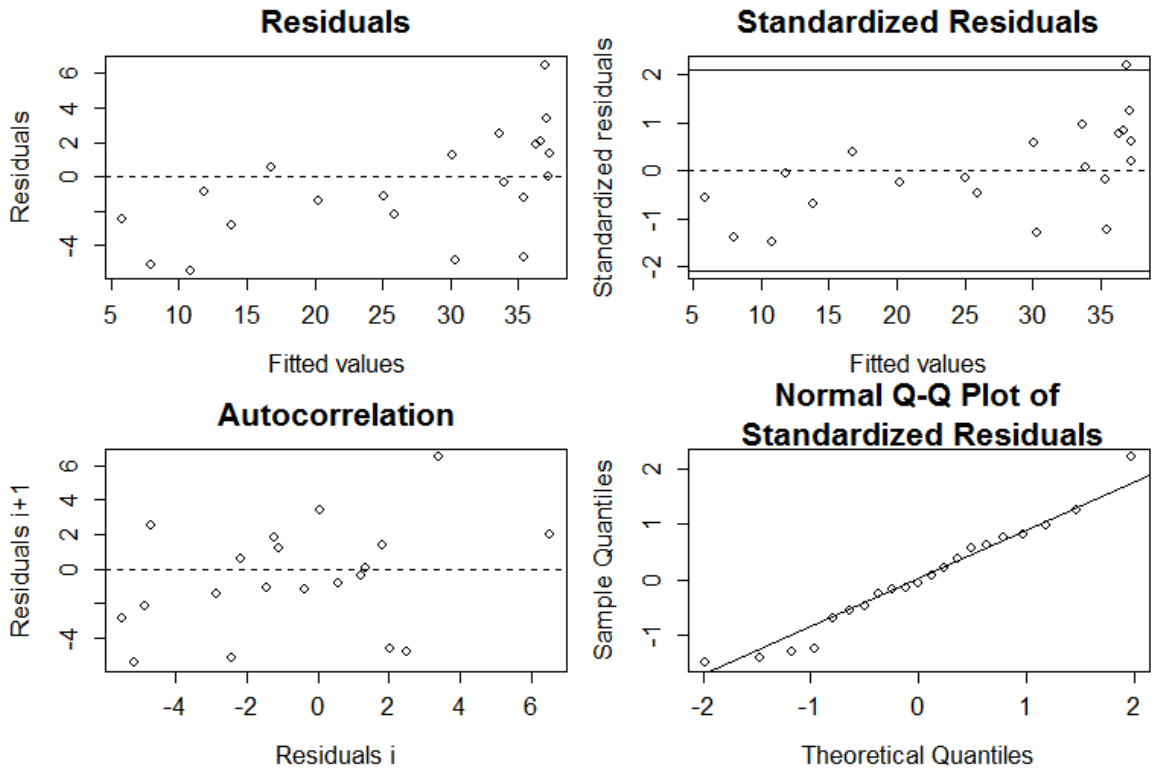


**Figure 3-8: Model diagnostic plots for the bioaccumulation model (Equation 313) in Figure 37 (*Example 1*), with transformation parameter λ = 0.3.**

### *3.4.4 Step 4: Decide on the appropriateness of the model and data transformations used in Steps 1, 2 and 3*

119. In this step, the results from the different runs are analysed. A decision is made on the most satisfactory model fit, based on visual inspection of the fit graphics, the four model diagnostic plots and the supporting statistical tests. It may be that one of the model fits from Step 1 or 2 (untransformed or ln-transformed) yields a good fit with errors that show a nearly normal distribution in the Q-Q plot and do not exhibit any trends in the residuals plots such as heteroscedasticity. Descriptors from that fit will then be used for further growth and lipid corrections (see Section 3.5 below).

120. In the example described above, the Step 1 analysis (see Section 3.4.1 above) indicates that data transformation may improve the fit. An ln-transformation seems a natural first choice, but this does not yield an improved fit of the model to the data. In these cases where measurement errors are both non-normally distributed and show variance heterogeneity (as in Figure 3-2, top panels), the Box-Cox transformation will be useful to find the optimum data transformation that will produce normally distributed errors with constant variance.

121. In the example described above, results from the Box-Cox transformed data are spaced between the previous two, as shown here as a function of the transformation parameter λ in Table 3-4. Note that λ = 1.0 corresponds to the untransformed fit, while λ = 0.0 denotes the ln-transformed fit. This example shows the considerable and relevant influence of the transformation on BCF estimation.

122. The combined result of the Box-Cox optimisation procedure, followed by graphical inspection of model diagnostics and statistical testing, provides confidence that in this case, a mild transformation improves the fit of the model to the data. This seems to be in line with many field observations that

indicate right-skewed distributions. As stated, rather than postulating the optimal transformation, this guidance encourages the user to find a suitable model fit and data transformation with the *R*-package that is made available, while following the steps outlined above.

**Table 3-4: $BCF_K$ estimates and uncertainty as a function of transformation parameter λ**

| λ | $BCF_K$ Estimate | Std. Error | 2.5% | 97.5% |
|---|---|---|---|---|
| 1.0 | 2529 | 180 | 2189 | 2982 |
| **0.3** | 2351 | 183 | 1993 | 2709 |
| 0.0 | 1882 | 264 | 1408 | 2548 |

## 3.5 $BCF_K$ estimation for growing fish

### *3.5.1 Mass-based modelling framework for exponential growth*

123. In order to take account of fish growth in the estimation of the $BCF_K$, a clear model definition is needed. This is done to make sure that the pseudo-elimination that is caused by fish growth, also known as 'growth dilution' is taken properly into account, and to estimate the growth rate parameter(s) with associated uncertainty. In Section 3.5.2, it will be discussed how this affects the estimation of overall $BCF_K$ uncertainty.

124. First, a mass-based dynamical system is defined for both fish wet weight, unit of kgW (kilograms of fish in wet weight), and chemical mass (X) in the fish, expressed in mgX. Then, the dynamics of internal chemical concentration in units of mgX/kgW are derived from these mass-oriented equations. This will work also when the mass-related equations are adapted to include growth in different ways (cf. Annex 3, A3.3).

125. With the method above, deriving the internal fish chemical concentration dynamics from equations of mass rate of change, with inclusion of exponential growth of the fish, is straightforward. More complicated growth models are discussed in Annex 3 (A3.3).

126. The dynamical system on the basis of mass rate of change becomes

$$\begin{cases} \dfrac{dX_{\text{fish}}}{dt} = \lambda_1 \cdot C_{\text{water}} \cdot W_{\text{fish}} - \lambda_2 \cdot X_{\text{fish}} \\ \dfrac{dW_{\text{fish}}}{dt} = \lambda_G \cdot W_{\text{fish}} \end{cases} \qquad \text{Equation 3-15}$$

Where $\frac{dX_{\text{fish}}}{dt}$: rate of change of fish chemical mass ($mgX \cdot d^{-1}$),
$X_{\text{fish}}(t)$: fish chemical mass over time (mgX),
$\frac{dW_{\text{fish}}}{dt}$: rate of change of fish biomass ($kgW \cdot d^{-1}$)
$W_{\text{fish}}(t)$: fish biomass over time ($kgW \cdot d^{-1}$),
$C_{\text{water}}$: exposure concentration constant ($mgX \cdot L^{-1}$),
$\lambda_1$: uptake rate constant ($L \cdot kgW^{-1} \cdot d^{-1}$),
$\lambda_2$: depuration rate constant ($d^{-1}$).
$\lambda_{G:}$ growth rate constant ($d^{-1}$)

The same constants from the basic BCF model (Equation 3-5) are used, but renamed as $\lambda_1$ and $\lambda_2$ to allow for their proper interpretation as being distinct from $k_1$ and $k_2$.

The second equation expresses the *exponential growth* of fish biomass with $\lambda_G$ the rate constant of fish growth ($d^{-1}$).

After some rearrangements (see Annex 3, A3.3) the dynamics for the fish chemical concentration $C_{fish}$ becomes:

$$\frac{dC_{fish}}{dt} = \lambda_1 \cdot C_{water} - (\lambda_2 + \lambda_G) \cdot C_{fish} \qquad \text{Equation 3-16}$$

The same *form* of the basic BCF equation results, but now overall loss rate includes *depuration rate plus growth rate*. Further information on fish growth and the assumptions made here can be found in Annex 3 (A3.3).

### *3.5.2 Example of Exponential Fish Growth in the BCF Equation*

127. The inclusion of exponential fish growth in the basic BCF equation is illustrated based on data from Crookes and Brooke ((36), Table 5.3, p.99), for exposure of rainbow trout to an unknown chemical.

128. Their estimated rate constants are used to obtain Figure 3-9: $k_1$ = 395 (L $kgW^{-1}$ $d^{-1}$), and $k_2$ = 0.0432 ($d^{-1}$) and $BCF_K$ = 9144.

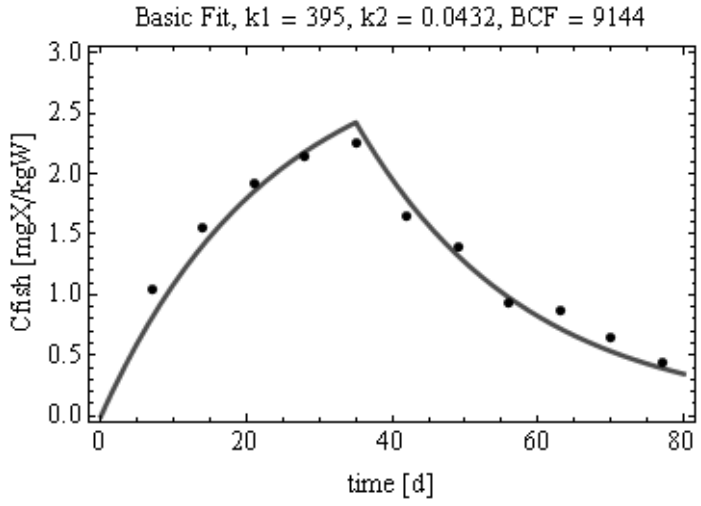


**Figure 3-9: Accumulation and depuration in fish, data from (36).**

129. Data plotted are mean chemical concentrations in fish. Figure 3-10 shows an exponential fit to the mean of fish weight data.

The growth rate constant (point estimate) is: $k_g$ = 0.0298 ($d^{-1}$). The growth corrected depuration constant ($k_{2g}$ in Annex 5 of OECD TG 305 (1)) can now be calculated. The growth constant $k_g$ relates to the overall depuration constant $k_2$ as:

$$k_2 = k_{2g} + k_g \qquad \text{Equation 3-17}$$

The depuration rate constant can now be corrected for growth to yield $k_{2g}$:

$k_{2g} = k_2 - k_g = 0.0432 - 0.0298 = 0.0134$ ($d^{-1}$).

Obviously, the growth corrected depuration rate constant $k_{2g}$ (0.0134) is substantially smaller than the 'overall depuration' rate constant (0.0432) including pseudo-elimination by growth.

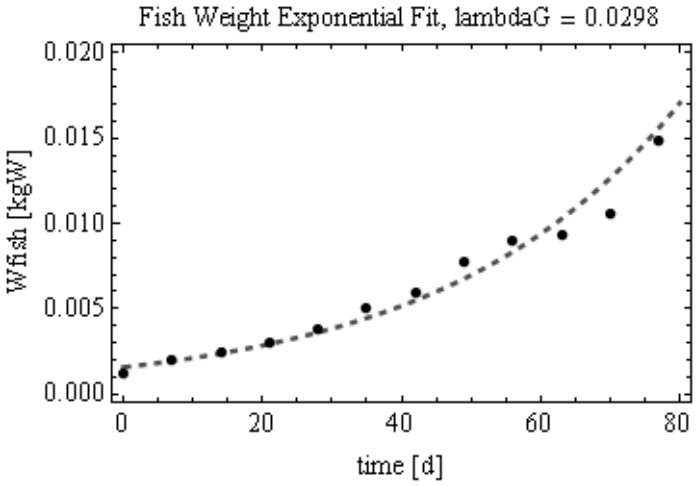


**Figure 3-10: Exponential growth fit, data from (36).**

The depuration rate constant can now be corrected for growth to yield $k_{2g}$:

$k_{2g} = k_2 - k_g = 0.0432 - 0.0298 = 0.0134$ ($d^{-1}$).

Obviously, the growth corrected depuration rate constant $k_{2g}$ (0.0134) is substantially smaller than the 'overall depuration' rate constant (0.0432) including pseudo-elimination by growth.

### *3.5.3 $BCF_K$ estimation, growth corrected and lipid normalised*

130. The standard BCF calculation can now be adapted for exponential growth of the fish. The kinetic BCF is now growth-corrected by using $k_{2g}$ instead of the overall depuration rate constant $k_2$.

$$BCF_{Kg} = \frac{k_1}{k_2 - k_g} \qquad (L \cdot kgW^{-1}) \qquad \text{Equation 3-18}$$

131. An additional complication is now that the uncertainty in estimating the growth rate constant influences the estimation of uncertainty in the uptake and depuration rate constants. Especially when substances are eliminated slowly, and growth rate dominates the overall depuration rate constant, special attention should be paid to avoid estimating negative "true" (growth corrected) depuration rate constants.

132. The estimated growth rate constant can now be inserted in the calculation of the $BCF_K$ and the ensuing growth correction. In this case, the growth rate $k_g$ was determined to be 0.0298.

133. Table 3-5 shows how this affects the estimate of the $BCF_K$ and its uncertainty for the case that was used in Section 3.5.2. The growth corrected $BCF_{Kg}$ in this case jumps from 9257 to 31415, with a standard error that strongly increases due to the additional uncertainty from the growth correction. It also illustrates that the growth correction has a large effect on $BCF_K$ estimates and its confidence interval, and is another reason why it is important to document the various $BCF_K$ estimates and their associated

confidence intervals. Furthermore, it illustrates that the use of slow growing fish in BCF testing can limit the uncertainty in $BCF_K$ estimates.

134. Correction of the BCF for differences in lipid content is described in OECD TG 305 (cf paragraph 67 and Annex 5 in (1)). Kinetic bioconcentration factors are expressed on a 5% lipid content, unless the test substance does not primarily accumulate in lipid (e.g. perfluorinated substances that may bind to proteins). If chemical and lipid analyses have been conducted on the same fish, this requires each individual measured concentration in the fish to be corrected for that fish's lipid content (see Equation A5-29 of OECD TG 305). This should be done *prior* to using the data to calculate the kinetic BCF. However in most cases, lipid analysis is not conducted on all sampled fish so this *a priori* correction for fish lipid content is not possible and a mean lipid value must be used to normalise the BCF.

The BCF is normalised according to the ratio between 5% and the actual (individual) mean lipid content[8] (in % wet weight) (cf. Annex 5 in (1)). In this example, the lipid correction is done for a growth corrected $BCF_{Kg}$:

$$BCF_{KgL} = \frac{0.05}{L_n} \cdot BCF_{Kg} \qquad \text{Equation 3-19}$$

Where $BCF_{KgL}$: lipid-normalised and growth corrected kinetic BCF (L $kg^{-1}$)
$L_n$: mean lipid fraction (based on wet weight); 13.76% for *Example 1* (Annex 5, A5.1, Table A – 6).
$BCF_{Kg}$: kinetic BCF, growth corrected (L $kg^{-1}$)

**Table 3-5: Parameter estimates and BCFK uncertainty from optimal Box-Cox fit to *Example 1* (Annex 5, A5.1, Table A – 6).**

| | Estimate | Std. Error | 2.5%[1)] | 97.5%[1)] |
|---|---|---|---|---|
| $k_1$ | 367.8 | 45.3 | 273.0 | 462.6 |
| $k_2$ | 0.1565 | 0.0194 | 0.1158 | 0.1971 |
| $k_{2g}$ | 0.119 | 0.0194 | 0.0811 | 0.157 |
| $BCF_K$ | 2351 | 183 | 1993 | 2709 |
| $BCF_{Kg}$ | 3087 | 301 | 2496 | 3677 |
| $BCF_{KgL}$ | 1122 | 109 | 907 | 1336 |

[1)] These *t*-based confidence intervals come from nlstools, and may slightly differ from previous confidence intervals.

## 3.6 Reporting on $BCF_K$ estimation

135. As indicated in OECD TG 305 (1) a list of substance uptake and depuration rate constants and bioconcentration factors (BCF) are required in the reporting of the results (see the list at the end of paragraph 81 of OECD TG 305). For those parameters related to the $BCF_K$ estimation the values will be derived from the procedure described here (see e.g. Table 3-5) and thus should be reported.

136. To provide transparency in the decisions on a certain transformation and model fit (and thus on values reported), the user is encouraged to report for each of the steps 1 to 3 (cf Sections 3.4.1 to 3.4.3) the estimates for $k_1$, $k_2$ and $BCF_K$, but also the model diagnostics plots, Shapiro-Wilk test results, and plots that

[8] If possible time-weighted average is recommended. This can be calculated in a similar way as a time-weighted mean fish weight (cf. Annex 4, A4.3)

show how the curves describe the data. For the Box-Cox transformation the transformation parameter λ and the plot of the log likelihood function for the Box-Cox parameter λ are relevant as well.

# 4 GENERAL GUIDANCE FOR 305-III: DIETARY EXPOSURE BIOACCUMULATION FISH TEST

137. The current OECD Test Guideline 305 (OECD TG 305 (1)) provides an alternative test design for a dietary bioaccumulation approach that estimates dietary biomagnification factors (BMFs). The alternative test design is recommended especially for bioaccumulation studies on superhydrophobic substances ($\log K_{OW} > 6$ with solubility below ~0.01 – 0.1 mg/L), where aqueous exposure may be technically impossible. This Chapter of the Guidance Document looks firstly at some practical issues around test selection and conduct: aqueous versus dietary exposure (Section 4.1), feed preparation (Section 4.2) and approaches to avoid test substance leaching from feed (Section 4.3). Secondly it looks at how "set" experimental parameters (feeding rate, fish size and age) can affect study results (Section 4.4), and finally it considers how data generated from a test should be treated (dealing with uncertainty; Section 4.5) and used (use of derived parameters from dietary studies, including for regulatory purposes; Section 4.6). This final section includes methods to estimate surrogate bioconcentration factors and a decision scheme for their use, illustrated with worked examples.

## 4.1 Further guidance on test selection: aqueous versus dietary exposure

138. OECD TG 305 states that there should be a preference for aqueous exposure bioaccumulation (i.e. bioconcentration) studies to be run when "technically feasible" (see (1), paragraph 10). This may involve using more advanced dosing systems involving stock solutions or passive dosing systems (*e.g.* column elution method; see Section 2.1.1).

139. This recommendation is made on a scientific as well as a pragmatic basis. It is commonly accepted that water-borne exposure will dominate over dietary exposure for many less hydrophobic test substances in the aquatic environment, and so it is more relevant that these substances should be tested using the aqueous exposure method. Where water-borne exposure becomes less important than exposure via the oral route, OECD TG 305 offers the option of dietary exposure in the laboratory as a surrogate for the real world situation. The goal is to use the appropriate test method, i.e. testing the exposure route that would make the greatest contribution to body burdens in the real world.

140. However, in OECD and non-OECD countries there is a lot of experience using the aqueous exposure method and more laboratories are equipped to run this study than the newer and less commonly-used dietary method. In addition, the bioconcentration factor seems to be the most widely used measure of aquatic bioaccumulation potential in regulatory regimes in OECD countries. This is not a discouragement for uptake of the new method, but simply a reflection of the current situation where the aqueous exposure method is much longer established as a standardised method.

141. OECD TG 305 does not give fixed numerical criteria for when the dietary study should be used (see paragraphs 5, 7 and 11 of (1)). OECD TG 305 (paragraph 7) recognises that "*information on water solubility is not definitive for these hydrophobic types of chemicals*", and, importantly, goes on to say "*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.*"

142. Investigations of water solubility and the preparation of viable test solutions are made when it is clear that aqueous exposure is the appropriate exposure regime for a particular chemical. The same investigations should also be made before aquatic testing can be discounted for more hydrophobic substances where test choice is less obvious. Such investigations should be formally documented as part of, or preparatory to, the bioaccumulation study report itself. Consultation with the relevant regulatory authority for whom the study is being conducted should also be carried out prior to commencing any testing.

143. Available information and preliminary experiments should be explored sequentially. Guidance similar to that which follows is likely to be common practice in many testing laboratories, but it is given here so that it is formally available.

144. When deciding which test to run, the first step should be an assessment of the available water solubility information. A study according to OECD TG 105 (37) or equivalent should be available; however, for hydrophobic chemicals this may only report a less than value (e.g. "< 1 mg/L") and so its usefulness may be limited. Several quantitative structure-property relationship (QSPR) programs are available to estimate water solubility and should be explored. Next, any available aquatic toxicity or aquatic environmental fate data for the substance should be reviewed to check if results were reported on a measured concentration basis; if so, information on analysis of test solutions during the course of a study may prove useful, especially considering the dosing technique employed in toxicity tests (semi-static or flow-through). In addition, information on exposure techniques (e.g. if a passive dosing system was used in any chronic testing) will be useful. It may be that for highly hydrophobic substances aquatic toxicity testing was conducted using the water-accommodated fraction technique (see (2)). If analytical monitoring was not carried out in these tests, this can only tell us that normal dosing techniques may not have been possible, indicating poor solubility or other confounding factors (adsorption to apparatus, etc.).

145. The next step should be preliminary aquatic dosing experiments in an aquarium that would be used for the bioaccumulation test, but with no test animals present and over a much shorter timescale than typically used in a bioaccumulation study. Special methods of dosing should be considered, for example solid phase desorption dosing systems as described in Section 2.3 and other methods discussed in the OECD Guidance Document 23 on aquatic toxicity testing of difficult substances and mixtures (2). Such methods also make it possible to avoid the use of solvents and dispersions (as is discouraged in OECD TG 305 (1) in the preparation of stock solutions) as well as increasing the ratio of dissolved to total concentrations.

146. At this stage work on sample extraction/preparation from water and a specific analytical technique for the test substance would be required (unless using radiolabelled test substance), if not already investigated. Methods for both water and fish tissue will be required (if a dietary study is carried out, methods for food and fish tissue is required). These should ideally have similar levels of quantification in order to prevent a situation where the method in water performs far better than that in tissue (otherwise fish tissue concentrations in depuration may be below limit of detection, and impact the $k_2$ estimation). Alternative analytical methods such as SPME should be considered (see Section 2.3), especially since they are able to better distinguish between truly dissolved (or bioavailable) and total concentrations in water, which is highly relevant for very hydrophobic test substances.

147. The results of these investigations will be largely dependent on the substance's characteristics, the test laboratory's capabilities and the sensitivity of the analytical technique. If investigations

demonstrate no measurable dissolved concentrations (with an analytical technique that is deemed of suitable sensitivity) or high variability in measured concentrations, then this would form the basis of a proposal to run the dietary study. It is suggested that the selected exposure scenario, especially the dietary exposure, is discussed by the commissioner and the relevant regulatory authority before the actual test is started.

## 4.2 Further guidance on feed preparation in fish dietary bioaccumulation studies

### *4.2.1 Experimental diet and concentrations*

148. Suitable feed constituents and pellet size are described in OECD TG 305 (1) (paragraph 117). For pellet size, diameters are indicated as "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 at the start of the test". Furthermore, "pellet size may be adjusted depending on fish growth in the depuration phase". The nutritional value of the food should be taken into account when setting a study's feeding rate (see also Section 4.4 below with regard to feeding rates). In Annex 7 to OECD TG 305, the table "*Example of constituent quantities of a suitable commercial fish food*" cites a raw fat content of ≤ 15%, but notes that in some regions such food may not be easily available. Where only fish food with a lipid concentration very much lower than this upper limit can be obtained, a study should be run with the lower lipid concentration food, the feeding rate adjusted appropriately to maintain fish health, without artificially increasing lipid content by adding excess oil. This means that if a study is being run with a diet low in lipid, then a higher food ration is likely to be needed (i.e. higher than the recommendation in paragraph 181 below). See also further discussion of this in OECD TG 305 (paragraph 117 and Annex 7 in (1)).

149. In such cases it may prove necessary to spread the daily ration over two feeds (as mentioned in OECD TG 305). For the dietary study, spiking the feed using the corn or fish oil technique should also be considered (see Section 4.2.2.2), since the low lipid content of such feeds may have a deleterious effect on bioavailability of the test substance. Note that this spiking technique necessitates the addition of oil to the diet, as opposed to addition with the intent of artificially increasing the food's lipid content.[9]

150. Less detailed information is given on suitable concentrations of test substance in food in OECD TG 305. Only guiding factors are given, including sensitivity of the analytical technique, palatability, relevant environmental concentrations, the substance's no observable effect concentration (NOEC), if known, and potential accumulative effects. OECD TG 305 (1) further cites a workable concentration range of 1 – 100 µg/g feed for hexachlorobenzene and of 1 – 1000 µg/g feed for test substances known not to have specific toxic mechanisms. In practice concentrations near the higher end of this range should be avoided if possible, because such concentrations represent what would be considered a maximum dose in mammalian toxicity testing, and for some substances such high concentrations may lead to difficulties in achieving sufficient homogeneity and bioavailability in the feed.

151. As is the case for the aqueous exposure test, the dietary study can be used to test more than one concentration of the test substance. Limited information is available on the potential for bioaccumulation concentration dependence via the dietary route in the laboratory, however if it is assumed that *in vivo*

[9] New, as yet unpublished, research in Japan suggests that adding lipid to fish food to raise the lipid level does not affect the BMF measured for hexachlorobenzene. Similar to food spiking (paragraph 154, 156 and 163), the assessor should be satisfied that food pellet integrity, and the homogeneity and bioavailability of their test chemical in the food is not compromised by adding additional lipid. Prior to initiating the test with fish, food can be tested without animals to check its integrity.

distribution following dietary uptake is to all intents and purposes the same as for uptake via the gill, the same processes will govern whether concentration dependence occurs.

152. As is the case for aqueous exposure, concentrations should differ ideally by a factor of ten but this factor can be smaller if necessary based on the sensitivity of the analytical technique (that limits the lower concentration), the substance's (known) chronic toxicity and palatability issues (that limit the higher concentration). Use of radiolabelled test substance (of the highest purity, *e.g.* preferably > 98%) should also be considered.

153. Use of a minimised design analogous with that described in OECD TG 305 for the aqueous method has been investigated by Springer (38). Initial investigations show that such a design may not be as robust when applied to the dietary study as for the aqueous study, especially if results are being used to estimate BCF values. This is because widths of confidence intervals in the minimised design dietary BMF are similar to the discriminatory range for whether a substance would be bioaccumulating or not (further details available in (38)).

### *4.2.2 Preparation of test feed*

#### *4.2.2.1 Solvent spiking*

154. Food can be spiked using a suitable organic solvent, but it is very important to ensure that homogeneity and bioavailability are not compromised. The most convenient way of solvent-spiking of the test feed is to dissolve the test chemical in an appropriate volatile solvent, mix the solution with the feed pellets and leave this mixture under a fume hood to let the solvent evaporate to visual dryness of the feed. With this approach, it is key to confirm that the food pellet matrix is not changed by the solvent, for instance through a loss of lipid or reduced stability. An improved process of solvent spiking feeding pellets can be performed using spray spiking as described by Goeritz et al. (39). This uses a vacuum spiking apparatus for spray application of a solvent/test substance mixture onto the surface of the food pellets using only a small volume of solvent. Please refer to (39) for a full protocol.

#### *4.2.2.2 Spiking with enriched oil*

155. If the substance is soluble and stable in triglycerides, the chemical can be dissolved in a small amount of fish oil or edible vegetable oil before mixing with fish food. 50 g of spiked feed pellets thoroughly mixed with 0.5 g of oil and incubated overnight on a shaking table provide an evenly distributed thin oil film on the pellets (1% oil coating)[10]. Generally, the amount of oil used as an adhesive should be kept as low as possible taking into account the natural lipid content of the spiked feed. In some experiments, coating with 3–4% of corn oil clearly affected the consistency of the spiked feed pellets, leading to a dripping oily surface of the pellets and to a rapidly formed oil film on the water surface when transferred to the fish tank. However, feed with lower levels of lipid and variations on the way in which the oil is added to the food (i.e. mechanical mixing) may negate this problem.

156. The spiked feed should be analysed to prove that a homogeneous distribution of the test item(s) was obtained confirming the suitability of the spiking procedure.

[10] Mörck et al. (40) assessed the solubility of decabromodiphenyl ether (DecaBDE) in different vehicles as part of preparation for oral gavage testing. This is considered equally relevant for the dietary food preparation. The authors investigated three different dosing vehicles: dimethyl sulphoxide:peanut oil (50:50 mixture), anisole/peanut oil (30:70 mixture) and a solution of soya phospholipone:Lutrol (16:34 w/w) in water (concentration 0.11 g/L). The mixture where the highest solubility of DecaBDE was obtained (in this instance soya phospholipone:Lutrol) was used for the mammalian testing.

### *4.2.3 Further guidance on fish size and age*

157. Intraspecies differences in fish size and age can have an effect on the results of a bioaccumulation study, because the relative surface area of the gills to the body size/weight in larger (older) fish may be lower than for smaller fish, and because smaller (younger) fish often have higher ventilation rates (41) (42). This can affect depuration rates. Smaller fish will also achieve a maximal body burden in a test more rapidly.

158. In BCF studies this effect may largely cancel out, because the same factors that will influence the rate of depuration by respiration will dictate the rate of uptake. In the case of the dietary study, higher rates of loss at the gill may be associated with smaller fish, so the overall depuration rate constant would be higher leaving aside other potential differences (metabolism and faecal egestion). It is not clear how uptake across the gut varies with size and age, and so this leads to the possibility that tests with small fish may result in a lower BMF than would be the case with larger fish of the same species (again, leaving aside potential differences in metabolic capacity between smaller (younger) and larger (older) fish). However, in practice the effect in larger fish may to some extent be circumvented by any increased capacity for metabolism compared with smaller, juvenile fish with gastro-intestinal tracts and livers that may not be fully developed with respect to biotransformation pathways. This latter point is also relevant for the aqueous study.

159. Therefore the dietary study should be run with fish towards the upper end of the ranges given in Annex 3 of OECD TG 305 (1), if possible. It is especially important that the size distribution is as tight as possible, preferably well within the OECD TG 305 recommendation that *the smallest fish is no smaller than two-thirds the weight of the largest*. This helps to ensure a similar level of uptake of the offered diet in a study (less competition in feeding) and also means that the calculated feeding rate is more applicable for individuals in the test. Using larger fish may also help with chemical analysis, as more tissue per individual will be available for analysis. This avoids the alternative of using (more) smaller fish, which has animal welfare implications due to increased fish numbers, as well as the need for analysis of pooled samples.

## 4.3 Accounting for leaching

160. The dietary bioaccumulation test may also be more feasible than a bioconcentration test with aqueous exposure for surfactants (1). Surfactants are surface acting agents that lower the interfacial tension between two liquids. Due to their amphiphilic nature, significant leaching of the test substances from the surface of spiked experimental diets might occur. As for dietary studies with superhydrophobic substances, a standard fish food should be used and spiked. However, with regard to the physical characteristics and solubility of water-soluble substances such as surfactants, no specific measures to prevent leaching of test substance into the water during the feeding studies are described in OECD TG 305 (1). Depending on the extent of the loss, leaching might lead to a significant uptake of the test substance by the test fish from the surrounding water and thus lead to inaccurate dietary BMF estimates. Therefore, the availability of sufficiently stable test diets is required for dietary studies with more water-soluble test compounds.

161. For dietary studies with highly hydrophobic test substances (i.e. with log $K_{OW}$ > 5) leaching losses after application of the spiked feed pellets should be of minor relevance due to the high adsorption of the test substance to organic matter and/or very low fugacity. However, feeding should still be observed to ensure that the fish are visibly consuming all of the food presented. The time until the applied feed ration is completely ingested by the experimental animals should be kept as short as possible. For more water-soluble test substances the stability of the spiked feed in water needs to be tested to avoid significant leaching losses prior to ingestion.

162. There is no standard method to determine the stability of spiked feed in water. In principle, a certain amount of feed needs to be incubated in a beaker of water and allowed to sit for a variable length of time with occasional shaking. Timing should be relevant to timing in the experiment. Leaching losses are measured in water samples taken from the test vessel after defined time intervals and expressed as a percentage of the test item concentration of the immersed feed sample. For instance, Erlenmeyer flasks (200 mL) can be filled with 100 mL of water (room temperature) and placed on a horizontal laboratory shaker. 1 g of spiked feed is then added to each flask that is gently shaken (75 rpm) imitating the water movement that would be generated by fish movement and by the flow-through system in real study conditions. After 0.25, 0.5, 1, 2, 5 minutes, one flask is sampled and the content of the flask is filtered through a paper filter to separate the feed from the water sample, which is collected in a glass vessel. The concentration of the test item in the filtered water is then measured and leaching losses are calculated for each sampling point. In addition the concentration of the test item in the filtered fish feed could be determined to enable calculation of a mass balance. OECD TG 305 defines no threshold (%) with respect to acceptable leaching losses. However, losses higher than 10% prior to feed uptake should certainly be avoided to ensure an accurate estimation of study results. If higher losses cannot be avoided an increased frequency of feeding times (with lower amounts of food) should be considered. Alternatively, the stabilisation of spray-spiked pellets by surface-coating could be considered. Different coating materials such as edible vegetable oil and fish oil as well as settable gel material such as sodium alginate are described in the literature and have shown a high potential to reduce leaching losses from larger (4 mm) pellets (39). However, it is important to notice that selection of an inappropriate coating agent can severely affect the bioavailability of the test item, the nutritional composition of the feed and may present palatability problems (43). In addition to that, coating of spray-spiked feed as used in dietary accumulation studies (small particles, 0.8 mm) may lead to clumping of the pellets. Therefore, coating of spray-spiked pellets should be carefully considered; alternatively an aqueous study may be conducted rather than a dietary study in case of high leaching losses.

### 4.4 The effect of varying study parameters on feeding rate, biology, calculations and study results

163. Since the ration given to the fish during the uptake phase is not adjusted, fish growth during the uptake phase can affect the true feeding rate. This is described in OECD TG 305 (see paragraph 160 and part 3 of annex 7 to OECD TG 305 (1); see also (44), pp 84), and has an impact on the calculated absorption efficiency α (i.e. α may be underestimated). However, it is only important if α is being used itself, e.g. to compare absorption efficiencies between studies, because the feeding rate terms cancel in the calculation of dietary BMF, as is shown below.

$$\alpha = \left(\frac{C_0 \cdot k_2}{I \cdot C_{\text{food}}}\right) \cdot \left(\frac{1}{1 - e^{-k_2 \cdot t}}\right) \qquad \text{Equation 4-1}$$

$$\text{BMF}_g = \frac{I \cdot \alpha}{k_{2g}} \qquad \text{Equation 4-2}$$

where: $C_0$: derived concentration in fish at time zero of the depuration phase (mg $kg^{-1}$),
$k_2$: overall (not growth-corrected) depuration rate constant ($day^{-1}$),
$I$: 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),

$BMF_g$: growth-corrected dietary biomagnification factor
$\alpha$: assimilation efficiency (absorption of test substance across the gut),
$k_{2g}$: growth-corrected depuration rate constant ($day^{-1}$)

Combining and rearranging these equations shows more clearly that the *I* terms cancel, as shown in a recent report (38) and explored further in Section 4.5:

$$BMF_g = \left(\frac{C_0}{C_{food}}\right) BMF_g = \left(\frac{C_0}{C_{food}}\right) \cdot \left(\frac{k_2}{k_{2g}}\right) \cdot \left(\frac{1}{1 - e^{-k_2 \cdot t}}\right) \qquad \text{Equation 4-3}$$

164. Equation 4-3 can be used to calculate BMF in one step from study results (see Section 4.5). However, α should be calculated using Equation 4-1, because it is an important parameter in its own right and provides an easy way of checking that the study conduct and its measurements are as expected (i.e. absorption is not negligible and α is ≤ 1). αα is also needed as part of the BCF estimation (see Section 4.6.3).

165. The main focus of this section is on how different set feeding rates (*I*) and resulting differences in fish biology (growth and lipid content), especially during the depuration phase, can affect the study parameters that are calculated in a dietary bioaccumulation study. The affected parameters are clearly interlinked and so this description tries to cover the effects holistically.

166. In summary, increased feeding rate may cause:

- a potential (marginal) decrease in absorption efficiency (α);
- increasing fish lipid during the depuration phase that in turn results in a decrease in calculated depuration rate constants ($k_2$ and $k_{2g}$) for hydrophobic substances that partition into fish lipids;
- a potentially higher calculated dietary BMF (considering the relationship between fish lipid and $k_2$ or $k_{2g}$ and the mathematical relationship between feeding rate, $k_2$ and BMF).

167. In the ring test report produced for OECD TG 305 (44), results from two studies run at a lower feeding rate (1.5%) were compared with those of the higher feeding rate (3%) that was used in the "main" ring test studies.

168. Table 4-1 adapted from (45), shows the mean growth- and lipid-corrected BMFs ($BMF_{gL}$), absorption efficiencies and growth corrected depuration rate constants ($k_{2g}$) for the two studies at the lower feeding rate versus the mean values for seven studies at the higher feeding rate, all reported with standard deviations.

169. On first inspection all of the absorption efficiencies at the higher feeding rate are lower than those at the lower feeding rate (except for methoxychlor), but analysis of these data (*t*-test with alpha = 0.05) does not indicate a statistical difference between each set of α values. Given the small sample size and potential natural variability in results, it is difficult to draw a conclusion from these data. In addition, conflicting evidence exists (see (46)). Note that there does not seem to be a relationship between concentration in the food and absorption efficiency (see (47) (48)).

170. It has been postulated that increased loss of substance via faecal egestion operates at higher feeding rates (49), so the apparent effect on absorption efficiency may in fact be due to increased faecal egestion, as is suggested by fugacity relationships between fish and food that have been developed (50).

The outcome of this preliminary analysis is that ideally feeding rates should be comparable between studies to ensure a meaningful comparison of resulting α values. The potential effect of fish growth on the actual feeding rate and its effect on α should be taken into account, as discussed above (paragraph 163).

171. Table 4-1 below also highlights another apparent effect of differing feeding rates, this time on growth-corrected depuration rate constants. It can be seen that $k_{2g}$ values for the lower feeding rate are roughly half of those for the higher feeding rate. Differences between paired values are all statistically significant at the $p = 0.05$ level (*t*-test). To put this in context, 172. As Table 4-2 shows, lipid contents on average were roughly half for the lower feeding rate studies, as were growth rate constants, compared with the higher feeding rate studies. This suggests an inverse relationship between lipid content and growth-corrected depuration rate constant (see (45) and (51) for further discussion) and demonstrates the importance of correcting results for lipid content for hydrophobic substances that partition into fish lipids.

Table 4-2 below shows the mean lipid contents and growth rate constants for the studies.

**Table 4-1: Comparison of mean calculated growth- and lipid-corrected BMF, absorption efficiencies and growth corrected depuration rate constants between OECD TG305 ring test studies run with a feeding rate of 1.5% and 3%.**

| | Mean $BMF_{gL}$ [1] | | | | Absorption efficiency (α) | | | | $k_{2g}$ ($d^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Chemical** | ***1.5% feeding rate*** [2] | ***rel. SD*** [3] | ***3% feeding rate*** [4] | ***rel. SD*** [3] | ***1.5% feeding rate*** [2] | ***rel. SD*** [3] | ***3% feeding rate*** [4] | ***rel. SD*** [3] | ***1.5% feeding rate*** [2] | ***SD*** [3] | ***3% feeding rate*** [4] | ***SD*** [3] |
| Hexachlorobenzene | 1.47 | 12% | 2.66 | 33% | 0.83 | 30% | 0.6 | 28% | 0.035 | 0.005 | 0.016 | 0.006 |
| Musk xylene | 0.43 | 16% | 0.67 | 40% | 0.62 | 48% | 0.51 | 47% | 0.089 | 0.001 | 0.052 | 0.016 |
| *o*-Terphenyl | 0.38 | 19% | 0.44 | 25% | 0.6 | 28% | 0.38 | 29% | 0.099 | 0.014 | 0.058 | 0.019 |
| Methoxychlor | 0.022 | [5] | 0.14 | 71% | 0.05 | [5] | 0.2 | 100% | n/a [6] | n/a [6] | n/a [6] | n/a [6] |

[1] lipid normalised according to depuration phase data only
[2] 1.5% feeding rate data comprises two labs
[3] SD = standard deviation
[4] 3% feeding rate comprises 7 labs
[5] single value only
[6] n/a: no meaningful comparison could be made for methoxychlor

172. As Table 4-2 shows, lipid contents on average were roughly half for the lower feeding rate studies, as were growth rate constants, compared with the higher feeding rate studies. This suggests an inverse relationship between lipid content and growth-corrected depuration rate constant (see (45) and (51) for further discussion) and demonstrates the importance of correcting results for lipid content for hydrophobic substances that partition into fish lipids.

**Table 4-2: Mean lipid contents and growth rate constants for OECD TG305 ring test studies at lower and higher feeding rates.**

| | **1.5% feeding rate (2 Laboratories)** | **3% feeding rate (7 Laboratories)** |
|---|---|---|
| Mean $k_g$ ($d^{-1}$) | 0.019 ± 0.002 | 0.036 ± 0.01 |
| Mean lipid (%) | 3.12 ± 1.73 | 6.03 ± 1.86 |

173. In the calculation of dietary BMFs (and analogously the BCF) this effect cancels in the calculation, since depuration as well as uptake operates during the uptake phase. In the dietary study, absorption efficiency is derived mathematically from the uptake rate in food ($k_f$) in the same way as the uptake rate constant in the aqueous exposure study. This is because both result from rearrangements of Equation 3-1, where depending on the test exposure method, either $k_1 \cdot\cdot C_{water}$ is zero or alternatively $k_f \cdot\cdot C_{food}$ is zero. Therefore $k_f$ is obtained by a rearrangement of Equation 3-4. Further explanation and derivation is included in Section 4.5.

$$k_1 = \frac{C_{\text{fish}} \cdot k_2}{C_{\text{food}}(1 - e^{-k_2 \cdot t})}$$ Equation 4-4

$$\alpha = \frac{C_0 \cdot k_2}{I \cdot C_{\text{food}}} \cdot \frac{1}{1 - e^{-k_2 \cdot t}}$$ Equation 4-5

If $k_1 = I \cdot \alpha$ (cf. Equation 3-2), then

$$k_1 = \frac{C_{0,\text{d}} \cdot k_2}{C_{\text{food}}(1 - e^{-k_2 \cdot t})}$$ Equation 4-6

174. In OECD TG 305 (1) Annex 5 (General Calculations) and Annex 7 (Equation section for Dietary Exposure Test) lay out how to correct BCFs and BMFs for fish lipid content in studies where fish lipids changed markedly during the depuration phase. In such cases, Annex 7 recommends that the mean (test) fish lipid concentration calculated from the measured values at the end of exposure and end of depuration phases is used.

175. Although dietary BMFs (and analogously BCFs) should be relatively unaffected, differences in $k_2$ and $k_{2g}$ between studies because of differing lipid contents are relevant when depuration rate constants are being compared between different studies, or a depuration rate constant is being used to estimate a BCF directly from dietary study data or as a direct measure of bioaccumulation potential.

176. In some studies lipid contents can change markedly during the course of a study. When this happens, the depuration rate constant is not "fixed", and may vary throughout the phases. This change in lipid content, and how to account for it in the derivation of depuration rate constants, has been explored by Brooke and Crookes (51). A correction for this effect based on the work of Brooke and Crookes (51) is described in Annex 4 to this guidance.

177. Table 4-1 above also highlights a potential relationship between feeding rate and BMF. BMFs in the studies run at the lower feeding rate appear lower than those at the higher feeding rate, especially for hexachlorobenzene. As was the case with the comparison of absorption efficiencies discussed above, none of the paired differences in BMF in On first inspection all of the absorption efficiencies at the higher feeding rate are lower than those at the lower feeding rate (except for methoxychlor), but analysis of these data (*t*-test with alpha = 0.05) does not indicate a statistical difference between each set of α values. Given the small sample size and potential natural variability in results, it is difficult to draw a conclusion from these data. In addition, conflicting evidence exists (see (46)). Note that there does not seem to be a relationship between concentration in the food and absorption efficiency (see (47) (48)).

170. It has been postulated that increased loss of substance via faecal egestion operates at higher feeding rates (49), so the apparent effect on absorption efficiency may in fact be due to increased faecal egestion, as is suggested by fugacity relationships between fish and food that have been developed (50). The outcome of this preliminary analysis is that ideally feeding rates should be comparable between studies to ensure a meaningful comparison of resulting α values. The potential effect of fish growth on the actual feeding rate and its effect on α should be taken into account, as discussed above (paragraph 163).

171. Table 4-1 below also highlights another apparent effect of differing feeding rates, this time on growth-corrected depuration rate constants. It can be seen that $k_{2g}$ values for the lower feeding rate are

roughly half of those for the higher feeding rate. Differences between paired values are all statistically significant at the $p = 0.05$ level (*t*-test). To put this in context, 172. As Table 4-2 shows, lipid contents on average were roughly half for the lower feeding rate studies, as were growth rate constants, compared with the higher feeding rate studies. This suggests an inverse relationship between lipid content and growth-corrected depuration rate constant (see (45) and (51) for further discussion) and demonstrates the importance of correcting results for lipid content for hydrophobic substances that partition into fish lipids.

Table 4-2 below shows the mean lipid contents and growth rate constants for the studies.

Table 4-1 are statistically significant (*t*-test with alpha = 0.05).

178. Mathematically a proportional relationship between feeding rate and dietary BMF is suggested (see Equation 4-3 above). However Equation 4-5, used to derive absorption efficiency, must also be considered since feeding rate effectively "cancels" between these two equations; it is likely that the reasons for any apparent relationship between *I* and BMF are not straightforward, since changes in feeding rate will affect other study parameters as described above, and all of these parameters are inter-related. Co-variance analysis in the OECD TG 305 ring test report (45) showed few statistically significant correlations, but it is possible that some correlations were simply "lost in the noise" of natural and uncontrolled variation between studies. Although no published study has been carried out that looks specifically at quantifying natural variation in results between similar dietary studies to date, the analysis of Hashizume et al. (52) on the potential variation in minimized design BCF studies in relation to definitive BCF studies may give useful context (see Section 4.6.3.1).

179. Given this uncertainty, it may be prudent to recommend a smaller range for acceptable feeding rates than is described in OECD TG 305 (1). This guidance is an interim measure while further experience is gained with this new method, until such time that enough data has been gathered to allow firmer conclusions on the factors discussed above and enable a revision to OECD TG 305 (and this guidance) to be made, if necessary.

180. OECD TG 305 (1) states (in paragraphs 45, 126 and 140) that "...the feeding rate should be selected such that fast growth and large increase of lipid content are avoided", and gives a range of "1 – 2% of body weight per day" as an example for rainbow trout. This wording recognises that studies are run with different species, at different temperatures and almost always with juvenile fish, so some change (increase) in lipid content will naturally occur when a food ration sufficient to maintain health and natural growth is provided. The reverse situation, where fish lipid content decreases over the course of a study because too low a food ration has been offered, is not acceptable on animal welfare grounds.

181. Overall, it is recommended that studies are run with a feeding rate at or near the upper boundary of the range given in OECD TG 305 (i.e. 2% of body weight per day (1)), unless a robust argument can be presented for a different value (a possible exception is described in paragraph 148).

## 4.5 Fitting BMF Models

### *4.5.1 Equations*

182. Models describing the dietary exposure method of fish chemical accumulation are very similar to those expressing the aqueous exposure test (see Section 3.2). However, the estimation methods differ, as in the dietary exposure test the accumulation in the uptake phase is usually less monitored, or completely neglected. Contrary to the aqueous exposure model, the depuration phase is therefore taken to start at $t = 0$.

183. The system for the rate of change of chemical concentration in the fish is given by the differential equations (Equation 4-7 reproduces Equation 3-4 for ease of reference):

$$\frac{dC_{\text{fish}}}{dt}(t) = k_{\text{f}} \cdot C_{\text{food}} - k_2 \cdot C_{\text{fish}}(t) \qquad \text{for } -t_{\text{f}} \le t \le 0 \qquad \text{Equation 4-7}$$

$$\frac{dC_{\text{fish}}}{dt}(t) = -k_2 \cdot C_{\text{fish}}(t) \qquad \text{for } t \ge 0 \qquad \text{Equation 4-8}$$

Where $\frac{dC_{\text{fish}}}{dt}(t)$: rate of change of whole-body fish chemical concentration ($\text{mgX}\cdot\text{kgW}_{\text{fish}}^{-1}\cdot\text{d}^{-1}$), where X is the test chemical,
$k_{\text{f}}$: uptake rate constant from food ($\text{kgW}_{\text{food}}\cdot\text{kgW}_{\text{fish}}^{-1}\cdot\text{d}^{-1}$),
$C_{\text{food}}$: food concentration ($\text{mgX}\cdot\text{kgW}_{\text{food}}^{-1}$),
$k_2$: depuration rate constant ($\text{d}^{-1}$),
$C_{\text{fish}}(t)$: whole-body fish chemical concentration over time ($\text{mgX}\cdot\text{kgW}_{\text{fish}}^{-1}$),
$t_{\text{f}}$: feeding period (d).

The first equation describes the uptake phase, with simultaneous depuration taking place; the second equation models the depuration phase with only depuration occurring.

If fish growth occurs during the test, it is considered exponential during both uptake and depuration phases as has become standard in the OECD TG 305 (1) data treatment for studies with juvenile fish (see Section 3.5.1). For growing fish, the depuration rate constant ($k_2$) is corrected for growth dilution ($k_{\text{g}}$), as per Equation 3-17:

$$k_{2\text{g}} = k_2 - k_{\text{g}} \qquad \text{Equation 4-9}$$

which explains the interpretation of the elimination rate constant as a *growth-corrected depuration* rate constant.

184. It is assumed that uptake from contaminated food proceeds with constant uptake rate and concentration in the food for a constant feeding rate and with exponential growth.

The *growth-corrected* kinetic BMF is now:

$$\text{BMF}_{\text{Kg}} = \text{BMF}_{\text{K}} \cdot \frac{k_2}{k_2 - k_{\text{g}}} \qquad \text{Equation 4-10}$$

185. When substituting the above equation for the kinetic BMF, uncorrected for growth, the growth-corrected kinetic BMF can be expressed in quantities readily estimated from the depuration phase (as also shown in Section 4.4 in the combined Equation 4-3):

$$\text{BMF}_{\text{Kg}} = \frac{C_{\text{fish}}(0)}{C_{\text{food}}} \cdot \frac{k_2}{k_2 - k_{\text{g}}} \cdot \left(\frac{1}{1 - e^{-k_2 \cdot t_{\text{f}}}}\right) \qquad \text{Equation 4-11}$$

186. The next section deals with this estimation process and the quantification of uncertainty from regression procedures.

187. Note again that the food ingestion rate constant $I$ ($kgW_{food} \cdot kgW_{fish}^{-1} \cdot d^{-1}$) is not necessary in the equations. It has the same units as $k_f$, the uptake rate constant. It can be shown (38) that $k_f$ suffices in the calculations, although the absorption efficiency coefficient, α, is a useful parameter and should be estimated (see Section 4.4). As shown in Equation 3-2, the absorption efficiency coefficient $\alpha$ is defined as

$$\alpha = \frac{k_f}{I} \qquad \text{Equation 4-12}$$

If $I$ is given, an estimate of $k_f$ can be used to estimate $\alpha$ as an alternative to using Equation 4-1.

### *4.5.2 Estimating parameters from the depuration phase*

188. Similar to what was presented earlier for the BCF (Chapter 3), it is proposed to analyse the bioaccumulation model in the depuration phase (Equation 4-8) in a stepwise fashion as detailed below:

1. Fit the model to the data[11], with ln model and data transformation, using a set of model diagnostics.
2. Fit the model to the data without transformation, using a set of model diagnostics.
3. Find an optimum data transformation using the Box-Cox optimisation procedure.
4. Decide on the appropriateness of the model and data transformations used in steps 1, 2 and 3. Provide justification (based on model diagnostics and graphical plots) for the preferred data transformation used to estimate the dietary BMF (and underlying parameters).
5. Correct the dietary BMF for fish and food lipid contents (ratio of fish to food lipid).

Similar to the BCF estimations in Chapter 3 the stepwise approach for BMF estimations can be automated through the use of the *R*-package '*bcmfR*'[12].

189. The default approach to estimate all BMF parameters from the depuration phase is to do linear regression on ln-transformed $C_{fish}(t_i)$ data at time moments $t_i$. Because of this, the default approach for estimating the BMF uncertainty is to start with the ln-transformed data. Similar to the BCF procedure, all

[11] In many cases chemical concentrations in fish at the end of the depuration phase in BMF studies will be very low and may fall below the limit of detection (l.o.d.). For these concentrations it will be difficult to decide on their true value, in particular when the l.o.d. is relatively high. For this reason it may be advisable to not use the time-points showing chemical concentrations in fish below the l.o.d. in data analysis. However, in some cases (e.g. when depuration is fast and many of the chemical concentrations in the fish fall below the l.o.d.), it may be advisable to allocating a specific value to those values below l.o.d. (e.g., 0.5 × l.o.d.). This would then allow a sensitivity analysis to be performed for the influence of these values below l.o.d. on the outcome of the test. An example where such a consideration may be needed is where both values below and above l.o.d. are observed at the same time point.

[12] The current version of the *R*-package (*bcmfR*, version 0.3-2) has been verified to work in *R* version 3.3.2. That does not mean that it will not work in newer versions of *R* or its packages, but as the *R* software is open source and continuously under development, some functionalities may disappear in newer versions, in particular in certain packages (further details on necessary packages and their version release numbers can be found in the accompanying "OECD-TG305 *R*-Package *bcmfR* User Guide (v0.3-2)". Potential future updates of the *R*-package bcmfR will be made available via the OECD public website (www.oecd.org/env/ehs/testing).

steps are calculated in one go in the *R*-package '*bcmfR*' and growth and lipid correction is done for all BMF estimations.

190. The solution of the fish accumulation over time in the depuration phase:

$$C_{\text{fish}}(t) = C_{0,\text{d}} \cdot \text{e}^{-k_2 \cdot t} \qquad \text{Equation 4-13}$$

is rewritten as:

$$\ln C_{\text{fish}}(t) = \beta_0 + \beta_1 \cdot \text{t} \qquad \text{Equation 4-14}$$

Linear regression yields estimates $\beta_0$ and $\beta_1$, as well as standard errors and covariance.

First, the initial fish concentration is estimated at the start of the depuration phase and the depuration rate constant as:

$$C_{0,\text{d}} = \text{e}^{\beta_0} \qquad \text{Equation 4-15}$$

$$k_2 = -\beta_1 \qquad \text{Equation 4-16}$$

Everything else follows from these two regression coefficients.

191. An estimate of the elimination rate constant becomes

$$k_{2\text{g}} = k_2 - k_{\text{g}} \qquad \text{Equation 4-17}$$

where the measured growth rate constant is taken as a constant, without error. One may estimate two half-lives, one based on the depuration rate including elimination and growth:

$$t_{1/2} = \frac{\ln(2)}{k_{2\text{g}}} \qquad \text{Equation 4-18}$$

and a growth-corrected half-live based on the elimination rate only:

$$t_{1/2} = \frac{\ln(2)}{k_2 - k_{\text{g}}} \qquad \text{Equation 4-19}$$

192. The kinetic BMF is estimated as

$$\text{BMF}_{\text{K}} = \frac{C_{0,\text{d}}}{C_{\text{food}}} \cdot \left( \frac{1}{1 - e^{-k_2 \cdot t_{\text{f}}}} \right) \qquad \text{Equation 4-20}$$

with the feeding period and food concentration both given.

193. Similarly, the growth-corrected kinetic BMF is calculated as

$$\mathrm{BMF_{Kg}} = \frac{C_{0,\mathrm{d}}}{C_{\mathrm{food}}} \cdot \frac{k_2}{k_2 - k_{\mathrm{g}}} \cdot \left( \frac{1}{1 - e^{-k_2 \cdot t_{\mathrm{f}}}} \right) \qquad \text{Equation 4-21}$$

194. The uptake rate constant can be back-calculated from the BMF:

$$k_{\mathrm{f}} = \mathrm{BMF_K} \cdot k_2 \qquad \text{Equation 4-22}$$

*4.5.2.1 Step 1: Ln-transformed fit and diagnostics*

195. Typical results for the dietary exposure test, recalculated for hexachlorobenzene from the OECD TG 305 ring test (45), are shown for the ln-transformation of the fish concentration over time in the depuration phase (Figure 4-1). The exposure concentration in food ($C_{\mathrm{food}}$), exposure period ($t_{\mathrm{f}}$), ingestion rate ($I$) and growth rate ($k_{\mathrm{g}}$) are summarised as:

| | | |
|---|---|---|
| $C_{\mathrm{food}}$: | 22.1 | $\mathrm{mgX \cdot kgW_{food}^{-1}}$ |
| $t_{\mathrm{f}}$: | 13 | d |
| $I$: | 0.03 | $\mathrm{kgW_{food} \cdot kgW_{fish}^{-1} \cdot d^{-1}}$ |
| $k_{\mathrm{g}}$: | 0.0373 | $\mathrm{kgW_{fish} \cdot d^{-1}}$ |

196. At first sight, the ln-transformed fish data and fitted model show good agreement on a straight line, indicating a first order depuration process. The BMF parameter statistics and dietary test parameter estimates are shown in Table 4-3 for HCB, based on data from the OECD TG305 ring test (44). Parameter estimates and their confidence intervals were generated using the *R* package '*bcmfR*'.

197. The BMF estimate is 0.368 (95% CI 0.334-0.402), and with growth correction it is much higher: 0.912 (95% CI 0.716-1.108). Similar to the findings for BCF growth correction, note that the standard error of the growth-corrected BMF estimate is almost 6 times larger than the standard error of uncorrected BMF estimate.

Ln-Transformation: Fit Characteristics

198. The fit characteristics are studied by looking at diagnostic plots as discussed for the BCF (Section 3.4), in conjunction with statistical tests, especially for deciding on variance homogeneity and normality assumptions (the output of the *R*-package includes such graphical plots):

- Correct mean function for bioaccumulation $C_{\mathrm{fish}}(t)$
- Checking (for heteroscedasticity) of measurement errors
- Normal distribution of measurement errors
- Mutually independent measurement errors

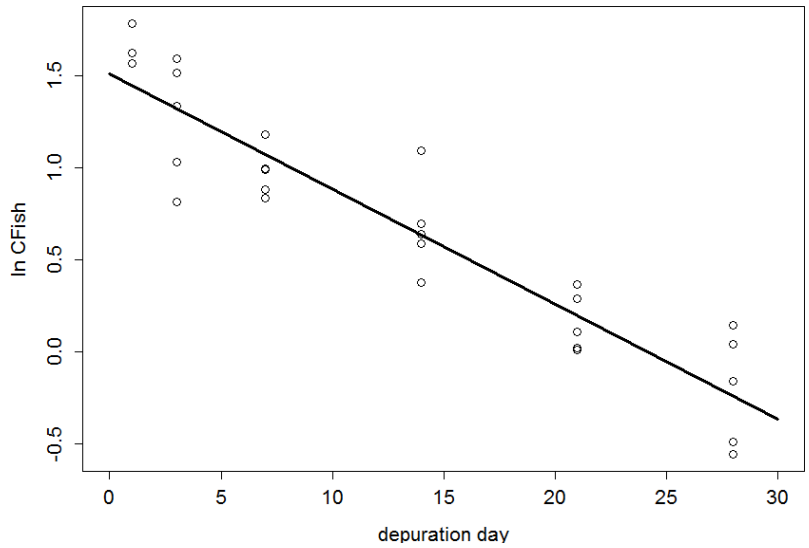


**Figure 4-1: The ln-transformed fish concentration data for hexachlorobenzene over time during the depuration phase after dietary uptake. Data from (44). The line represents the linear fit to the ln-transformed data.**

**Table 4-3: BMF parameter estimates and uncertainty for hexachlorobenzene (OECD TG305 ring test (44))**

| BMF parameter | Estimate | Std. Error | 2.5% | 97.5% |
|---|---|---|---|---|
| $C_{0,d}$ | 4.52 | 0.35 | 3.84 | 5.21 |
| $k_d$ | 0.0625 | 0.0048 | 0.0532 | 0.0719 |
| $k_e$ | 0.0252 | 0.0048 | 0.0159 | 0.0346 |
| $k_f$ | 0.0230 | 0.0023 | 0.0185 | 0.0275 |
| $\alpha$ | 0.766 | 0.077 | 0.616 | 0.917 |
| $BMF_K$ | 0.368 | 0.017 | 0.334 | 0.402 |
| $BMF_{Kg}$ | 0.912 | 0.100 | 0.716 | 1.108 |

199. The residual plot for Figure 4-2 (top left) shows that the values go up and down without a clear pattern. The standardised residuals (see Figure 4-2, top right) are between –2 and 2 and shows a relatively well-balanced error over the data range, but with a relatively high variation.

200. The residuals do not show any specific pattern over time, which can be visually inspected by looking at the autocorrelation diagnostic plot (Figure 4-2, bottom left). This plot does not indicate a pattern over time. The runs test (34) can be used to indicate if the residuals are independent (random) or not. The null-hypothesis is that they are, and at low *p*-values this hypothesis is rejected. For Figure 4-2, the runs test indicates $p = 0.4552$, confirming the interpretation of independent errors in the autocorrelation plot.

```
Runs Test

Standard Normal = -0.74677, p-value = 0.4552
alternative hypothesis: two.sided
```

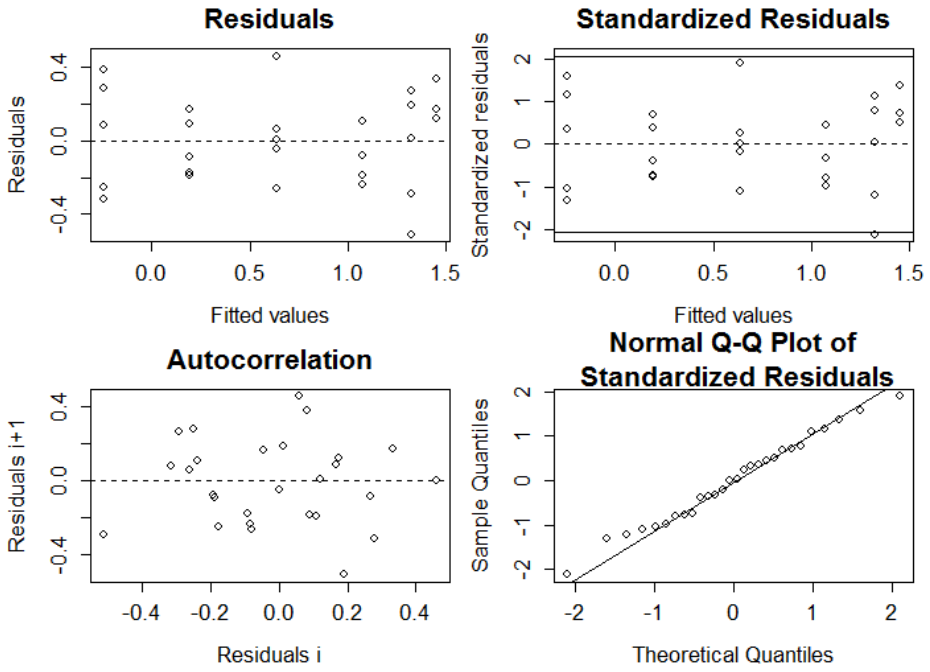


**Figure 4-2: Fit diagnostics for the ln-transformed data for hexachlorobenzene (37).**

201. The normal Q-Q plot (Figure 4-2, bottom right) plots the measurement errors against a straight line that indicates the standardised normal distribution. Departures from normality are indicated by deviations of the residuals from the straight line, but here that is hardly the case. The Shapiro-Wilk test is

done to indicate the plausibility of the normal distribution (see Section 3.4.1). The Shapiro-Wilk test *p*-value for Normality is very high, and the hypothesis that the error distribution is Normal is not rejected.

```
Shapiro-Wilk normality test

data: stdres
W = 0.9855, p-value = 0.9557
```

This seems to indicate that the natural log-transformation is quite effective in this case.

*4.5.2.2 Step 2: Untransformed Nonlinear Fit to the $C_{fish}$ Data in the Depuration Phase*

202. The application of the log-transformation is usually done, because the tools for linear regression are readily available. However, low values of $C_{\text{fish}}$ at the end of the depuration phase can have large influence on the determination of the depuration rate constant, which may unduly influence the estimates of initial and growth-corrected BMF, as is discussed in Annex 7 to TG 305 (1).

203. To study the effect of possible data transformations on estimates of the BMF and rate constants, the untransformed model is used again:

$$C_{\text{fish}}(t) = C_{0,\text{d}} \cdot \text{e}^{-k_2 \cdot t} \qquad \text{for } t \geq 0 \qquad \text{Equation 4-23}$$

204. Now the depuration rate constant enters in a nonlinear fashion. The parameter estimates derived from the ln-transformed linear regression can be used as starting point for the nonlinear regression. The nonlinear exponential fit on untransformed $C_{\text{fish}}$ data is shown in Figure 4-3.

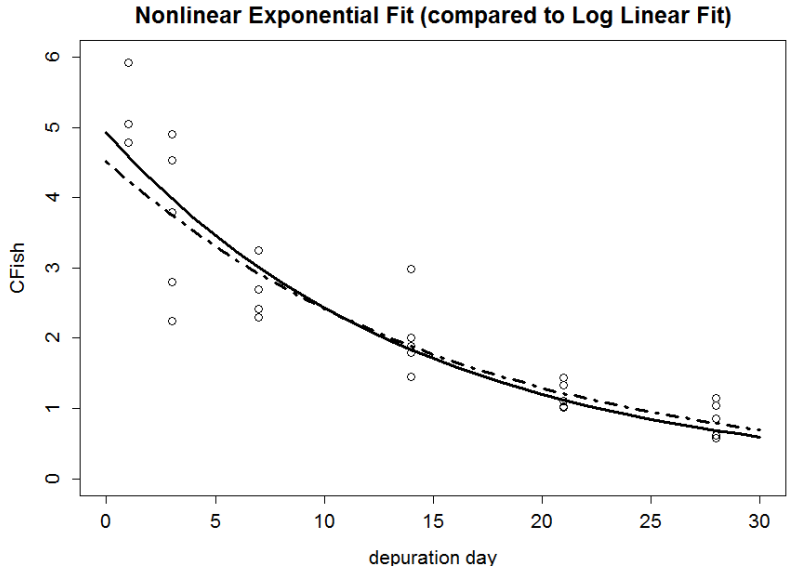


**Figure 4-3: Nonlinear exponential fit on untransformed $C_{\text{fish}}$ data over time (solid line). Back-transformed log-linear model fit plotted on the same vertical axis.**

205. The nonlinear regression parameter estimates and resulting BMF estimates are given in the 206.

The fit characteristics are not as nice as in the log-transformed case. The residuals seem to be larger for higher fitted values for $C_{\text{fish}}$ (Figure 4-4, top two panels), indicating heteroscedasticity. The

standardised residuals also indicate outliers for the highest fitted values (Figure 4-4, top right panel). A trend over time does not seem indicated by the autocorrelation plot (Figure 4-4, bottom left panel).

Table 4-4. Note that the parameter estimates are somewhat higher, e.g. the initial fish concentration $C_{0,d}$ = 4.93 instead of 4.52, while $k_2$ = 0.0705 compared to 0.0625 in the ln-transformed linear fit. (cf. Table 4-3).

206. The fit characteristics are not as nice as in the log-transformed case. The residuals seem to be larger for higher fitted values for $C_{fish}$ (Figure 4-4, top two panels), indicating heteroscedasticity. The standardised residuals also indicate outliers for the highest fitted values (Figure 4-4, top right panel). A trend over time does not seem indicated by the autocorrelation plot (Figure 4-4, bottom left panel).

**Table 4-4: BMF parameter estimates and uncertainty for hexachlorobenzene (OECD TG305 ring test (44)), untransformed fit.**

| BMF parameter | Estimate | Std. Error | 2.5% | 97.5% |
|---|---|---|---|---|
| $C_{0,d}$ | 4.93 | 0.30 | 4.34 | 5.52 |
| $k_d$ | 0.0705 | 0.0085 | 0.0538 | 0.0872 |
| $k_e$ | 0.0332 | 0.0085 | 0.0165 | 0.0499 |
| $k_f$ | 0.0262 | 0.0026 | 0.0211 | 0.0313 |
| $\alpha$ | 0.873 | 0.0867 | 0.703 | 1.040 |
| $BMF_K$ | 0.372 | 0.020 | 0.332 | 0.411 |
| $BMF_{Kg}$ | 0.790 | 0.137 | 0.521 | 1.060 |

207. The runs test confirms that there is no time trend.

```
Runs Test

Standard Normal = 0, p-value = 1
alternative hypothesis: two.sided
```

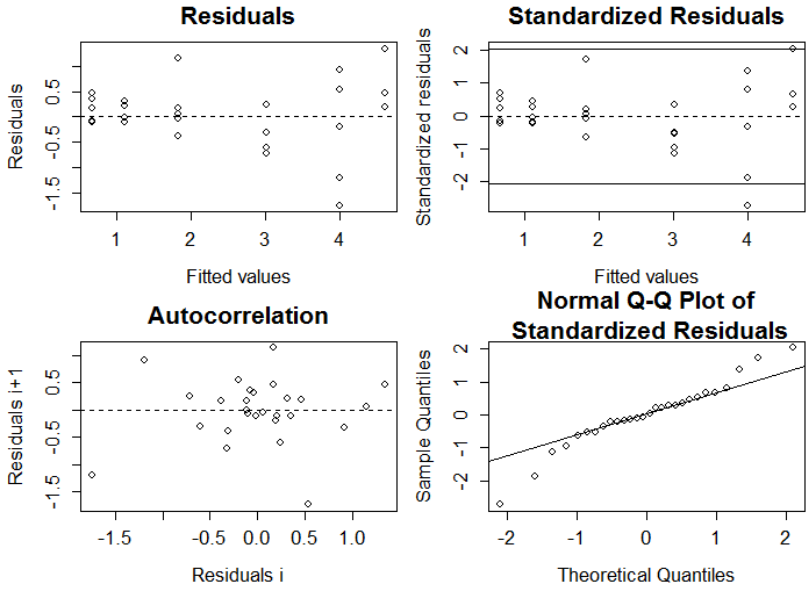


**Figure 4-4: Fit diagnostics for the untransformed data for hexachlorobenzene (44).**

The tails in the Q-Q plot are less attractive than for the ln-transformed case (Step 1, Section 4.5.2.1), and the Shapiro-Wilk *p*-value is less than in the ln-transformed case, but not significantly rejecting Normality.

Shapiro-Wilk normality test

```
data: stdres
W = 0.9533, p-value = 0.24
```

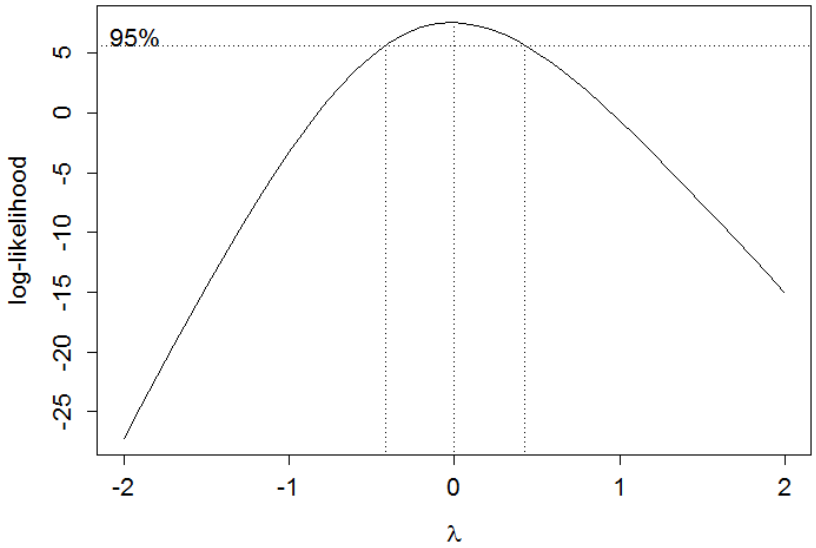


**Figure 4-5: Plot of the log likelihood function for the Box-Cox parameter λ applied to both $C_{fish}$ data and model prediction.**

*4.5.2.3 Step 3: Find an optimum data transformation using the Box-Cox optimisation procedure*

Optimal Box-Cox transformation for the Depuration Phase

208. To check if other (milder) transformations might improve the fit, the Box-Cox procedure is used in this step. From the untransformed non-linear fit, the optimal Box-Cox transformation parameter can be derived. The Box-Cox transformation and the profile log-likelihood method to study it are described in Section 3.4.3. When the optimal Box-Cox transformation is plotted, the optimum in this case is $\lambda = 0$, which corresponds to the natural logarithm transformation (Figure 4-5). The confidence interval for the Box-Cox parameter is (–0.41, 0.43), which is nearly symmetric. In this specific example, the optimal Box-Cox transformation suggests that the log-transformation provides a satisfactory fit to the data. Hence the diagnostic plots and *p*-values are those of the ln-transformed fit of Section 4.5.2.1.

*4.5.2.4 Step 4: Decide on the appropriateness of the model and data transformations used in steps 1, 2 and 3*

209. The untransformed fit showed a larger departure from normality as for the ln-transformed fit, indicating that indeed a data transformation will probably improve the fit.

210. The combined result of the steps 1–3, followed by graphical inspection model diagnostics and statistical testing provides confidence that in this case, a logarithmic transformation improves the fit of the model to the data. As is stated for the BCF estimate case, rather than postulating a "default" optimal transformation, the guidance user is encouraged to find a suitable BMF model fit and data transformation with the supplied *R*-package.

#### *4.5.2.5 Step 5: BMF estimation, growth and lipid corrected*

211. Analogous to Section 3.5.3 for the aqueous exposure method, the growth–corrected dietary BMF, $BMF_{Kg}$, must be corrected for fish lipid if the test substance is primarily associated with lipid storage in fish. Correction of the dietary BMF for differences in lipid content is described in OECD TG 305 (Annex 7 section 4) (1). If chemical and lipid analyses have been conducted on the same fish, one option is to correct each individual measured concentration in the fish for that fish's lipid content. This should be done *prior* to using the data to calculate the dietary BMF. However in most cases, lipid analysis is not conducted on all sampled fish so this is not possible and a mean lipid value must be used to correct the BMF.

212. As detailed in OECD TG 305 Annex 7, section 4 (1), the $BMF_{Kg}$ is also corrected to the mean lipid content of the food[13] (in % wet weight). Uptake of a chemical from the food is driven by the digestion of the food, a process during which the fugacity capacity of the food is decreased. As a result the fugacity of the chemical in the food increased during digestions, as a result of which the chemical is taken up from the food (54). The normalisation to the initial lipid content of food is thus not supported by the mechanistic explanation of absorption of a chemical from the food. The lack of a correlation between lipid content of the food and $BMF_{Kg}$ is also supported by the outcome of the ring test (44; pp. 139-140) and research with carp at varying lipid contents of the food (53). In accordance with the latter publication a $BMF_{Kg}$ normalised to 5% fish lipids may be considered as additional output.

### *4.5.3 Reporting on $BMF_K$ estimation*

213. As indicated in OECD TG 305 a list of parameters and biomagnification factors (BMFs) are required in the reporting of the results (see the list at the end of paragraph 167 of OECD TG 305 (1)). For those parameters related to the $BMF_K$ estimation the values will be derived from the procedure described here (see e.g. 206. The fit characteristics are not as nice as in the log-transformed case. The residuals seem to be larger for higher fitted values for $C_{fish}$ (Figure 4-4, top two panels), indicating heteroscedasticity. The standardised residuals also indicate outliers for the highest fitted values (Figure 4-4, top right panel). A trend over time does not seem indicated by the autocorrelation plot (Figure 4-4, bottom left panel).

Table 4-4) and thus should be reported.

214. To ensure that the decision for a certain transformation and model fit is transparent, the user is encouraged to report for each of the steps 1 to 3 the estimates for $C_{0,d}$, $k_d$, $k_e$, $k_f$, α, and $BMF_K$, but also the model diagnostics plots, Shapiro-Wilk test results, and plots that show how the curves describe the data. For the Box-Cox transformation the transformation parameter λ and the plot of the log likelihood function for the Box-Cox parameter λ are relevant as well.

## 4.6 Using Dietary Study Results

215. This section of the Guidance Document suggests how data generated in dietary studies according to OECD TG 305 (1) can be used by regulatory authorities and other assessors.

[13] New, as yet unpublished, research in Japan, however, suggests normalisation of BMF for fish lipid alone, using 5% as for the aqueous study (53). This is based on results for one chemical, hexachlorobenzene. This is still being considered, therefore at present it is suggested to also include this value when reporting the study results.

### *4.6.1 Overview of parameters derived from the dietary study*

216. The dietary exposure method allows the determination of the depuration rate constant ($k_2$), growth-corrected depuration rate constant ($k_{2g}$), the substance-specific half-life ($t_{1/2}$), the absorption efficiency (absorption across the gut; α), the kinetic dietary biomagnification factor ($BMF_K$), the growth-corrected kinetic dietary biomagnification factor ($BMF_{Kg}$), the lipid-corrected kinetic dietary biomagnification factor ($BMF_{KL}$), and the combined growth-and lipid-corrected kinetic dietary biomagnification factor ($BMF_{KgL}$) for the test substance in fish. As additional output parameters, the kinetic dietary biomagnification factor normalised to 5% fish lipids ($BMF_{K5\%L}$) and the growth-corrected kinetic dietary biomagnification factor normalised to 5% fish lipids ($BMF_{Kg5\%L}$), as well as an uptake rate constant ($k_f$, see Equation 3-2) can be estimated from the data. All of these parameters can be useful in assessing the bioaccumulation potential of a substance.

217. The combined growth- and lipid corrected kinetic dietary biomagnification factor ($BMF_{KgL}$) will usually be the preferred biomagnification parameter (as recommended in paragraph 162, OECD TG 305 (1))[14]. This preference is for organic substances that partition to lipids tested in studies in which fish growth occurs (i.e. use of juvenile fish).

218. The BMF calculated in the OECD TG 305 study, termed a "dietary BMF", is not the same as a BMF derived in the field. BMFs from the field are usually derived in a way similar to the steady state approach in the aqueous method, in that they represent the ratio of the concentration of the substance in the organism to the concentration in its diet at steady state. However, field BMFs account for all routes of uptake, including uptake via the water phase as well as via the diet, and more important both prey and predator are exposed to the same environmental concentrations whereas only food has been contaminated in the dietary test, while fish are kept in an otherwise clean environment (flow through with clean water). The relative contributions from the different uptake routes will depend on how the substance ends up in the environment, the substance's intrinsic properties, the properties of the aqueous environment (i.e. factors affecting bioavailability like dissolved organic carbon and pH), diet (prey species), and species-specific attributes (e.g. (55)). Generally for the kinds of substances that require testing according to the dietary method (e.g. low solubility and high log $K_{ow}$), aqueous exposure is thought to become less important but still not negligible compared with the dietary route (56). Therefore, it follows that feeding study BMFs will usually be lower than field BMFs, because one of the uptake routes is missing. In some dietary OECD TG 305 studies, it may not be possible to derive a dietary BMF. For example, substances that are depurated so quickly that reliable estimation of the parameters $C_{0,d}$ (the concentration of test substance in the fish at the start of the depuration phase) and $k_2$ is not possible because concentrations reached the limit of quantification after only one or two sampling points into the depuration phase. Similar issues may be faced when using the kinetic approach in the aqueous exposure method. In such cases, the high rate of depuration suggests that the substance is unlikely to be bioaccumulative in aquatic organisms. Guidance for how to deal with this situation in the dietary study is given in section 6 of Annex 7 to OECD TG 305 (1).

219. In most regions bioaccumulation data are required for the purposes of classification (according to the Globally Harmonised System), risk assessment (for the derivation of chemical concentrations in biota/food items for the estimation of exposure through the diet, bioconcentration and biomagnification data are needed) and PBT assessment. Regulatory (numerical) criteria are usually derived relating to bioconcentration (BCF), as derived according to the aqueous exposure study in OECD TG 305 (1). This

[14] In the light of mechanistic considerations (54), supported by the outcome of the ring test (44; pp. 139-140) as well as recent Japanese research (53), the kinetic dietary biomagnification factor normalised to 5% fish lipids ($BMF_{K5\%L}$) and the growth-corrected kinetic dietary biomagnification factor normalised to 5% fish lipids ($BMF_{Kg5\%L}$) may become more important in the future. It is, therefore, strongly recommended to report these values as well.

results in the problem of how to use the results from a dietary study for the purposes of regulation or (quantitative) assessment when the resulting metric is different. The following sections of this guidance explore this.

### *4.6.2 The Dietary BMF and its relationship to BCF*

220. The OECD TG 305 dietary test set up is essentially the same as the aqueous test, the obvious differences being the exposure route, the duration of exposure and lack of analytical monitoring during the uptake phase (1). The OECD TG 305 dietary test, like the aqueous study, generally uses juvenile fish that have faster rates of respiration and can reach maximal body burdens of test chemicals over a relatively short period of time. Consequently, chemical loss via respiration can also be fast.

221. It has been recognised that regulatory trigger values based on BCF (e.g. 2000 or 5000 L/kg) do not necessarily correspond to dietary BMFs from the dietary study greater than 1, especially in very small fish in the exponential phase of growth. It is likely that higher BMFs (and higher BCFs for aqueous studies) would result from laboratory studies with larger, adult fish of the same species. For example, the regression analysis comparing measured laboratory dietary BMFs and BCFs for nine bioaccumulative substances by Inoue et al. (57) (see Section 4.6.3.3 below) has shown that a BCF value of 5000 L/kg corresponds to a dietary $BMF_{KgL}$ of around 0.3 in juvenile carp. Some of the substances that had dietary BMFs < 1 but measured BCFs > 5000 (lipid normalised to 5%) are already identified as bioaccumulative substances in regulatory regimes in OECD member countries. A discussion of how trigger levels are set and what protection goals they represent is out of the scope of this guidance. Nevertheless, it should be noted that the EU PBT guidance for the REACH regulation (58) identifies organisms similar to the test species (i.e. wild fish at the same trophic level) as a protection goal of PBT assessment and not just predators at the top of the food chain.

222. For reliable dietary BMF studies where the resulting $BMF_{KgL}$ is 1 or greater, it is clear that this result should allow the categorisation of the test chemical as highly bioaccumulative (i.e. the BCF would be > 5000 L/kg) according to many regulatory schemes in OECD member countries that allow the use of surrogate bioaccumulation data other than the BCF. Biomagnification would have been shown to occur in the tested species under the conditions of the test.

223. For cases where the dietary $BMF_{Kgl}$ is < 1, but the results still indicate an appreciable level of accumulation, the ability to express the results of both aqueous and dietary studies on the same scale that can be used directly for regulatory purposes is an important need. It is also important that, as far as possible, chemicals tested according to the different OECD TG 305 protocols are treated in the same way in terms of regulation.

224. Using dietary study data to generate a BCF estimate is one option, and may be the most pragmatic since BCF is the metric used in most OECD countries to measure bioaccumulation potential. The hierarchical ordering of bioaccumulation measures in terms of scientific insight into a chemical's bioaccumulative behaviour and their use in regulation has been the subject of some debate and a lot of work in recent years. This includes the derivation and use of fugacity ratios as a way of representing measures of bioaccumulation on one scale. However no agreement in OECD member countries on the use of this method in regulation has been reached to date and certain aspects of the approach rely on many assumptions (for example the use of log $K_{OW}$ as a surrogate for prey lipid when developing a fugacity ratio equivalent to the BCF).

225. Although uptake routes may differ in the process of bioaccumulation, similar factors may influence the relative rates and efficiencies of uptake by passive diffusion via the different routes. Following prolonged exposures, in vivo distribution and subsequent excretion should in theory be the same

regardless of uptake route. This means that different measures of bioaccumulation should in theory be related to one another.

226. Inoue et al. (59) have provided convincing evidence that the assumption holds that depuration is independent of uptake route. They exposed carp to nitrofen (and hexachlorobenzene) via water or spiked food in separate studies according to OECD TG 305 to compare bioaccumulation parameters and tissue distributions. The BCF for nitrofen was 5,100 L/kg, and the $BMF_{KgL}$ was 0.137. Growth-corrected depuration half-lives were 2.1 – 3.0 days for the aqueous exposure study and 2.7 – 2.9 days for the dietary study. Tissue distributions followed the same trend for nitrofen for both study types, with highest concentrations in the head, followed by muscle, viscera, dermis, digestive tract and hepatopancreas, being highly correlated with the tissue lipid content. Inoue et al. (59) concluded that the route of uptake (via the gill or gastro-intestinal tract) had no influence on tissue distribution of nitrofen, and that the accumulation potential in tissues depended on the lipid content.

227. Mackay et al. (60) showed that it is possible mathematically to relate bioaccumulation metrics such as the biomagnification factor to the kinetic BCF. The relationship includes the use of an "equilibrium multiplier", made up of two ratios (the diet-to-water concentration ratio and the ratio of uptake rate constants for respiration and the diet). It is a factor by which the concentration in the fish exceeds its steady state or near-equilibrium value as a result of food uptake and digestion. Mackay et al. in summary state that "the kinetic BCF and the values of equilibrium multiplier can be regarded as the fundamental determinants of bioaccumulation and biomagnification in aquatic food webs" (60).

228. This approach should be seen as complementary to other approaches that relate bioaccumulation metrics to one another, like fugacity ratios. Mackay et al. (60) demonstrated that theoretically the BMF is proportional to the (lipid-normalised) ratio of the predator/prey kinetic BCFs and the ratio of equilibrium multipliers (for the two species). To prevent derivations becoming overly complicated and relying on too many assumed values, they derived relationships based on the assumption that chemicals partition to the lipid fraction of the fish, that there is no fish growth (and so no growth dilution) and that no metabolism is occurring.

229. They derived a relationship for BMF between a predatory fish (species 2) and a prey fish (species 1) as follows (60):

$$BMF_{21} = BMF_{K2} \cdot \frac{\left(1 + \frac{k_{D2}}{k_{R2} \cdot C_{D2}/C_W}\right)}{(C_{F1}/C_W)} \qquad \text{Equation 4-24}$$

Where: $BMF_{21}$: the BMF for the relationship (predatory) species 2 and (prey) species 1
$BCF_{K2}$: the kinetic BCF for species 2
$k_{D2}$: the dietary uptake rate constant (for predatory species 2) ($m^3$food.$d^{-1}$)
$k_{R2}$: the gill respiration uptake rate constant (for predatory species 2) ($m^3$water.$d^{-1}$)
$C_{D2}$: the concentration in the diet for species 2 (mol.$m^{-3}$)
$C_{F1}$: the concentration in species 1 (mol.$m^{-3}$)
$C_W$: the concentration in water (mol.$m^{-3}$)

Since for this two-species relationship $C_{F1} = C_{D2}$, Mackay reduced the equation in terms of the predator's BCF and two equilibrium multipliers:

$$BMF_{21} = BMF_{K2} \cdot \frac{C_W}{C_{F1} + (k_{D2}/k_{R2})} \qquad \text{Equation 4-25}$$

230. While this was derived for a predator/prey relationship, it is logical that it will also hold for the fish/spiked feed situation in the dietary BMF test. This equation can be related to that situation by

substituting species 1 (prey) for the spiked fish food diet used in the study and setting the concentration in water ($C_W$) to zero ((38), personal communication). Hence the equation further reduces to:

$$\mathrm{BMF} = \mathrm{BCF}_k \cdot \frac{k_{D2}}{k_{R2}} \qquad \text{Equation 4-26}$$

In the context of OECD TG 305 (1), such a relationship has been indicated by the work of Inoue et al. (57), in which dietary study BMFs were correlated with aqueous exposure BCFs (see Section 4.6.3.3 below).

### *4.6.3 BCF estimations from dietary study data*

231. This section summarises three available approaches that can be used to estimate a BCF from dietary study data. A decision scheme for using dietary study data in this way, illustrated with some worked examples, is also presented. The methods are:

1. Uptake rate constant estimation method
2. Relating depuration rate constant directly to BCF
3. Correlating dietary BMF with BCF

At the end of each description, the pros and cons are evaluated. A separate Excel spreadsheet that automates the BCF estimations is available in Annex 4.

232. Gobas and Lo (61) have recently suggested a further option which would require additional measurements of the reference substance during depuration. Their premise is that some of the variables in equations used to calculate BCF and BMF can be assumed to be constant or extremely small in relation to other variables. Using the additional information from the reference substance, these constants can be calculated, and a BCF derived for the test substance. It should be noted that these methods are designed for substances which partition to lipid. None of these methods are considered applicable to substances which do not partition to lipid, for example chemicals thought to bind to protein.

233. The first two approaches to estimating a BCF are related in that, in terms of measured study kinetic data, they use only the depuration rate constant. The third approach considers a study's derived BMF, i.e. uptake as well as depuration kinetics are included. The assumption that depuration is independent of uptake route is implicit in all approaches, whilst the third approach implicitly includes the assumption that rates of uptake via different exposure routes are correlated. These assumptions are discussed below. Estimation of uncertainty, as described above for the aqueous method and dietary BMF, is not possible for these BCF estimates based on dietary study data. An estimate of uncertainties in the measured parameters used in the estimates (depuration rate constant and BMF) can be derived, but estimates of uncertainty in the predicted parameters (uptake rate constant and BCF) are not possible to derive, because they are related both to the dietary study measured data and the models used in the prediction, including their underlying training sets.

#### *4.6.3.1 Method 1 – Uptake rate constant estimation method: Use of models to estimate $k_1$, combined with dietary $k_2$ to provide BCF*

234. In this approach, an uptake rate constant is estimated for hypothetical uptake from water. This estimated uptake rate constant is then compared with the depuration rate constant measured in the dietary BMF study to give a surrogate kinetic BCF. This was also discussed as a method to estimate a BCF in the

background document to the dietary protocol on which the dietary method in OECD TG 305 was based (62).

235. Several mathematical relationships have been derived that relate the theoretical uptake rate constant for gill respiration ($k_1$) to either a substance parameter (e.g. log $K_{OW}$) or a biological parameter (that is measured in a dietary bioaccumulation study, e.g. fish weight). The ratio of this estimated uptake rate constant ($k_1$) to the measured depuration rate constant ($k_2$) from the dietary study gives a "tentative" BCF. Assumptions in this approach are discussed below. It is this approach that is outlined in Annex 8 to OECD TG 305 and referred to in paragraph 14 and 106 of OECD TG 305 (1), with caveats for its use.

236. A number of equations or QSAR-type approaches are available in the literature that have been derived to relate the uptake rate constant for gill respiration to fish weight, log $K_{OW}$, or both, amongst other parameters. These equations and approaches include Sijm et al. (63) (64) (42), Opperhuizen (65), Hendriks et al. (66), Campfens and Mackay (67), Arnot and Gobas (68), Arnot and Gobas (24), Thomann (50), and Barber (69). A thorough review of these approaches was produced by Crookes and Brooke (36), and a summary of this review was published in a peer-reviewed journal (70). It is this review that forms the basis of the description of the approach here. The review should be referred to for further detail.

237. The majority of the available models (12 out of 22) depend solely on fish weight. These are the allometric approaches of Sijm et al. (42), Barber (69), and ten allometric regression equations within Barber (69). The equations and brief details of the data used to derive these relationships are included in Table 4-5 below. Refer to the publication of Crookes and Brooke (36) for further details. These methods vary in the magnitude of the $k_1$ value predicted for a given fish weight, although all of these methods predict that the $k_1$ value should decrease with increasing fish weight. For example, the $k_1$ values predicted for a fish weight of 0.1 g are in the approximate range 190 to 6,200 L $kg^{-1}$ $day^{-1}$ for the range of models; the $k_1$ values predicted for a fish weight of 16 g are in the approximate range 80 to 1,240 L $kg^{--1}$ $day^{-1}$.

238. The Sijm et al. (42) model did not use data for identified outliers phenol, octachloronaphthalene and octachlorodibenzo-*p*-dioxin. The measured uptake rate constant for phenol (log $K_{OW}$ 1.8) was lower than predicted by the equation, whilst the other two substances showed low measured uptake. The authors postulated that this was due to their large molecular size limiting diffusion across gill membranes, although the relative molecular weights of these substances are well below thresholds used to indicate such an effect in some OECD countries' regulations. Log $K_{OW}$ data are not available for the other derived models, but the Sijm equation was derived for substances in the range log $K_{OW}$ 3.6 – 8.3.

239. Unfortunately, only limited detail on how most of the equations were derived is available. However an assumed applicability domain for these models can be estimated from the more detailed information included by Sijm et al. (42) and the information on substance types included in Barber's (69) reanalysis of models. Broadly speaking, this approach should be useable for aromatic hydrocarbons, those that are chloro-, bromo-, nitro- substituted, and may be suitable for organochlorine and organophosphate pesticides, triarylphosphates and alcohol ethoxylates with log $K_{OW}$ in the range around 3.5 – 8.5. Particular care, however, must be taken when using these equations for larger, or higher molecular weight, molecules where there is an indication that uptake may be over-predicted.

240. Five of the 22 methods that were considered depend on both the fish weight and the log $K_{OW}$ of the test substance. These are the approaches of Hendriks et al. (66), Arnot and Gobas (68), Arnot and Gobas (24), Thomann (50) and the (calibrated) Gobas and Mackay (71)/Barber (69) method. The equations and brief details of the data used to derive these relationships are included in Table 4-6 below. Refer to the publication of Crookes and Brooke (36) for further details.

241. For a log $K_{OW}$ of five, these methods predict a $k_1$ value in the approximate range 1,180 to 3,980 L $kg^{-1}$ $day^{-1}$ for a fish weight of 0.1 g and 330 to 520 L $kg^{-1}$ $day^{-1}$ for a fish weight of 16 g. Three of the methods (66) (68) (24) predict that $k_1$ should increase with increasing log $K_{OW}$ up to a limit, after which the $k_1$ becomes independent of log $K_{OW}$. The log $K_{OW}$ value at which this occurs is approximately log $K_{OW} \geq 6$ (66) or log $K_{OW} \geq 4$ (68). The Thomann (50) approach predicts a different dependence of $k_1$ on log $K_{OW}$, with an increase in $k_1$ with increasing log $K_{OW}$ being predicted up to around a log $K_{OW} \approx 5$, the predicted $k_1$ being independent of log $K_{OW}$ in between approximately log $K_{OW} \approx 5 - 6.5$, and the predicted $k_1$ value decreasing with increasing log $K_{OW}$ above a log $K_{OW} \approx 6.5$.

242. Very limited information relevant for the applicability domain of these models is available. On the basis of the types of chemicals used to calibrate the Gobas and Mackay (71) model, it is likely that the models can be applied to chemicals with log $K_{OW}$ in the range 3.5 – 8.5. Again, particular care must be taken when applying these models to larger or higher molecular weight chemicals.

243. Four of the 22 methods depend on the log $K_{OW}$ of the substance alone. These are the approaches of Hawker and Connell (72), Hawker and Connell (73), Spacie and Hamelink (74) and Tolls and Sijm (75). The equations and brief details of the data used to derive these relationships are included in Table 4-7 below. Refer to (36) for further details.

244. Three of these approaches predict that $k_1$ should increase exponentially with increasing log $K_{OW}$, whereas the Hawker and Connell (73) predicts that the $k_1$ value would reach a constant maximum of around 35 L $kg^{-1}$ $day^{-1}$ at log $K_{OW}$ values around six and above. The three other methods predict that for a log $K_{OW}$ value of 10, the $k_1$ would be in the approximate range 1,000 – 2,700 L $kg^{-1}$ $day^{-1}$. In a review paper, Barber (69) commented that careful evaluation is needed before using these equations for predictions as they are based on very limited databases and they implicitly assume that biological determinants of uptake are either insignificant or constant across species or body sizes. Based on the available information and this comment, these methods should apply to chlorinated aromatics with log $K_{OW}$ in the approximate range of 2.6 – 6.5. Again, particular care must be given to larger or higher molecular weight chemicals.

245. The method of Campfens and Mackay (67) is different from the other methods in that it depends on the elimination rate constant as well as the lipid content of the fish and the log $K_{OW}$ of the substance (Table 4-8). This method predicts that the $k_1$ value should increase markedly with log $K_{OW}$ above a log $K_{OW}$ of around five. It is difficult to describe an applicability domain for the model given the lack of information on underlying data, partly due to the focus of the paper from which the method is taken (estimation of accumulation in different organisms within a food web).

**Table 4-5: Allometric equations used to estimate uptake rate constants.**

| Equation[1)] | Summary | Chemicals in training set | Log $K_{ow}$ | Ref |
|---|---|---|---|---|
| $k_1 = 520 \times W^{-0.32}$ | Derived using 29 data points; $r^2$ 0.85; rate constant data from a combination of gill perfusion studies in rainbow trout and *in vivo* studies in guppy. Perfused gill studies were carried out at 12ºC using fish of average weight 54 g or 109 g. Uptake rate constants were obtained *in vivo* in guppy from experiments carried out by Opperhuizen (65), Opperhuizen and Voors (76) and de Voogt (77) with a fish weight around 0.1 g. Data for phenol, octachloronaphthalene and octachlorodibenzo-*p*-dioxin were not used in equation derivation ($k_1$ for phenol was lower than predicted using the equation; apparent lack of uptake of octachloronaphthalene and octachlorodibenzo-*p*-dioxin was thought to result from their large molecular size limiting diffusion across gill membranes). | Phenol (not used)<br>Anthracene<br>1,2,3,4-Tetrachlorobenzene<br>Pentachlorobenzene<br>Hexachlorobenzene<br>Hexabromobenzene<br>2,2',5,5'-Tetrachlorobiphenyl<br>Decachlorobiphenyl<br>2,3,5-Trichloroanisole<br>2,3,6-Trichloroanisole<br>2,3,4,5-Tetrachloroanisole<br>Pentachloroanisole<br>Octachloronaphthalene (not used)<br>Octachlorodibenzo-*p*-dioxin (not used)<br>Tetrachloroveratrole | 1.8<br>4.7<br>4.6<br>5.2<br>5.7<br>7.8<br>6.1<br>8.3<br>3.9<br>3.6<br>4.5<br>5.5<br>8.5<br>8.5<br>4.7<br>(Range 3.6 – 8.3) | (42) |
| $k_1 = 445 \times W^{-0.197}$ | Equation derived using 517 data points; $r^2$ 0.11; data set consisted of uptake and depuration rate constants from published literature for wide range of freshwater fish species; chemicals in data set either neutral organic chemicals or weakly ionisable organic chemicals ($pK_a$ indicated these substances could be treated as neutral substance at test and physiological pHs. Data set covered 284 substances and 22 species of fish; fish weight ranged from 0.015 g to 1,060 g. | identities and properties not given but substances included:<br>brominated benzenes<br>brominated toluenes<br>chlorinated anisoles<br>chlorinated anilines<br>chlorinated benzenes<br>hexachlorocyclohexanes<br>isopropyl polychlorinated biphenyls<br>nitrobenzenes<br>nitrotoluenes<br>organochlorine pesticides<br>organophosphorus pesticides<br>polyaromatic hydrocarbons<br>polyaromatic heterocyclic hydrocarbons<br>polybrominated biphenyls<br>polychlorinated alkanes<br>polychlorinated biphenyls<br>polychlorinated dibenzo-*p*-dioxins and furans<br>triaryl phosphates<br>alcohol ethoxylates | Not known | (69) |
| $\ln k_1 = -0.192 \times \ln W + 7.343$ – routine<br>$\ln k_1 = -0.161 \times \ln W + 6.541$ – standard | $R^2 = 0.733$<br>$R^2 = 0.512$<br>Barber (69) analysed the relationship between predicted $k_1$ and fish weight assuming routine and standard respiratory demands[2)]; predictions were made for the same data set as above; models were parameterised for fish species included in the data set; the allometric regression equations were derived based on predictions. | See Barber (69) entry above | Not known | Model 1: (78) in (69) |

| Equation[1)] | Summary | Chemicals in training set | Log $K_{ow}$ | Ref |
|---|---|---|---|---|
| $\ln k_1 = -0.241 \times \ln W + 7.279$ – routine<br>$\ln k_1 = -0.182 \times \ln W + 6.523$ – standard | $R^2 = 0.843$<br>$R^2 = 0.591$<br>See Barber (69) entry above | See Barber (69) entry above | Not known | Model 2: (79) in (69) |
| $\ln k_1 = -0.183 \times \ln W + 7.259$ – routine<br>$\ln k_1 = -0.157 \times \ln W + 6.511$ – standard | $R^2 = 0.594$<br>$R^2 = 0.480$<br>See Barber (69) entry above | See Barber (69) entry above | Not known | Model 3: (80) in (69) |
| $\ln k_1 = -0.274 \times \ln W + 6.795$ – routine<br>$\ln k_1 = -0.228 \times \ln W + 6.345$ – standard | $R^2 = 0.854$<br>$R^2 = 0.736$<br>See Barber (69) entry above | See Barber (69) entry above | Not known | Model 4: (81) in (69) |
| $\ln k_1 = -0.394 \times \ln W + 7.135$ – routine<br>$\ln k_1 = -0.394 \times \ln W + 7.135$ – standard | $R^2 = 0.912$<br>$R^2 = 0.912$<br>See Barber (69) entry above | See Barber (69) entry above | Not known | Model 5: (71) in (69) |
| $\ln k_1 = -0.317 \times \ln W + 8.003$ – routine<br>$\ln k_1 = -0.317 \times \ln W + 8.003$ – standard | $R^2 = 0.904$<br>$R^2 = 0.904$<br>See Barber (69) entry above | See Barber (69) entry above | Not known | Model 6: (82) and (83) in (69) |
| $\ln k_1 = -0.234 \times \ln W + 6.769$ – routine<br>$\ln k_1 = -0.234 \times \ln W + 6.769$ – standard | $R^2 = 0.759$<br>$R^2 = 0.636$<br>See Barber (69) entry above | See Barber (69) entry above | Not known | Model 7: (84) in (69) |
| $\ln k_1 = -0.157 \times \ln W + 5.873$ – routine<br>$\ln k_1 = -0.126 \times \ln W + 5.071$ – standard | $R^2 = 0.065$<br>$R^2 = 0.041$<br>See Barber (69) entry above | See Barber (69) entry above | Not known | Model 8: (85), (86), (50) and (87) in (69) |
| $\ln k_1 = -0.185 \times \ln W + 6.771$ – routine<br>$\ln k_1 = -0.158 \times \ln W + 6.011$ – standard | $R^2 = 0.638$<br>$R^2 = 0.494$<br>See Barber (69) entry above | See Barber (69) entry above | Not known | Model 9: (88) in (69) |
| $\ln k_1 = -0.196 \times \ln W + 5.682$ – routine<br>$\ln k_1 = -0.165 \times \ln W + 4.880$ – standard | $R^2 = 0.649$<br>$R^2 = 0.449$<br>See Barber (69) entry above | See Barber (69) entry above | Not known | Model 10: (89) in (69) |

1) W = fish weight in g. $k_1$ = uptake rate constant (L $kg^{-1}$ $day^{-1}$).

2) Barber (78) considered that under laboratory conditions (limited swimming space and scheduled feedings) the fish's actual respiratory demands may be more accurately reflected by its standard respiratory demand than its routine respiratory demand. In most cases, standard respiration was assumed to be half of routine respiration.

**Table 4-6: Methods relating uptake rate constant to fish weight and log $K_{OW}$**

| Equation[1)] | Summary | Chemicals in training set | Log $K_{OW}$ | Ref |
|---|---|---|---|---|
| $k_1 = \frac{W^{-\kappa}}{\rho_{H_2O} + \frac{\rho_{CH_2}}{K_{OW}} + \frac{1}{\gamma}}$<br>Where $k_1$: Uptake rate constant ($L \cdot kg^{-1} \cdot day^{-1}$).<br>W: Fish weight in kg.<br>κ: Rate exponent = 0.25.<br>$\rho_{H2O}$: Water layer diffusion resistance = $2.8 \cdot 10^{-3}$ $day \cdot kg^{-1}$.<br>$\rho_{CH2}$: Lipid layer permeation resistance = 68 $day \cdot kg^{-1}$.<br>$K_{OW}$: Octanol-water partition coefficient.<br>γ: Water absorption – excretion coefficient = 200 $kg \cdot day^{-1}$ | Based on fugacity concept. More complex parameters for the model were obtained through fitting rate constants on literature data. Model also includes methods for estimating depuration rate constant – collectively known as OMEGA (optimal modelling for ecotoxicological assessment); used in several studies to investigate bioaccumulation potential of organic chemicals. | Not available | Not available | (66) |
| $k_1 = \frac{1}{\left(0.01 + \frac{1}{K_{OW}}\right) \times W^{0.4}}$ | Taken from a BAF model based on a non-steady state mass balance approach. | Not available | Not available | (68) |
| $k_1 = \frac{E_W \times G_V}{W}$<br>$E_W = \frac{1}{\left(1.85 + \frac{155}{K_{OW}}\right)}$<br>$G_V = \frac{1{,}400 \times W^{0.65}}{C_{OX}}$<br>Where $k_1$: Uptake rate constant ($L\ kg^{-1}\ day^{-1}$).<br>$E_W$: Gill uptake efficiency – assumed to be a function of $K_{OW}$.<br>$G_V$: Gill ventilation rate ($L\ day^{-1}$).<br>W: Weight of the organisms (kg).<br>$K_{OW}$: Octanol-water partition coefficient.<br>$C_{OX}$: Dissolved oxygen concentration ($mg\ O_2\ L^{-1}$). This can be estimated as $C_{OX} = (-0.24 \times T + 14.04) \times S$, where T is the temperature in °C and S is the degree of oxygen saturation in water. For water at 12 °C and a minimum 60 % oxygen saturation (as may typically be found in a laboratory BCF test with rainbow trout), the $C_{OX}$ would be 6.7 $mg\ O_2\ L^{-1}$. | Rate constant for uptake via gills is assumed to be a function of the ventilation rate and the diffusion rate of the chemical across the respiratory surface. Arnot and Gobas (24) indicate that this model is applicable to non-ionisable organic chemicals with a log $K_{OW}$ in the approximate range one to nine. | Not available | Not available | (24) |
| $k_1' \approx \frac{10^3 \times W^{-\gamma} \times E}{\rho}$<br>Where E: Transfer efficiency of the chemical.<br>γ: The value is a function of the specific organism and ecosystem function. Recommended values vary between 0.2 and 0.3 for routine metabolism.<br>ρ: Lipid fraction of the organism<br><br>$\log E = -2.6 + 0.5 \times \log K_{OW}$ for log $K_{OW}$ in the range 2 to 5.<br>$\log E = 0.8$ for log $K_{OW}$ in the range 5 to 6.<br>$\log E = 2.9 - 0.5 \times \log K_{OW}$ for log $K_{OW}$ in the range 6 to 10. | From a model for calculating the concentration of a chemical in a generic aquatic food chain. Uptake rate constant equation is a combined and simplified derivation of separate equations for ventilation volume, respiration rate amongst others using certain assumptions for parameters within these equations. Author considered transfer efficiency (E) across gill membranes to depend on chemical properties (log $K_{OW}$), steric properties and molecular weight; transfer efficiency expected to decrease with increasing log $K_{OW}$. Equations for <10 – 100 g fish given here for E. | Not available | Not available | (50) |

| Equation[1)] | Summary | Chemicals in training set | Log $K_{OW}$ | Ref |
|---|---|---|---|---|
| $k_1 = 0.343 \times \left(\frac{1{,}400 \times W^{-0.4} \times K_{OW}}{100 + K_{OW}}\right)^{1.048}$ – routine respiratory<br>$k_1 = 0.401 \times \left(\frac{1{,}400 \times W^{-0.4} \times K_{OW}}{100 + K_{OW}}\right)^{1.025}$ – standard respiratory | Equation based on Barber's (69) calibration of the Gobas and Mackay (71) model (Model 5 above) using the same experimental database. | See (69) | See (69) | (71)<br>(69) |

1) $k_1$ = uptake rate constant (L kg$^{-1}$ day$^{-1}$), W = fish weight in g. $K_{OW}$ = octanol-water partition coefficient.

**Table 4-7: Methods that related uptake rate constant to log $K_{OW}$**

| Equation[1)] | Summary | Chemical in training set | Log $K_{OW}$ | ref |
|---|---|---|---|---|
| $\log k_1 = 0.337 \times \log K_{OW} - 0.373$ | Based on an equation relating the fish BCF to log $K_{OW}$ derived by Mackay (90) and a regression equation developed related the depuration rate constant to log $K_{OW}$. Fish species included guppy, goldfish and rainbow trout. | Included:<br>chlorinated benzenes<br>chlorinated biphenyls<br>tetrachloroethane<br>carbon tetrachloride<br>diphenyl ether<br>biphenyl | range 2.60 - 6.23 | (72) |
| $k_1 = \frac{0.048 \times K_{OW}}{(0.00142 \times K_{OW} + 12.01)}$ | Re-analysis of the above data. | See above | See above | (73) |
| $\log k_1 = 0.147 \times \log K_{OW} + 1.98$ | Fish species included guppy and rainbow trout | Not available | Not available | (74) |
| $\log k_1 = 0.122 \times \log K_{OW} + 2.192$ | | included polychlorinated biphenyls<br>chlorobenzenes. | | (75) |

1) $k_1$ = uptake rate constant (L kg$^{-1}$ day$^{-1}$), $K_{OW}$ = octanol-water partition coefficient.

**Table 4-8: Method relating uptake rate constant to elimination rate constant, fish lipid and log $K_{OW}$**

| Equation | Summary | Chemical in training set | Log $K_{OW}$ | ref |
|---|---|---|---|---|
| $\frac{1}{k_1} = \frac{V_F}{Q_W} + \frac{\left(\frac{V_F}{Q_L}\right)}{K_{OW}} = \frac{1}{(L \times K_{OW} \times k_2)}$<br>Where $k_1$: Uptake rate constant from water (L kg$^{-1}$ day$^{-1}$).<br>$k_2$: Elimination (depuration) rate constant (day$^{-1}$).<br>$V_F$: Fish volume (L).<br>L: Fish lipid content (as a fraction).<br>$K_{OW}$: Octanol-water partition coefficient.<br>$Q_W$: Transport parameter that expresses water phase conductivity (L day$^{1}$).<br>$Q_L$: Transport parameter that expresses lipid phase conductivity L day$^{-1}$). | Fugacity-based mass balance model developed for food webs where uptake occurs via diffusion from water and from diet, and depuration occurs via respiration, egestion and metabolism, taking into account growth dilution.<br>Simplified form can be run to simulate uptake from water into a single organism. Rate constants for uptake via the gill ($k_1$) and elimination rate constants ($k_2$) are estimated in the model using the indicated correlation equation derived by Gobas and Mackay (71) and Gobas (91).. | Not available | Not available | (67) |

246. Crookes and Brooke (36) took available bioconcentration test data from three datasets and compared the studies' calculated uptake rate constants with the uptake rate constants estimated using each method. The first dataset included 169 data points for 108 substances (log $K_{OW}$ range 0.19 – 8.2), for 14 fish species with weights varying between 0.01 and 700 g (majority in the 0.1 – 18 g range). The second dataset included 18 data points for nine substances (log $K_{OW}$ range 2.59 – 5.1), for four fish species with weights varying between 0.2 and 7 g. The third dataset included 23 data points for 18 substances (log $K_{OW}$ range 3.3 – 5.73), for three fish species with weights varying between 0.19 and 2.5 g (this set also included duplicate data with the first dataset, so these duplicates were removed). Further information on the three datasets can be found in Annex 4 and Annex 5. Any ionisable substances were only included in the first dataset if their p$K_a$ indicated they were predominantly in the unionised form at pH values relevant for bioaccumulation testing.

247. To further investigate the comparison, for each model the ratio of the estimated $k_1$ to the measured $k_1$ was plotted against log $K_{OW}$ (so a ratio > 1 shows that the model over-predicted the $k_1$, and < 1 indicates an under-prediction). Statistical analysis was carried out by converting the estimated $k_1$:measured $k_1$ ratios to log values for each study and method, and calculating the mean, median and standard deviation for the log ratios for each model (ideally a method would have a mean log ratio near zero with a low standard deviation to provide a good uptake rate constant estimate). Because it was found that the models performed relatively poorly for low $K_{OW}$ substances, the dataset was divided so that the analysis was also carried out on a reduced dataset for chemicals with a log $K_{OW}$ > 3.5 only (maximum log $K_{OW}$ 8.2). Since most studies used juvenile test fish and information on actual growth was not available, a second dataset was developed to account for fish growth during the uptake phase. For this second dataset a set of log ratios was developed using $k_1$ predictions based on twice the initial fish weight for models with weight as an input parameter (again with chemicals with log $K_{OW}$ >3.5).

248. The review (36) found that the correlation between predicted $k_1$ and measured $k_1$ was relatively poor, with $r^2$ values for the regression analysis being very low and there being a large scatter of data in the regression analysis. The log ratio analysis for the reduced dataset of chemicals (i.e. log $K_{OW}$ >3.5) was used to rank the models in order of performance. 12 models (or 13, depending on which measure of fish weight was used as the input) out of the 22 equations tested, showed log ratio values between -0.15 and 0.15 (corresponding to actual ratios of 0.70 to 1.41). This ranking is shown in in 249. Despite the exercise above, no one model stood out as being more applicable than any other for the test data. Both the type of models that were included as well as their ranking order showed differences, depending on whether the initial weight or twice initial weight was used for the models that used weight as an input parameter; no obvious pattern was identified. The standard deviations for all the model predictions were rather high (for example a mean log10 ratio of zero with a standard deviation of 0.5 log units is equivalent to a range of the actual ratio ± one standard deviation of 0.32 to 3.2, that is, under- or overestimation by a factor of three). This means that although the mean ratio from these methods is close to one, for any one substance there will be a large uncertainty in the predicted *k1*. Thus, estimated BCFs for a particular test substance using models in this approach could span a large range (two or three orders of magnitude). The Excel spreadsheet that accompanies this guidance includes BCF estimation for the 13 models identified according to initial fish weight.

250. More recently, Hashizume et al (52) conducted a similar exercise with 149 chemicals with measured bioconcentration data collected under Japan's Chemical Substances Control Law. Hashizume and co-workers found that estimations according to Arnot and Gobas (68), Arnot and Gobas (24) (both depending on log *KOW* and fish weight) and Tolls and Sijm (75) (depending on log *KOW*) gave relatively good ratios of predicted *k1*: measured *k1* (i.e. close to 1). Their findings were rather similar to those of Crookes and Brooke (36) (summarised in (92)). They also found that linear regressions of plots of predicted BCF (for the selected methods) with measured BCF were heavily influenced by data for hexachlorobenzene, because hexachlorobenzene had greatly higher BCFs than the other substances in the

training set they used. If the hexachlorobenzene data were removed, correlations became statistically non-significant (i.e. no statistical relationship between the estimated and measured BCFs was apparent).

Table 4-9 for initial fish weight and twice initial fish weight. For each method, the required input parameters are identified.

249. Despite the exercise above, no one model stood out as being more applicable than any other for the test data. Both the type of models that were included as well as their ranking order showed differences, depending on whether the initial weight or twice initial weight was used for the models that used weight as an input parameter; no obvious pattern was identified. The standard deviations for all the model predictions were rather high (for example a mean $\log_{10}$ ratio of zero with a standard deviation of 0.5 log units is equivalent to a range of the actual ratio ± one standard deviation of 0.32 to 3.2, that is, under- or overestimation by a factor of three). This means that although the mean ratio from these methods is close to one, for any one substance there will be a large uncertainty in the predicted $k_1$. Thus, estimated BCFs for a particular test substance using models in this approach could span a large range (two or three orders of magnitude). The Excel spreadsheet that accompanies this guidance includes BCF estimation for the 13 models identified according to initial fish weight.

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**Table 4-9: Ranking of Methods used to estimated $k_1$ that had log values ($k_1$ predicted:$k_1$ measured) between -0.15 and 0.15. Taken from Crookes & Brooke (35)**

| | Input based on initial fish weight | | Input based on twice initial fish weight | |
|---|---|---|---|---|
| **Rank** | **Model and summary statistics (Mean log ratio ± standard deviation)** | **Model Input parameters** | **Model and summary statistics (Mean log ratio ± standard deviation)** | **Model Input parameters** |
| 1. | Hayton and Barron (84)<br>–0.04 ± 0.48 | weight | Erickson and McKim (80)<br>0.04 ± 0.48 | weight |
| 2. | Erickson and McKim (80)<br>0.08 ± 0.48 | weight | Barber et al. (79)<br>0.04 ± 0.48 | weight |
| 3. | Barber et al. (79)<br>0.09 ± 0.48 | weight | Barber (78)<br>0.05 ± 0.48 | weight |
| 4. | Barber (69) – observed<br>–0.09 ± 0.48 | weight | Hayton and Barron (84)<br>–0.10 ± 0.48 | weight |
| 5. | Barber (78)<br>0.10 ± 0.48 | weight | Barber (69) – observed<br>–0.15 ± 0.48 | weight |
| 6. | Streit and Sire (88)<br>–0.13 ± 0.48 | weight | Erickson and McKim (81)<br>–0.05 ± 0.49 | weight |
| 7. | Erickson and McKim (81)<br>0.02 ± 0.49 | weight | Hendriks et al. (66)<br>–0.02 ± 0.50 | weight, log $K_{OW}$ |
| 8. | Hendriks et al. (66)<br>0.05 ± 0.50 | weight, log $K_{OW}$ | Tolls and Sijm (75)<br>0.13 ± 0.50 | log $K_{OW}$ |
| 9. | Tolls and Sijm (75)<br>0.13 ± 0.50 | log $K_{OW}$ | Spacie and Hamelink (74)<br>0.06 ± 0.51 | log $K_{OW}$ |
| 10. | Sijm et al. (42)<br>–0.02 ± 0.51 | weight | Sijm et al. (42)<br>–0.12 ± 0.51 | weight |

| | | | | |
|---|---|---|---|---|
| 11. | Spacie and Hamelink (74)<br>0.06 ± 0.51 | log $K_{OW}$ | Barber (69) – calibrated<br>–0.03 ± 0.54 | weight |
| 12. | Barber (69) – calibrated<br>0.10 ± 0.54 | weight | Thomann (50)<br>–0.07 ± 0.55 | weight, log $K_{OW}$ |
| 13. | Thomann (50)<br>–0.15 ± 0.55 | weight, log $K_{OW}$ | – | – |

251. In conclusion, no one model can be recommended over the others and results must be used with caution, with reference to assumed applicability domains. If this method is used, estimates of $k_1$ should be derived according to all the methods selected above to give a range of BCFs.

252. For those models that use fish weight as an input, the most representative value should be used for studies in which growth occurs. Since fish weights are only measured before the test is conducted, at the end of the uptake phase and during the depuration phase, a study's overall growth rate can be used to estimate the time-weighted mean fish weight during the uptake phase (cf. Annex 4). This estimated mean value should be used in the $k_1$ models with weight as an input.

253. This method (and method 2 below) may be less reliable for substances that are:

- molecularly large or bulky (e.g. more than two aromatic rings and fully halogenated or have molecular weights > 1100 or maximum molecular lengths > 4.3 nm)
- high log $K_{OW}$ (approx. 9 or above)
- low assimilation efficiency[15] observed in the study

This is because these test substances are likely not within their indicative applicability domains. This includes substances like octachloronaphthalene, decabromodiphenylether and octachlorodibenzo-*p*-dioxin that were discounted from training sets. This will be apparent because $k_1$ and BCF estimates according to most method 1 models and method 2 will be orders of magnitude higher than the method 3 estimate. This results from an over-estimation of $k_1$ as factors affecting passive diffusion are not accounted for in the models.

254. When using results from the method 1, results according to the various models should be reported as a range, excluding outliers. Outliers can be identified by comparison with estimates from methods 2 and 3 (> a factor of 2 larger or smaller) and by considering the substance's log $K_{OW}$. If the log $K_{OW}$ is towards the higher end of the indicative applicability domain, some models (e.g. (66) and (50)) may give markedly lower estimates. Consideration should also be given to the test substance structure in relation to those structures in the models. If the method 1 range is narrow and spans a criterion of concern for bioaccumulation, the information can be used to build the case that the substance may fulfil that criterion. As stated in Section 4.6.3.1, the mean of the various models should not be used since the different models work in different ways. However, in these cases where the range spans a regulatory criterion the median value from the range could be used, or a specific model estimate if it can be justified.

255. For both this method (method 1) and the following method (method 2), the $k_2$ used must be normalised for lipid (to 5%) as well as growth corrected, as it has been shown that depuration rates can depend on fish lipid content (see Section 4.4). How to go about this data manipulation is described in Annex 4.

[15] As starting working guide a value of $\alpha <$ approx. 0.1 is proposed based on expert judgement

*Pros and Cons of the uptake rate constant estimation method*

256. Some of the issues for this method are discussed in Annex 8 to OECD TG 305 (1). The paragraphs below summarise these and other issues relevant for the uptake rate constant estimation method.

257. Pros of the uptake rate constant estimation method include:

- A general approach that can be used with readily available input data from a dietary study..
- The large number of available models with differing input parameters, allow flexibility..
- In many models large and varied underlying datasets covering different ranges and types of substances and sizes and species of fish.

258. Cons of the uptake rate constant estimation method include:

- The large number of available models give a wide range of results for $k_1$, differing by a factor of two to three for those assessed by Crookes and Brooke (36), with no reliable way of discriminating between estimates based on combinations of substance and model.
- Limited information for some models on training and validation datasets and so limited ways of judging a model's applicability domain with respect to test substance.
- Respiratory uptake is taken to be a thermodynamic process largely driven by passive diffusion across the gill. Since the models do not take account of test substance-related factors that may affect passive diffusion like molecular weight and size, or ionisability, resulting uptake rate constants may be overestimated unless care is taken with regard to a substance's structure and properties. In extreme cases, substances that only very poorly absorb across the gut and so have very low dietary BMFs may however have high predicted BCFs based on high estimated uptake rate constants using this method. Hence information on the applicability domain is critical.
- Low correlation when models were tested with data from available BCF study datasets.
- For high log $K_{OW}$ substances, Gobas and Lo (61) make some assumptions about gill respiration. They assume $k_1$ will be low, and to be identical or approach zero as log $K_{OW}$ approaches infinity. Therefore this contradicts the current approach of method 1. This requires further consideration.

259. The uptake rate constant estimation method, like the two methods that follow, may also be affected by issues around bioavailability in terms of the (BCF) training sets used to derive each equation, since it is not clear if BCFs were based on dissolved or total concentrations in water.

*4.6.3.2 Method 2 – Relating depuration rate constant directly to BCF: Using available BCF data to determine $k_2$ values equivalent to regulatory thresholds of 2000 and 5000*

260. Studies have attempted to relate the laboratory depuration rate constant directly to the laboratory BCF so that the depuration rate constant (or elimination half-life) can be used as a determinand of bioaccumulation in its own right (see (92) (93)). This approach can thus be used to estimate a BCF from dietary study depuration data alone; the approach is similar to that described above in that the estimation of an uptake rate constant is implicit in the derived relationship between depuration rate constant and BCF.

261. To investigate the relationship between various measures of depuration rate constant and BCF, Brooke and Crookes (92) took three BCF datasets (as used previously by the same authors (36) for testing the uptake rate constant estimation method – see Section 4.6.3.1 and Annex 4 for further information on the three datasets). They used the largest dataset as the training set to derive equations relating depuration directly to BCF, and used the other two datasets for validation.

262. Depuration rate constants (either "raw": $k_2$; or lipid normalised to a lipid content of 5%: $k_{2L}$) and BCF values (either "raw": BCF or lipid normalised to a lipid content of 5%: $BCF_L$) were log transformed and plotted against one another. Analysis by linear regression of each plot yielded a number of equations (see Table 4-10 below). An obvious outlier in the regression analyses, octachlorodibenzo-*p*-dioxin, was identified early on and was excluded in subsequent analysis.

263. Lipid normalisation was carried out to try to remove some of the variability in the analysis; growth rate could not be taken into account for the depuration rate constants, since little information was available (it was thought that many of the studies were conducted before growth correction of kinetic data became usual practice).

264. Table 4-10 below lists the equations derived by Brooke and Crookes (92), and what depuration rate constant values (with 95% confidence intervals derived from the regression analysis) would constitute BCF values of 2000 or 5000 L/kg, as these are commonly used trigger values for bioaccumulative chemicals. Equivalent elimination half-lives are given in parentheses.

**Table 4-10: Relationships between depuration rate constants ($k_2$) and BCF**

| | | | Depuration rate constant corresponding to a BCF (L/kg) of: | | | |
|---|---|---|---|---|---|---|
| | | | 2000 | | 5000 | |
| Relation-ship[1)] | Equation | | $k_2$ ($d^{-1}$) | 95% CI | $k_2$ ($d^{-1}$) | 95% CI |
| $k_2$ & BCF | log BCF = –1.2394 × log $k_2$ + 2.3706<br>$R^2$ = 0.78 | Equation 4-27 | 0.178<br>[$t_{½}$: 3.9 d] | 0.171 – 0.185 | 0.085<br>[$t_{½}$: 8.2 d] | 0.083 – 0.086 |
| $k_2$ & $BCF_L$ | log $BCF_L$ = –1.1892 × log $k_2$ + 2.2889<br>$R^2$ = 0.77 | Equation 4-28 | 0.141<br>[$t_{½}$: 4.9 d] | 0.138 – 0.144 | 0.065<br>[$t_{½}$: 10.7 d] | 0.062 – 0.068 |
| $k_{2L}$ & $BCF_L$ | log $BCF_L$ = –1.2220 × log $k_{2L}$ + 2.3935<br>$R^2$ = 0.76 | Equation 4-29 | 0.181<br>[$t_{½}$: 3.8 d] | 0.175 – 0.188 | 0.085<br>[$t_{½}$: 8.2 d] | 0.083 – 0.088 |

[1)] $BCF_L$: Lipid normalised BCF; $k_{2L}$: Lipid normalised $k_2$

265. The equations were then tested using the three datasets separately and together (bearing in mind that the first dataset, used to derive the equations, did not constitute an independent validation set). The authors included two main caveats:

- It was probable that the $k_2$ value was dependent on the fish species and the weight of fish; this would effectively set the applicability domain of the model.
- The confidence intervals related only to uncertainty in the regression analysis, and did not capture uncertainty in the underlying database of BCF values.

266. The suitability of BCF values predicted from depuration rate constants according to each equation was investigated by comparing the paired predicted and measured BCFs against the PBT BCF triggers used in EU member countries for a bioaccumulative ("B": 2000 L/kg) or very bioaccumulative ("vB": 5000 L/kg) substance. The following paragraphs summarise this exercise as it is illustrative of the method's predictive utility. Each equation gave a number of "false negatives" and "false positives" for each of the three datasets. False negatives and positives break down into six possibilities:

| | | Predicted | | |
|---|---|---|---|---|
| | | **not B** | **B (BCF >2000<5000)** | **vB (BCF >5000)** |
| **Measured** | **not B** | – | B (not B) | vB (not B) |
| | **B (BCF >2000<5000)** | not B (B) | – | vB (B) |
| | **vB (BCF >5000)** | not B (vB) | B (vB) | – |

267. The number of substances in the datasets with experimental BCFs >2000 (or lipid normalised BCFs > 2000) is as follows:

- First dataset (used for training): 49 (or 44 for $BCF_L$) out of 169 data points
- Second dataset (used for validation): 7 (or 6 for $BCF_L$) out of 18 data points
- Third dataset (used for validation): 3 (or none for $BCF_L$) out of 23 data points

The number of substances in the data sets with experimental BCFs > 5,000 (or lipid normalised BCFs > 5000) is as follows:

- First dataset (used for training): 33 (or 28 for $BCF_L$) out of 169 data points
- Second dataset (used for validation): 2 (or 3 for $BCF_L$) out of 18 data points
- Third dataset (used for validation): none (and none for $BCF_L$) out of 23 data points

268. Table 4-11 summarises the analysis carried out in relation to the BCF triggers used in the EU for the first equation and each of the datasets (summarised from (92))), using the abbreviations listed above for each of the six combinations of incorrect predictions. Further details are available in (92), where the authors looked at each incorrect prediction in terms of the specific data point (i.e. test chemical).

**Table 4-11: Analysis results from the relationship between $k_2$ and BCF (excluding outlier). B: BCF = 2000 ($k_2$ = 0.178 $d^{-1}$); vB: BCF = 5000 ($k_2$ = 0.085 $d^{-1}$).**

| **Dataset** | **Number of data point [substances] predictions miscategorised against the EU PBT BCF criteria with respect to test result** | | | | | |
|---|---|---|---|---|---|---|
| | **Not B (B)** | **Not B (vB)** | **B (not B)** | **B (vB)** | **vB (not B)** | **vB (B)** |
| Dataset 1 (training set); data points = 169 [substances = 108] | 13 [9] **Error! eference source not found.** | 1 [1] | 5 [2] **Error! eference source not found.** | 6 [6] | 2 [1] | 1 [1] |
| Dataset 2 (validation); data points = 18 [substances = 9] | – | – | 7 [4] [a] | – | 1 [1] | – |
| Dataset 3 (validation); data points = 23 [substances = 18] | 5 [3] | – | – | 2 [1] | – | – |

[1)] In the report (92), four predictions or measured data are borderline as to whether they would fulfil the EU B or vB criteria. In these cases, the values are considered B rather than vB for simplicity.

Overall, with respect to EU categorisations of B or vB, Equation 4-27 would miscategorise 43 out of 210 data points (equating to 29 out of 135 substances), or 20% of the data points (equating to 21% of the substances). Similar exercises with the Equation 4-28 and Equation 4-29 gave similar results.

269. The analysis by Brooke and Crookes (92) shows that the incorrect predictions arise from a similar subset of the database in each case, as might be expected given the similarity in the $k_2$ trigger values. It appears that in total, across the three equations, 30 distinct chemicals with 47 discrete data points are predicted incorrectly. A number of these chemicals were esters, or were substances with BCF values that meant assignment into the EU PBT categories of either B or vB was equivocal. Brooke and Crookes (92) concluded that this method for identifying substances as B or vB against the PBT criteria used in the EU appeared to show promise, with a large proportion of the available data set being correctly categorised.

However, it was noted that the large number of false positive and false negative assignments was a problem and that the approach might best be used as part of a weight of evidence/expert judgement-type approach.

270. Brooke and Crookes (92) then compared estimated BCFs using Equation 4-27 (relating non-lipid normalised values of BCF and $k_2$; see Table 4-10) with those estimated using the uptake rate constant estimation method, discussed above in Section 4.6.3.1, for four of the substances tested in the OECD TG 305 ring test (44). Overall, the estimated BCFs were similar according to both methods and compared reasonably well with the available measured data.

271. The applicability domain of the derived equation will be related to both the type of substance and the fish species and weight. A wide range of chemicals was used in the training set, spanning a log $K_{OW}$ range of 0.19 – 8.2. The majority of fish weights fell in the 0.1 – 18 g range, whilst the majority of studies were conducted in fathead minnow, carp, guppy, and rainbow trout.

272. For use in estimating a BCF from dietary study data in general, the recommendation of Brooke and Crookes (92) to use it as part of a weight of evidence approach is echoed here. Although in theory the equations mean that any $k_2$ value can be translated to an equivalent BCF value, the approach described here is principally to be used to assess whether the $k_2$ is above/below a regulatory BCF threshold such as 2000 or 5000.

273. For both this method and the previous one (see Section 4.6.3.1), the $k_2$ used must be normalised (to 5%) for lipid as well as growth corrected, as it has been shown that depuration rates can depend on fish lipid content (see Section 4.4). This means the Equation 4-29, relating $k_{2L}$ and $BCF_L$, should be used. How to go about this data manipulation is described in Annex 4 and this is incorporated in the Excel spreadsheet that automates BCF estimation calculations.

*Pros and Cons of Relating depuration rate constant directly to BCF*

274. Because this second method is closely related to the first method described above (see Section 4.6.3.1) many of the pros and cons overlap.

Pros of relating depuration rate constant directly to BCF include:

- A general and simple approach that can be used with readily available input data from a dietary study.
- Large underlying datasets, covering different ranges and types of substances, and sizes and species of fish.
- Possibility to derive relationships for specific fish species and sizes that are being tested, assuming BCF studies for these species and fish sizes are available.

275. Cons of relating depuration rate constant directly to BCF include:

- The large variety of fish species and sizes may not relate well to dietary study species and fish size and may result in low accuracy of predictions.
- Uncertainties in the underlying datasets owing to limited test conduct information.
- Respiratory uptake is taken to be a thermodynamic process largely driven by passive diffusion across the gill. As was described above for the uptake rate constant estimation method, this method does not take account of test substance-related factors that may affect passive diffusion like molecular weight and size, or ionisability. This may result in overestimated BCF values. In

extreme cases, substances that only very poorly absorb across the gut and so have very low dietary BMFs may however have high BCFs predicted because of the underlying basis of this method. Hence, information on the applicability domain is critical.

276. Relating depuration rate constant directly to BCF, like the previous and next method, may also be affected by issues around bioavailability in terms of the (BCF) training sets used to derive each equation, since it is not clear if BCFs were based on dissolved or total concentrations in water.

*4.6.3.3 Method 3 – Correlating BMF with BCF: Using a correlation of dietary BMF and BCF results to interpolate other BMF results*

277. Instead of estimating an uptake rate constant or correlating the depuration rate constant directly to a BCF, a different approach is to correlate the measured dietary BMF itself to a BCF. Highly relevant to this approach are the theoretical relationships of Mackay et al. (60) described above (Section 4.6.1).

278. Although based on limited data points, Inoue et al. (57) demonstrated a relationship between dietary BMF and BCF in common carp for studies following the dietary OECD TG 305 method and the aqueous OECD TG 305 (previous OECD TG 305E; i.e. BCF is based on steady state) method for nine poorly water-soluble chemicals. The chemicals considered, their octanol-water partition coefficients, and measured BCFs and BMFs are shown below in Table 4-12.

279. Full details of the dietary studies are given in (57), and appear to have been well conducted. It is worth noting the following: dietary exposure to five substances (Binox M, pentachlorobenzene, 2,4-dichloro-1-(4-nitrophenoxy)benzene, Solvent blue 36 and *N,N'*-di-2-naphthyl-p-phenylenediamine) was carried out in separate tests, whereas exposure to the remaining substances (musk xylene, *o*-terphenyl and methoxychlor) was carried out in one test with combined exposure (as was employed in the OECD TG 305 ring test (44)). Hexachlorobenzene was included in each test. This enabled its absorption efficiency being used as a way of verifying the suitability of the feed spiking technique (as recommended in OECD TG 305 (1)). This means that there were six results for hexachlorobenzene in total. In each study, the feeding rate was 3% and feeding was split over two rations per day, offered 30 minutes apart. Growth and lipid content were taken into account in the calculated results.

**Table 4-12: Summary of dietary BMF and BCF values used in the comparison by Inoue et al. (57).**

| :Substance | Log $K_{OW}$ | Lipid normalised (5%) $BCF_L$ (L/kg) | Lipid and growth corrected $BMF_{KgL}$ |
|---|---|---|---|
| Hexachlorobenzene | 5.86 | 27000 | 1.39 [1)] |
| Binox M | 8.99 | 8100 | 1.43 |
| Pentachlorobenzene | 5.22 | 7400 | 0.41 |
| Musk xylene | 4.45 | 6900 | 0.377 |
| 1,4-bis(isopropylamino)anthraquinone (Solvent blue 36) | 6.07 | 5300 | 0.316 |
| *N,N'*-di-2-naphthyl-p-phenylenediamine (DNPD) | 6.39 | 1500 | 0.0802 |
| 2,4-dichloro-1-(4-nitrophenoxy)benzene (NIP) | 4.32 | 4900 | 0.179 |
| *o*-terphenyl | 5.52 | 1200 | 0.0912 |
| Methoxychlor | 5.67 | 810 | 0.034 |

[1)] Inoue et al averaged (mean) the six values for hexachlorobenzene.

**Table 4-13: Key results from dietary BMF studies for nine poorly water soluble substances (taken from (57))**

| | Single chemical exposure studies | | | | |
|---|---|---|---|---|---|
| **Parameter** | **Binox M [hexachloro-benzene]** | **pentachloro-benzene [hexachloro-benzene]** | **N,N'-di-2-naphthyl-p-phenylenediamine [hexachlorobenzene]** | **Solvent blue 36 [hexachloro-benzene]** | **2,4-dichloro-1-(4-nitrophenoxy)benzene [hexachlorobenzene]** |
| $k_2$ ($d^{-1}$) | 0.0472 [0.0918] | 0.213 [0.0871] | 0.255 [0.068] | 0.200 [0.0686] | 0.269 [0.0846] |

| | | | | | |
|---|---|---|---|---|---|
| absorption efficiency | 0.336 [0.86] | 0.777 [0.76] | 0.19 [0.75] | 0.564 [0.728] | 0.497 [0.776] |
| $k_{2g}$ ($d^{-1}$) | 0.0241 [0.0686] | 0.196 [0.070] | 0.226 [0.0392] | 0.170 [0.0389] | 0.240 [0.0555] |
| $BMF_{KgL}$ | 1.43 [1.28] | 0.41 [1.12] | 0.0802 [1.83] | 0.316 [1.79] | 0.179 [1.21] |

| **Parameter** | **Combined exposure study[1)]** | | | |
|---|---|---|---|---|
| | **musk xylene** | ***o*-terphenyl** | **methoxychlor** | **hexachlorobenzene** |
| $k_2$ ($d^{-1}$) | 0.130 | 0.372 | 0.376 | 0.0571 |
| absorption efficiency | 0.541 | 0.43 | 0.162 | 0.488 |
| $k_{2g}$ ($d^{-1}$) | 0.106 | 0.348 | 0.353 | 0.0333 |
| $BMF_{KgL}$ | 0.377 | 0.0912 | 0.034 | 1.08 |

[1)] Inoue et al. (57) included variances with the results, but these have been omitted here.

280. Table 4-13 summarises the results for key parameters from the dietary study for each substance separated by whether studies were conducted with single or combined exposure.

281. Results for BCF studies were taken from the Japanese Chemical Substances Control Law test reports and normalised to a lipid fraction of 5%. Few further details were available on the BCF studies in Inoue et al. (57). While it is possible to estimate the error related to the fitting of the linear regression, the error associated with the individual studies themselves is not known.

282. The values in Table 4-13 have been ordered with decreasing lipid normalised $BCF_L$; the dietary BMFs follow the same trend apart from the results for hexachlorobenzene and Binox M and for 2,4-dichloro-1-(4-nitrophenoxy)benzene and *o*-terphenyl. In the case of hexachlorobenzene, it is worth noting that the value reported in the table is a mean of the six reported hexachlorobenzene values which ranged 1.08 – 1.83. Two measured dietary BMFs for hexachlorobenzene were higher than that reported for Binox M (1.79 and 1.83).

283. Inoue et al (57) plotted the log transformed BCF and dietary BMF data and analysed the plot by linear regression. They found a reasonable correlation, with the following equation. This equation is included in the Excel spreadsheet that automates BCF estimation.

$$\log BCF_L = 0.828 \cdot \log BMFK_{gL} + 4.12 \ (r^2 = 0.873) \qquad \text{Equation 4-30}$$

In the regression analysis the log value of the mean $BMF_L$ value for hexachlorobenzene was used, as opposed to each of the six individual results. Using the mean for hexachlorobenzene rather than the individual points may have an influence on the regression, but this has not been investigated.

284. From this regression, it can easily be estimated that a $BCF_L$ of 2000 (the B trigger in the EU) will correspond to a $BMF_{gL}$ of around 0.1 and a $BCF_L$ of 5000 (the vB trigger in the EU, bioaccumulation trigger in Canada and highly bioaccumulative trigger in the US and Japan) will correspond to a $BMF_{gL}$ of around 0.3. For a $BMF_{gL}$ of 1, a $BCF_L$ of around 13,000 would be indicated. These correlations are discussed further below.

285. Reported BCFs in Inoue et al. (57) are based on the steady state approach. Theoretically a steady state BCF and a kinetic BCF should be the same. However, an important difference that can influence a steady state BCF compared with a kinetic BCF is fish growth, as growth dilution is not taken into account in a steady state BCF. In cases where fish growth is more rapid and uptake is slower (possibly because depuration is relatively rapid), steady state may be approached but not actually reached (see the discussion in (36)). This means in practice kinetic and steady state BCFs may not always be the same (steady state

BCFs would tend to be lower than kinetic BCF). Since kinetic BCFs were not available for these chemicals in study set ups similar to that used by Inoue et al. (57), it is not possible to investigate the possible effect this might have on the derived correlation between BCF and BMF.

286. Mackay et al. (60), in giving illustrative food web relationships for their derivations (see paragraphs 227–230), assume a gill respiration uptake rate constant of 200 $d^{-1}$ and a dietary uptake rate constant of 0.01 $d^{-1}$ for a small fish. Using these assumed values in Equation 4-26, BCF values of 2000 and 5000 return BMF values of 0.10 and 0.25, respectively. It can be seen that these values compare relatively well with those from the regression analysis according to Inoue et al. (57) (bearing in mind that the Mackay et al derivation does not consider fish growth or metabolism).

287. In terms of applicability domain, the main limiting factors are that the equation was derived for carp only and the dataset is fairly limited, for example, there are significant structural differences between the substances tested. As the equation essentially relates rates of uptake via different routes (see discussion below under pros and cons), differences between dietary and gill uptake for other species may affect the relationship between dietary BMF and BCF for those other species. Since applicability should relate to biological factors alone (i.e. bioavailability and uptake rates as they depend on substance-specific factors are accounted for in the dietary test), in theory this method is less sensitive to different test chemical identities than the previous two methods.

*Pros and Cons of correlating dietary BMF with BCF*

288. Pros of correlating dietary BMF with BCF include:

- Estimations following this approach take account of uptake in the dietary study, unlike the two approaches described above where uptake and depuration are "decoupled". This means that situations where uptake in reality would be very low based on issues with bioavailability and passive diffusion but predicted uptake rate constants and BCFs are overestimated, are likely to be avoided with this approach.
- This approach could be considered more of a "metrics conversion" than an extrapolation, which is the case with the preceding two methods. If the assumption holds that depuration is the same regardless of uptake route given sufficient time for in vivo distributions to normalise, this method is basically comparing uptake rates between different exposure routes (Mackay et al.'s "equilibrium multiplier" (60), see Section 4.6.2). This could be considered a more "transparent" data transformation.

289. Cons of correlating dietary BMF with BCF include:

- The training set for the regression is very small and generally includes more bioaccumulative chemicals. The latter point may mean that the linear regression may be "skewed"..
- The intercept of the correlation is not x,y = 0. Instead if BMF = 0, BCF is a positive value. One reason for this is the difference in uptake routes. This is because the comparison is between diffusion across aqueous layer at the gill vs. assimilation efficiency in the gut as well as metabolism in the gastro-intestinal tract vs. at the gill.
- The extents of gut metabolism of the different chemicals in the correlation (training set) are not known, which could affect the slope of the correlation. This is also a consideration when applying the correlation to test substance results.
- Error associated with correlation (due to the test results themselves) is not known, which may be significant for the correlation given the small number of data points.

#### *4.6.3.4 Method 4 – Relating to reference substance: Co-dosing with a reference substance with known |BCF*

290. Gobas and Lo (61) propose an alternative way of estimating a BCF value from the dietary test. This builds on earlier work by Lo et al (94). They argue that elimination rates in fish can be related to $K_{OW}$. Based on this relationship, they propose including a non-biotransformable reference substance in the dietary bioaccumulation study to provide a rate constant representing the non-metabolic elimination. By subtracting this from the overall depuration rate constant, a somatic biotransformation rate constant can be calculated for a test chemical. If the $K_{OW}$ of the reference substance is the same as the test substance, it would be possible to use the non-metabolic elimination rate constant directly. Alternatively, a range of non-biotransformable reference substances with log $K_{OW}$ values that span the test chemicals could be included to allow the regression coefficients of the $K_{OW}$–elimination rate relationship to be determined. In Gobas and Lo (61), they extend this thinking to include a relationship between $k_1$ and log $K_{OW}$, which then allows calculation of a BCF value from the dietary test. The effect of their assumptions is that for chemicals with log $K_{OW} \geq 3$, $k_1$ would essentially have the same value in a test. They argue that this is because $k_1$ is largely controlled by the gill ventilation rate of the fish for highly hydrophobic chemicals.

291. In a second aspect related to the use of the depuration rate constant, they propose that the dietary study is performed with a co-dosed reference substance that has a known BCF value established in an OECD TG 305 bioconcentration study that is equivalent to the regulatory threshold (e.g. pentachlorobenzene, BCF ~ 5000). This provides a $k_2$ value in the test that is equivalent to 5000, therefore if the test substance $k_2$ is larger, the BCF will be < 5000, or if it is smaller, the BCF value will be > 5000. The authors highlight that one aspect not addressed by the approach is when gastro-intestinal transformation is significant in the bioaccumulation behaviour. This is because of the difference in uptake routes; while the dietary BMF will be affected, the BCF value would have been unaffected. If such an approach were used it would require analysis of the reference substance during depuration. This is beyond the requirements of the current OECD TG 305 (1), where the reference substance (hexachlorobenzene) is measured only at the start and end of uptake to assess assimilation efficiency. This also means that the method cannot be retrospectively applied, as the additional reference substance feed spiking and analysis will not have been performed[16]. Provided the reference substance is spiked in the same feed as the test substance, no additional fish would be required.

292. The authors' ideas are still very new, and further reflection is needed before deciding on how far to recommend the approach, particularly with respect to $k_1$ estimation. However it is considered that the guidance should include potential methods and therefore the papers have been described here. If the approach is used, the $k_1$ and BCF results should be presented alongside those derived from methods 1-3 above (Sections 4.6.3.1 to 4.6.3.3).

### *4.6.4 Using BCF estimations based on dietary study results*

293. This section of the guidance lays out how the data from a dietary study can be used and includes a decision scheme taking into account these limitations and uncertainties. Worked examples to illustrate the approach are included.

294. For all chemicals tested in the dietary study, BMF (including the growth- and lipid-corrected $BMF_{KgL}$) and associated parameters should be calculated according to OECD TG 305 (1) and Section 4.5 of this guidance. In cases where the dietary BMF indicates that the substance is highly bioaccumulative ($BMF_{KgL} > 1$), or conversely in cases where the dietary BMF is very low ($BMF_{KgL} < 0.01$), and a numeric

[16] This also indicates that ring-testing would be needed to assure the use of the reference substance in this approach.

BCF is not required for the purposes of classification or risk assessment it may not be necessary to estimate a BCF. In most other cases (i.e. $BMF_{KgL}$ falls in the region 0.01 – 1, or risk assessment or classification are necessary), it is likely that a BCF will need to be estimated.

295. When a BCF needs to be estimated, estimates according to all three methods described in Sections 4.6.3.1 – 4.6.3.3 above should be made using the spreadsheet that accompanies this guidance[17]. Due to the uncertainty in the three methods' domains of applicability, it is better to produce predictions according to all methods and then decide which is the most relevant, rather than discount methods first.

296. Section 4.6.3 above shows that currently there is no one method that allows the results of the dietary BMF study to be described in terms of a BCF without some limitations and uncertainties. This means that the models' domains of applicability can at best be described as indicative, because much information is lacking on how models were built and uncertainties remain on how they perform. Table 4-14 summarises the indicative domains of applicability for the three methods (suitable molecular structures, log $K_{OW}$ ranges and species are taken from Sections 4.6.3.1 – 4.6.3.3; assimilation efficiencies are based on observation and expert judgement).

297. This means that for the purposes of regulation BCF estimates from dietary studies cannot be considered definitive and therefore do not have the same status as BCFs from aqueous exposure studies. The estimates' uncertainty also means that in cases where a chemical's bioaccumulative properties are not "clear cut" (i.e. estimated BCF is neither very far below nor very much above a regulatory criterion, and the dietary BMF is neither > 1 nor < 0.01), the data should be used in a weight of evidence approach, especially in PBT assessment. This involves considering other information relevant for bioaccumulation, or in cases where no other bioaccumulation data are available but a substance is toxic and (very) persistent, it could mean taking a precautionary approach to the B criterion.

**Table 4-14 Summary of indicative applicability domains for the three BCF estimation methods**

| Method and reference | Indicative applicability domain | | | |
|---|---|---|---|---|
| | test substance type | Test substance log $K_{ow}$ | Absorption efficiency | Fish species |
| **Method 1** | | | | |
| Hayton and Barron (84)<br>Erickson and McKim (80)<br>Barber et al. (79)<br>Barber (69) – observed<br>Barber (78)<br>Streit and Sire (88) | (cyclic) aliphatics and aromatics; substituents may include halogen, amine, alkyl, alkoxy, nitro, hydroxy groups.<br><br>*Fully halogenated aromatics ≥ 2 rings may be out of the applicability domain.* | Approx. 3.5 – 8.3 | Not available[1)] | Training sets include guppy, rainbow trout other temperate and tropical species |
| Erickson and McKim (81) | | | | |
| Hendriks et al. (66) | | | | |
| Tolls and Sijm (75)<br>Sijm et al. (42)<br>Spacie and Hamelink (74)<br>Barber (69) – calibrated<br>Thomann (50) | | | | |
| **Method 2** | | | | |
| Brooke and Crookes (70) | (cyclic) aliphatics and aromatics; substituents may include halogen, (alkyl)amine, alkyl, alkoxy, nitro, hydroxyl, | Approx. 3 – 8.2 | Not available[1)] | Training set includes rainbow trout, carp, guppy, catfish, bluegill sunfish, flagfish, |

17 In many cases no reference substance will be used in the test (it is not required in OECD TG 305 (1)), which will render the fourth method (Section 4.6.3.4) non-applicable in these cases.

| | | | | |
|---|---|---|---|---|
| | cyanate, sulphonate, phosphonate, carboxylate groups.<br><br>*Esters may be out of the applicability domain.* | | | goldfish, medaka fathead minnow, sheepshead minnow, zebrafish. |
| **Method 3** | | | | |
| Inoue et al. (57) | Because of the basis of the model most hydrophobic and highly hydrophobic chemicals should be in the applicability domain<br><br>*(training set included cyclic aliphatics and aromatics; substituents may include chloro, (alkyl or aryl)amine, alkyl, alkoxy, nitro, hydroxy groups)* | Approx. 4.3 - 9 | Likely applicable for very low to very high values on basis of how the model works<br><br>*(training set 0.16 > α < 0.8)* | Carp<br><br>*applicability unknown for other species* |

[1)] an applicability domain for assimilation efficiency is not available from the literature for these methods. Neither method accounts for factors affecting passive diffusion, therefore the applicability of these methods for substances where very low assimilation efficiency was observed in the dietary study is limited (see also paragraph 258).

298. In cases where method estimates straddle a regulatory bioaccumulation criterion, reanalysis of how $k_{2g}$ and absorption efficiency were derived may be necessary, especially with respect to the use of "non-detect" concentrations in the latter stages of the depuration phase (generally these should not be used to derive these values). Reanalysis of data according to Section 4.5 should be conducted. Factors influencing effective feeding rate and absorption efficiency should also be considered (see Section 4.4).

299. Overall the following are relevant when considering the different BCF estimate(s) in a weight-of-evidence:

- Consider the test substance's structure (and mass and molecular dimensions if available), its log $K_{OW}$ and the absorption efficiency calculated from the dietary study:
    - Is the test substance within the indicative applicability domain of all the methods?
    - Is the substance's absorption efficiency within the indicative applicability domain of all methods?
- How do the BCF (and $k_1$) estimates compare between the methods (and between the model estimates within method 1)?
- What test species was used for the dietary test?
- Was $k_{2g}$ calculated appropriately?

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## ANNEX 1 SOLID-PHASE MICROEXTRACTION (SPME)

Below, exemplary calculations are given on the critical sample volume ($V_C$) that is needed to avoid depletive extraction that may be encountered when freely dissolved concentrations of highly hydrophobic substances are under equilibrium conditions analysed in small volume samples obtained from the system (cf. Section 2.3.2.4). The theoretical considerations are extensively discussed by Zeng and Noblet (17). Some of the useful partitioning equations are reproduced from this publication below to elaborate on this issue.

The degree of depletion that is desired is defined by the critical ratio $r_c$ which is the ratio of the concentration in water after SPME to concentration in water prior to SPME ($r_C = C_W/C_W^0$). Generally the degree of depletion should be minimised to less than 10% ($r_C = 0.9$) of the mass of material in the system, ideally less than 5% ($r_C = 0.95$). This is necessary if one wants to measure the $C_{free}$ that the organisms were exposed to during the study, rather than an erroneous measurement due to the shift in the equilibrium between $C_{total}$ and $C_{free}$.

The critical (sample) volume ($V_C$) that is needed to avoid depleting greater than the desired $r_C$ can be estimated via (17):

$$V_C = \alpha \cdot K_f \cdot V_f - \theta \qquad \text{Equation A – 1}$$

Where $\alpha$: $r_C/(1 - r_C)$
$K_f$: equilibrium partition coefficient of the analyte between the SPME fibre and the aqueous phase
$V_f$: volume of the sorbing fraction of the SPME fibre
$\theta$: a matrix sorption term reflecting the effects on SPME from suspended solids and dissolved organic matter (DOM; see (17) for details).

It can be seen that in the situation (hypothetical) where there is no dissolved or particulate carbon in the system, the sample volume required to prevent sample depletion can be calculated from $V_C = \alpha \cdot K_f \cdot V_f$ and becomes very large for hydrophobic substances. In reality, the water in the test system will contain small quantities of dissolved and particulate organic carbon, which will change the critical volume needed to avoid depletion of the $C_{free}$. Zeng and Noblet (17) provide the theory behind this and the means to estimate it.

The matrix sorption term $\theta$ is estimated from the $K_{OC}$ of the test chemical and the concentration and organic carbon content of the dissolved and particulate organic carbon in the system. Two equations are needed to estimate the critical volume (taken from (17)).

$$V_C = \frac{\alpha \cdot K_f \cdot V_f}{1 + \beta} \qquad \text{Equation A – 2}$$

$$\beta = \frac{K_{OC} \cdot C_{SS} \cdot f_{OC} + K_{DOC} \cdot \mathrm{DOC}}{1 - \frac{C_{SS}}{\delta_{SS}} - \frac{\mathrm{DOC}}{\delta_{DOM} \cdot f_{DOC}}} \qquad \text{Equation A – 3}$$

Where $K_{OC}$: equilibrium partition coefficient of the analyte between the solid phase (normalised to organic carbon) and the aqueous phase
$K_{DOC}$: equilibrium partition coefficient of the analyte between the DOM (normalised to organic carbon) and the aqueous phase
$f_{OC}$: organic carbon fraction in the solid phase
$f_{DOC}$: dissolved organic carbon in the DOM phase
$\delta_{SS}$: density of the solid phase
$\delta_{DOM}$: density of the DOM phase
$C_{SS}$: concentration of suspended solids in the system
DOC: concentration of dissolved organic carbon in the system

Using estimates for $K_f$, $K_{OC}$, $K_{DOC}$ based on relationships with $K_{OW}$ (Table A – 1), examples for the calculation of critical volumes ($V_C$) are given below for two hydrophobic substances ($K_{OW}$ values of 6.24 and 7.40), three different SPME fibres and two different concentrations of suspended solids and dissolved organic carbon.

**Table A – 1: Estimates of $K_f$, $K_{OC}$, $K_{DOC}$ for two hydrophobic substances based on relationships with $K_{OW}$. $K_f$ estimated as 0.123 × $K_{OW}$; $K_{OC}$ estimated as 0.41 × $K_{OW}$; $K_{DOC}$ estimated as 0.11 × $K_{OC}$.**

| Estimate | substance 1 | substance 2 |
|---|---|---|
| log $K_{OW}$ | 6.24 | 7.40 |
| log $K_f$ | 5.33 | 6.49 |
| log $K_{OC}$ | 5.85 | 7.01 |
| log $K_{DOC}$ | 5.28 | 6.44 |

For this exercise it is assumed that the exposure solutions contain 5 mg/L suspended solids (fraction carbon = 0.3) and 5 mg/L dissolved organic carbon (fraction carbon = 0.5), i.e. 4 mg/L total organic carbon. This will then result in the following estimates of critical water volumes (assuming measurements are made at equilibrium, Table A – 2) for fibres of 1 cm in length and three different diameters (7, 30, and 100 µm).

**Table A – 2: Estimates of $V_C$ assuming 5 mg/L suspended solids and 5 mg/L dissolved organic carbon.**

| Estimate | substance 1 (log $K_{OW}$ = 6.24) | | | substance 2 (log $K_{OW}$ = 7.40) | | |
|---|---|---|---|---|---|---|
| Fibre thickness (µm) | 7 | 30 | 100 | 7 | 30 | 100 |
| Fibre volume (µL) (length 1 cm) | 0.028 | 0.132 | 0.612 | 0.028 | 0.132 | 0.612 |
| $V_C$ at 5% depletion (mL) | 32 | 152 | 705 | 53 | 252 | 1168 |
| $V_C$ at 10% depletion (mL) | 15 | 72 | 334 | 25 | 119 | 553 |

For this exercise it is assumed that the exposure solutions contain 10 mg/L suspended solids (fraction carbon = 0.3) and 10 mg/L dissolved organic carbon (fraction carbon = 0.5), i.e. 8 mg/L total organic carbon. This will then result in the following estimates of critical water volumes (assuming measurements are made at equilibrium, Table A – 3) for fibres of 1 cm in length and three different diameters (7, 30, and 100 µm).

**Table A – 3: Estimates of $V_C$ assuming 10 mg/L suspended solids and 10 mg/L dissolved organic carbon.**

| Estimate | substance 1 (log $K_{OW}$ = 6.24) | | | substance 2 (log $K_{OW}$ = 7.40) | | |
|---|---|---|---|---|---|---|
| Fibre thickness (µm) | 7 | 30 | 100 | 7 | 30 | 100 |
| Fibre volume (µL) (length 1 cm) | 0.028 | 0.132 | 0.612 | 0.028 | 0.132 | 0.612 |
| $V_C$ at 5% depletion (mL) | 20 | 97 | 448 | 27 | 129 | 598 |
| $V_C$ at 10% depletion (mL) | 10 | 46 | 212 | 13 | 61 | 283 |

The above exercise shows that for highly hydrophobic substances, the critical volume needed generally exceeds the volumes used in automated SPME procedures under equilibrium conditions.

Therefore, the use of automated SPME is recommended under non-equilibrium conditions (cf. paragraph 31), although sensitivity/accuracy problems may occur (cf. paragraphs 40–45). Alternatively, (disposable) SPME fibres could be left in situ during the BCF test, and analysed after equilibration. During flow-through conditions where the freely dissolved concentration is continuously replenished, issues of depletion due to the partitioning to the fibre-phase should not occur.

## ANNEX 2 PREDICTING THE NEED FOR TWO TEST CONCENTRATIONS IN A DEFINITIVE FISH BCF TESTS[18]

In OECD TG 305 (1) (paragraph 91) the option is given to use a minimised test design at two test concentrations as a pilot test for determining the need for testing at two test concentrations in a subsequent definitive test, if a possible concentration dependence cannot be excluded (cf. Section 2.5.1). Here this option is further explored.

2) A set of proprietary data that had been accepted for regulatory purposes and were provided in an anonymised form by the German Environmental Agency (UBA) ("the UBA database") were used as test set (95). The UBA database contains data for 40 chemicals that were tested at two exposure concentrations (cf. Annex 5, A5.2,

18 Based on "An Analysis of the Use of the OECD TG 305 Minimised Design to Predict the Need for Use of Two Test Concentrations In Definitive Fish Bioconcentration Tests" by T.A. Springer, Ph.D, Wildlife International, September 17, 2014, unpublished.

Table A – 10 and Table A – 11). One study was eliminated from the dataset because the chemical did not bioconcentrate. BCF values for these chemicals were determined from the apparent steady state concentration of chemical in the fish ($BCF_{SS} = C_{fish}/C_{water}$ at steady state) as well as by using the uptake ($k_1$) and depuration ($k_2$) rate constants ($BCF_K = k_1/k_2$). For some exposure concentrations of some chemicals, it was not possible to calculate both $BCF_{SS}$ and $BCF_K$ values, whereas in other cases both could be calculated. When both methods could be used, the BCF value that appeared subjectively to be the best representation of the data was selected as the "best" available BCF estimate (96). When only one method of calculation of the BCF value could be used, the resulting estimate was considered to be the "best" available estimate. As these "best" BCF estimates were calculated using all available data from each uptake and depuration curve, these estimates are designated as $BCF_{best}$ estimates from the "full" data set, and are considered the definitive BCF estimates in subsequent analyses.

In principle, the minimised test design involves taking tissue samples only twice, at the beginning and at the end of a 14-d depuration period (96). OECD TG 305 (1) recommends to take additional samples in the middle of both the uptake and the depuration periods as additional checks, but these have not been considered in this calculation. The data representing the depuration curves in the UBA dataset were resampled to provide the same data that would have been obtained if the test had been performed using the minimised design (i.e. two sample points)[19]. The kinetic BCF estimates for each exposure concentration for each chemical were estimated using the kinetic rate constants derived from the reduced data sets as described in OECD TG 305 (1). The BCF values calculated using the reduced data set from the simulated minimised tests are identified as $BCF_{Km}$ estimates.

Even when BCF tests are performed using the same exposure concentrations under identical conditions, BCF estimates will vary between trials due to random variability and unknown uncontrolled factors. When differences between pairs of $BCF_{best}$ estimates from the two exposure concentrations used for each chemical in the UBA database are examined, these paired results appear to be randomly distributed around a mean of zero. Figure A – 1 shows the distribution of differences for $BCF_{best}$. These differences are calculated as:

$$\text{PctDiff}_{\text{BCFbest}} = \frac{\text{BCF}_{\text{best,highC}} - \text{BCF}_{\text{best,lowC}}}{\text{BCF}_{\text{best,highC}}} \times 100\% \qquad \text{Equation A – 4}$$

Where $BCF_{best,highC}$: the best BCF estimate from the highest exposure concentration
$BCF_{best,lowC}$: the best BCF estimate from the lowest exposure concentration.

[19] When resampling to simulate the minimized test, the length of the uptake period was set at 28 d, and length of depuration period was set at 14 d whenever possible. If no samples were available for these days, the concentrations of chemicals in the fish tissues that would have existed on these days were estimated by interpolation or projection if such calculations were feasible (96).

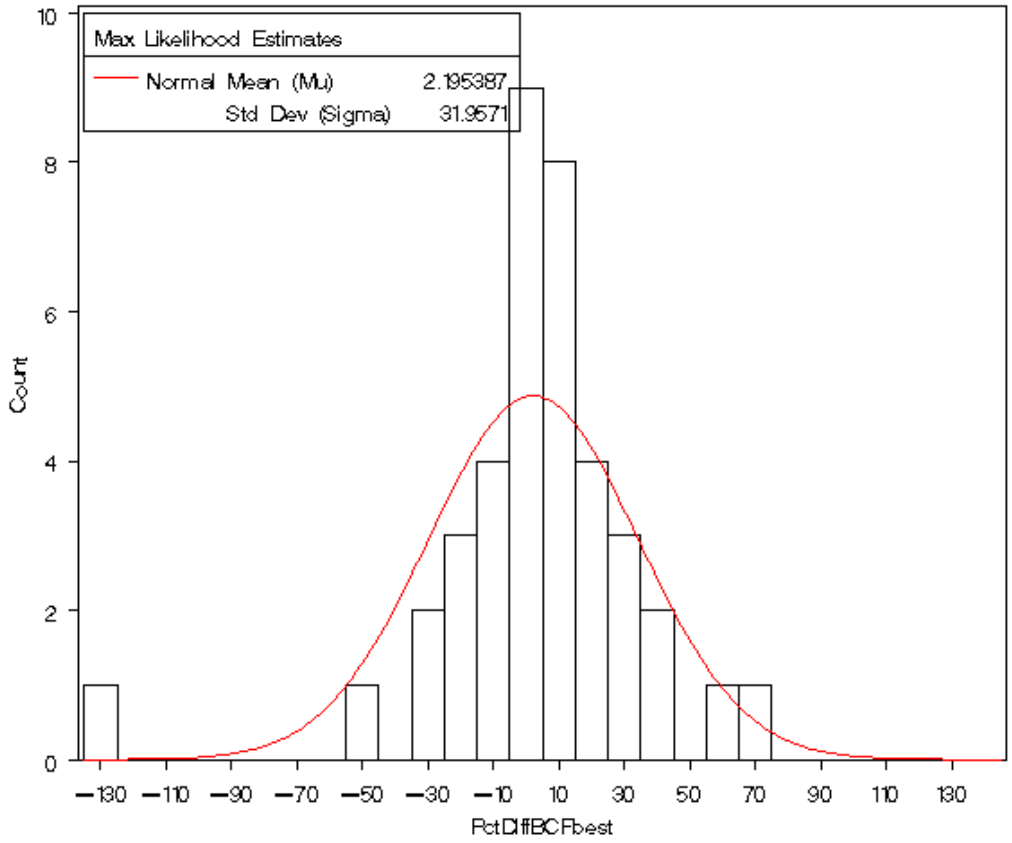


**Figure A – 1: Comparison of $BCF_{best}$ values determined at two exposure concentrations. The variable $PctDiff_{BCFbest}$ is the difference between $BCF_{best}$ estimates from two exposure concentrations determined using the full test design. Differences greater than zero indicate that the higher exposure concentration gave the higher $BCF_{best}$ estimate.**

Differences in BCF values between two exposure concentrations might arise where the (organic) chemical in question requires metabolisation before it can be eliminated. Saturation of the metabolic mechanisms in the fish could result in dramatic increases in the BCF value when the exposure concentration is increased (conversely, BCF values at intermediate concentrations might decrease if a certain body burden is required before relevant metabolic pathways start to operate). If a BCF study is conducted at two concentrations with the same chemical, subtracting the BCF estimate obtained using the lower exposure concentration from the BCF estimate obtained using the higher exposure concentration would yield a positive value if the higher test concentration resulted in metabolic saturation (and a negative value if metabolism was only initiated at the higher concentration).

If we assume for the sake of discussion that this mechanism is a dominant source of differences between BCF values determined at two test concentrations, then we should find that the distribution of differences of $PctDiff_{BCFbest}$ should be strongly skewed toward positive values. This does not appear to be the case as the mean of the distribution of $PctDiff_{BCFbest}$ values was only 2.2% (Figure A – 1) and not significantly different from zero ($p$ = 0.67). One might conclude that the differences between $BCF_{best}$ estimates from the higher and lower concentrations mainly reflect apparently random influences.

Nonetheless, there is still reason to expect that some chemicals exist where exposure concentration might indeed have a significant influence on the resulting BCF estimates because of factors other than experimental variability. Therefore, we must still decide how large a difference between BCF estimates from two exposure concentrations must be before we decide that a single BCF value cannot

adequately describe the bioconcentration potential of a chemical. Clearly, there should be no concern for differences of a few percent, but providing an answer to this question is difficult.

The distribution of $PctDiff_{BCFbest}$ values in Figure A – 1 has a standard deviation of about 32%. However, note that the empirical distribution is more sharply peaked around zero than a normal distribution would be. In other words, the distribution shows less variability than would be expected if differences were normally distributed. Indeed, the tenth and ninetieth percentiles of the observed differences are –27.9% and 36.0% respectively (Table A – 4). Thus, one might consider differences outside the range –27.9 to 36% as being large enough to warrant further investigation. However, this would flag one in every five chemicals, assuming that the empirical distribution shown here is representative. However, the symmetry of the distribution of differences suggests that many of the differences flagged would reflect random variation.

**Table A – 4: Statistical properties of $PctDiff_{BCFbest}$ estimates from the UBA dataset. See text for details.**

| | | | |
|---|---|---|---|
| **N** | 39 | **Sum Weights** | 39 |
| **Mean** | 2.1953869 | **Sum Observations** | 85.6200893 |
| **Std Deviation** | 31.9570998 | **Variance** | 1021.25623 |
| **Skewness** | -1.733044 | **Kurtosis** | 8.0876012 |
| **Uncorrected SS** | 38995.7058 | **Corrected SS** | 38807.7366 |
| **Coeff Variation** | 1455.64774 | **Std Error Mean** | 5.11723139 |

**Quantiles (Definition 5)**

| Quantile | 100% Max | 99% | 95% | 90% | 75% Q3 | 50% Median | 25% Q1 | 10% | 5% | 1% | 0% Min |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Estimate | 71.03 | 71.03 | 59.09 | 36.00 | 17.14 | 3.30 | -10.06 | -27.94 | -50.00 | -133.17 | -133.17 |

Given that pairs of BCF estimates from the full test differ, we expect that to some degree, the minimised test performed at two test concentrations can predict $PctDiff_{BCFbest}$. Let us define the term $PctDiff_{BCFKm}$ for the percent difference between $BCF_{Km}$ estimates (i.e. for pairs of kinetic BCF estimates from the minimised test) similar to the definition of $PctDiff_{BCFbest}$:

$$PctDiff_{BCFkm} = \frac{BCF_{Km,highC} - BCF_{Km,lowC}}{BCF_{Km,highC}} \times 100\% \qquad \text{Equation A – 5}$$

Figure A – 2 enables a comparison by showing $PctDiff_{BCFbest}$ and corresponding $PctDiff_{BCFkm}$ estimates. It appears that differences between BCF values from the two exposure concentrations estimates are generally similar (i.e. for each test pair both estimates are close together) regardless of whether estimated from the full or minimised test.

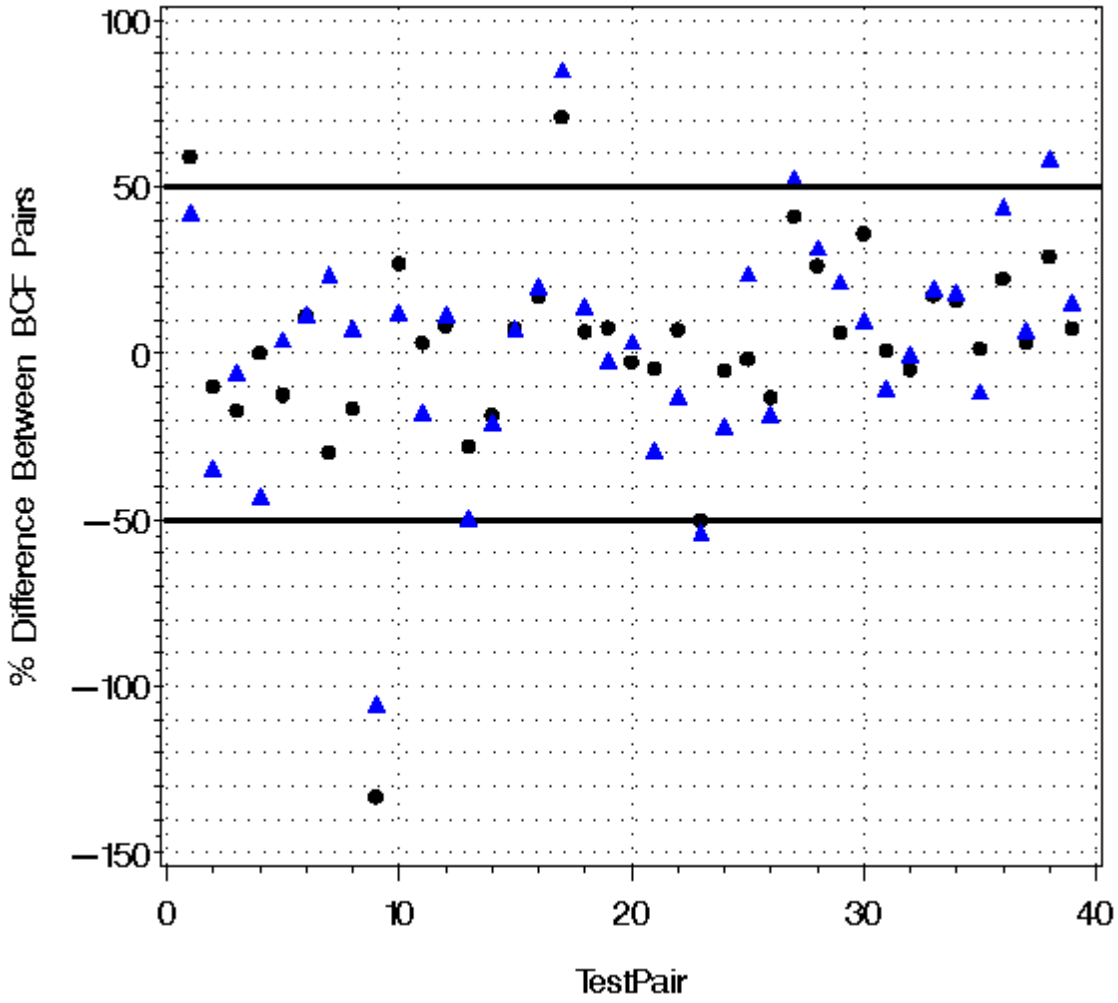


**Figure A – 2: Percent difference between pairs of BCF estimates (from low exposure concentration and high exposure concentration) estimated using the full test design and minimised design.**
**Values of $PctDiff_{BCFbest}$ (black dots) are differences between BCF values for the same chemical tested at two exposure concentrations using the full test. Values of $PctDiff_{BCFKm}$ estimates (blue triangles) are differences between the BCF estimates from the two test concentrations that would be obtained from the minimised test design. For each test pair of high and low concentrations (individually indicated by a number on the x-axis) both a $PctDiff_{BCFbest}$ and a $PctDiff_{BCFKm}$ estimate is depicted. Negative values indicate a higher BCF from the low exposure concentration, positive values indicate a higher BCF from the high exposure concentration. The solid horizontal lines indicate 50% difference levels (see text for further details).**

Let us also define the term 'maximum permissible percent difference' (MPD) to mean that any value of $PctDiff_{BCFbest}$ above the MPD would indicate that, for regulatory purposes, a single BCF estimate cannot be used to represent the bioconcentration potential of the chemical. As long as $PctDiff_{BCFbest}$ is below the MPD, for regulatory purposes, the difference in BCF between the two test concentrations is considered to be the result of random influences, and a single BCF estimate can be used to represent the bioconcentration potential of the chemical.

Suppose that the MPD is set at 50% (i.e. the high exposure concentration results in a BCF value that is twice as high as the BCF value from the low exposure concentration). If a horizontal line is drawn across Figure A – 2 at the 50% level (upper solid horizontal line), one finds that for 2 of the 39 chemicals (5.1% of chemicals in the data set), $PctDiff_{BCFbest}$ exceeds the MPD of 50%. Note also that $PctDiff_{BCFkm}$

estimates for three chemicals exceed the MPD of 50%, including one of the two identified as exceeding the MPD according to the $PctDiff_{BCFbest}$ estimates.

From a precautionary stance, it is preferable that the minimised test identifies a high percentage of cases where $PctDiff_{BCFbest}$ would exceed MPD. This is because it is of much less concern if the minimised test falsely predicts that $PctDiff_{BCFbest}$ exceeds the MPD, as this error leads only to performing the full test with two exposure concentrations, which is what would have happened anyway in absence of the minimised test. The following discussion focuses on failure of $PctDiff_{BCFkm}$ to predict exceedance of the MPD by $PctDiff_{BCFbest}$.

Returning to Figure A – 2, we can determine the number of chemicals where $PctDiff_{BCFkm}$ fails to predict exceedance of the MPD by $PctDiff_{BCFbest}$ for any value of the MPD (i.e. determine for a specific MPD how many test pairs have a $PctDiff_{BCFbest}$ above the MPD, but a $PctDiff_{BCFkm}$ below the MPD). Figure A – 3 shows the number of such failures in relation to the number of $PctDiff_{BCFbest}$ values that exceed MPD values chosen in 10% steps between 0 and 60%. Clearly lower MPD values result in a higher number of $PctDiff_{BCFbest}$ values that exceed the MPD.

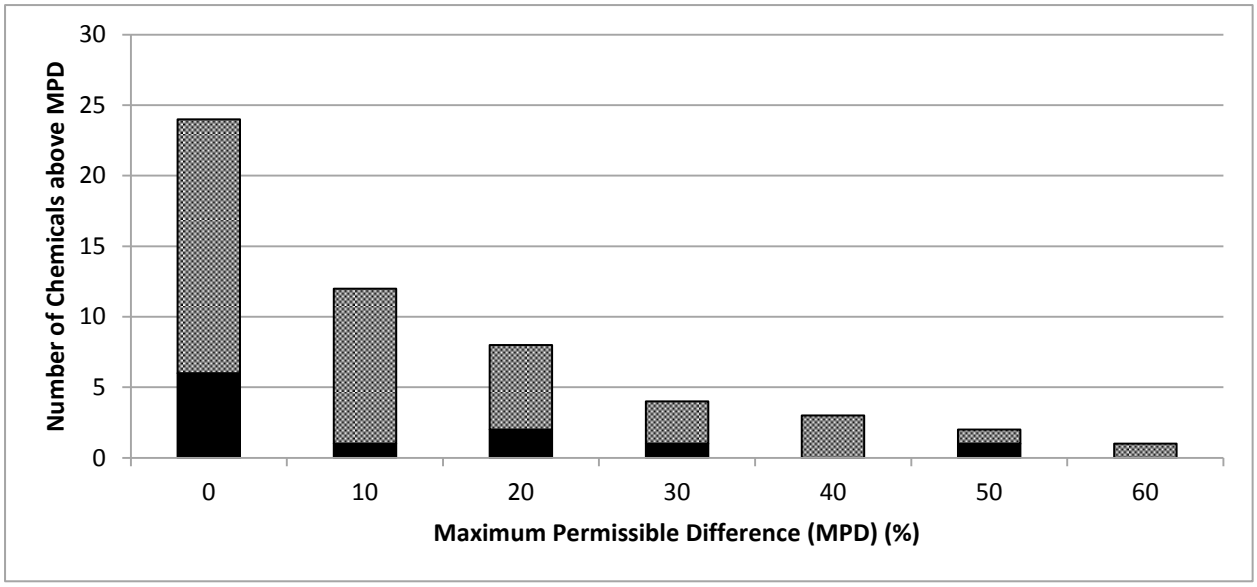


**Figure A – 3: Comparing different Maximum Permissible Difference (MPD) values for exceedance by $PctDiff_{BCFbest}$ values.**
**The top of the vertical bars indicate the number of $PctDiff_{BCFbest}$ values (out of 39) that exceed Maximum Permissible Difference (MPD) values given on the x-axis. The tops of the black portions of the vertical bars indicate the number of chemicals where $PctDiff_{BCFbest}$ for a chemical is above the MPD but the corresponding $PctDiff_{BCFkm}$ is not (i.e. the number of cases where $PctDiff_{BCFKm}$ fails to predict exceedance of the MPD by $PctDiff_{BCFbest}$).**

We can add an 'offset' to the $PctDiff_{BCFkm}$ value (e.g. $PctDiff_{BCFkm}$ + 10%) to try to eliminate these errors. Table A – 5 shows the effect of adding various offsets on the frequency of error. For the available data, adding an offset of 30% to $PctDiff_{BCFkm}$ ensures that exceedance of the MPD by $PctDiff_{BCFbest}$ is properly predicted for any value of MPD. This is true because $PctDiff_{BCFbest}$ and $PctDiff_{BCFkm}$ never differed by more than 30% for any chemical in the dataset.

**Table A – 5: Reduction of discordance resulting from addition of an offset percentage to $PctDiff_{BCFkm}$.**

| | Number of cases where $PctDiff_{BCFKm}$ fails to predict exceedance of the MPD by $PctDiff_{BCFbest}$ [1)] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Maximum Permissible Difference (MPD) | | | | | | |
| $PctDiff_{BCFkm}$ Offset | 0% | 10% | 20% | 30% | 40% | 50% | 60% |
| 0% | 6 | 1 | 2 | 1 | 0 | 1 | 0 |
| 10% | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| 20% | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 30% | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[1)] These are cases where $PctDiff_{BCFKm}$ falls below a given Maximum Permissible Difference (MPD) but $PctDiff_{BCFbest}$ is above the MPD. The number of such cases can be reduced if an offset percentage is added to the $PctDiff_{BCFKm}$ before comparison to the MPD. Numbers in cells are the numbers of chemicals (out of 39) where $PctDiff_{BCFkm}$ fails to predict exceedance of the MPD by $PctDiff_{BCFbest}$ for the MPD values given in column headings after application of the offset associated with each row.

A smaller offset can be used if a non-zero rate of discordance is acceptable or if the MPD is set at higher levels. For example, exceedance of MPD = 40% is properly predicted if an offset of 20% is used. In other words, if $PctDiff_{BCFkm}$ is greater than 60%, the data here suggest that there is near certainty that $PctDiff_{BCFbest}$ is greater than 40%.

Thus, it appears that $PctDiff_{BCFkm}$ has the potential to provide dependable information needed to decide on the need for the use of two test concentrations in definitive tests. However, for such an approach to be used there must be prior agreement on the size of the MPD and of the size of offset applied to $PctDiff_{BCFkm}$.

It is suggested to use an MPD of 50 % with no offset for cases where the results of a minimised test with two concentrations are not far from a regulatory level of concern. To define the term "not far from a regulatory level of concern", the analysis of Hashizume et al. (23) is useful (cf. paragraph 55). In this analysis, margins for $BCF_{Km}$ were estimated that correspond to regulatory values of concern. Should the result of one concentration of a two (or more) concentration minimized BCF test where the MPD is $\geq$ 50% fall into the relevant margin (depending on OECD country criteria, e.g. 1,400 to 2,700 for the 2,000 criterion (23)), a full bioconcentration test with two or more concentrations should be performed. For minimised tests with two concentrations that demonstrate a concentration dependence (i.e. where the MPD is $\geq$ 50%) but both $BCF_{Km}$ are very low (e.g. < 10 and < 100 L/kg), then conduct of a definitive test should not normally be necessary, depending on the requirements of the relevant regulatory authority (cf. (1), paragraphs 94 and 95).

# ANNEX 3 BCF ESTIMATION

## A3.1 Sequential BCF estimation

In Sections 3.3 and 3.4 guidance is given on the simultaneous fitting procedure to estimate uptake and elimination rate constants ($k_1$ and $k_2$) in one fitting procedure from data for both uptake and elimination phases. As indicated in OECD TG 305 (1) sequential fitting is another option for fitting these rate constants. Sequential BCF estimation involves estimation of $k_2$ first. Subsequently, this estimate is used to fit the uptake phase using Equation 3-11. This yields the estimate for $k_1$. As stated in paragraph 81, this is a robust procedure, but it ignores the fact that $k_1$ and $k_2$ are correlated. In addition, $k_2$ co-determines the uptake phase (as seen in Equation 3-6), resulting in differences in the uncertainty estimates between uptake and depuration regression procedures with no obvious way of combining the two measures of uncertainty.

Traditionally, to estimate $k_2$, a linear regression is usually done of *ln*(concentration in fish) versus time. As a consequence, also for the $k_1$ estimate *ln*(concentration in fish) versus time should be used in the fitting procedure. As explained in Section 3.4.3, however, an ln-transformation may not necessarily be the optimal transformation to correct for asymmetry in either, or both, explanatory and response variables, and for non-constant standard deviations. Also in sequential fitting procedures it may therefore be worthwhile to explore different data transformations and use model diagnostics to evaluate the model fit.

Two options for sequential BCF estimation are further detailed in this Annex. The first option is *not constraining* the estimated fish concentration at the end of the uptake phase, and let $k_1$ be determined only by the least-squares fit on the data of the uptake phase (Section A3.1.1). The second option is *constraining* the estimated fish concentration at the end of the uptake phase by calculating $C_{fish}$ by extrapolation from the linear depuration model (OECD TG 305 (1) Annex 5, equation A5.21) (Section A3.1.2). The calculated $C_{fish}$ is then used as the average $C_{fish}$ at the latest measurement time where the non-linear regression of the uptake phase should then 'end'. Finally in Section A3.1.3 pros and cons of both sequential fitting methods are compared with those of the simultaneous fitting procedure that is outlined in Section 3.4.

### *A3.1.1 Unconstrained estimated fish concentration at the end of the uptake phase*

The $k_2$ estimate on the depuration phase only can be used for an estimate of $k_1$ based on the least-squares fit on the data of the uptake phase. It is clear from Figure A – 4 that this sequential fitting strategy presents an apparent discontinuity[20], indicating that the model interpretation does not match reality.

The freedom of separately fitting the two parameters in the two phases does not guarantee that the model assembled from the two sub-models is continuous over time, for the whole duration of the experiment. This can only be accomplished by a joint $k_1$-$k_2$ regression model, as discussed in Section 3.3. The sequential fit shown in Figure A – 4 leads to the following parameter estimates:

| | | |
|---|---|---|
| $k_1$: | 66.12 | $(L \cdot kg^{-1} \cdot d^{-1})$ |
| $k_2$: | 0.01965 | $(d^{-1})$ |
| BCF (i.e. $k_1/k_2$): | 3365 | $(L \cdot kg^{-1})$ |

[20] Note that ln-transformation is needed to obtain a straight line for the depuration phase. When using the $k_2$ derived from this straight line to estimate the $k_1$ from the uptake phase, also data from the uptake phase should be ln-transformed.

The estimators of $k_1$ and $k_2$ are correlated. However, the uncertainty is hard to calculate either analytically, or with the numerical Delta method (see below in this Section A3.1.1), as the models do not really share the parameters. The $k_2$ point estimate from the depuration model is reused in the uptake model, without letting the uptake phase data influence its value[21]. The only way to estimate the uncertainty here is through bootstrapping (not shown here). For this reason, sequential fitting is not used in the *R*-package '*bcmfR*'.

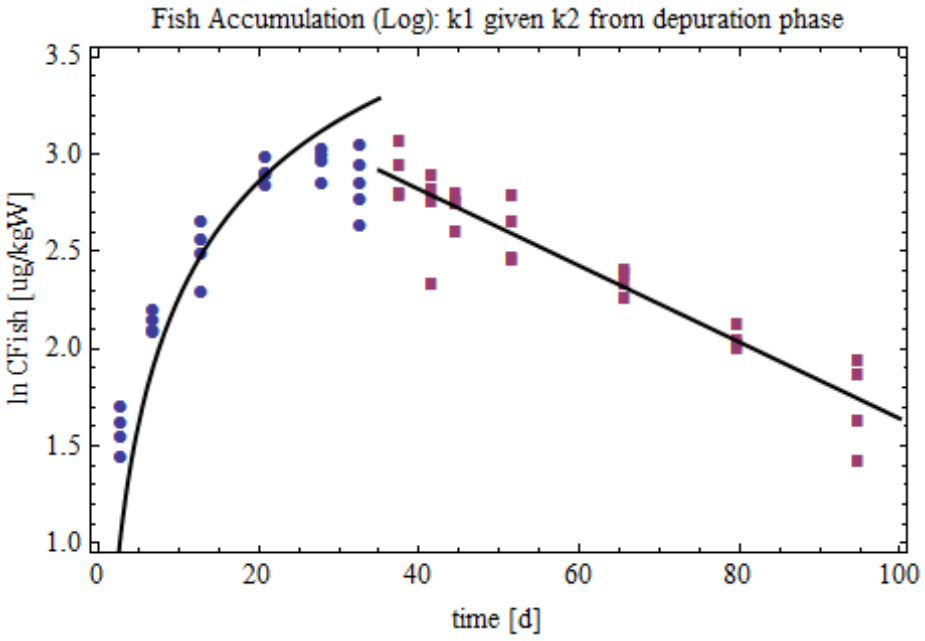


**Figure A – 4: Sequential fit of $k_2$ and $k_1$, showing discontinuity for *Example 2* (Annex 5, A5.1, Table A – 7)**

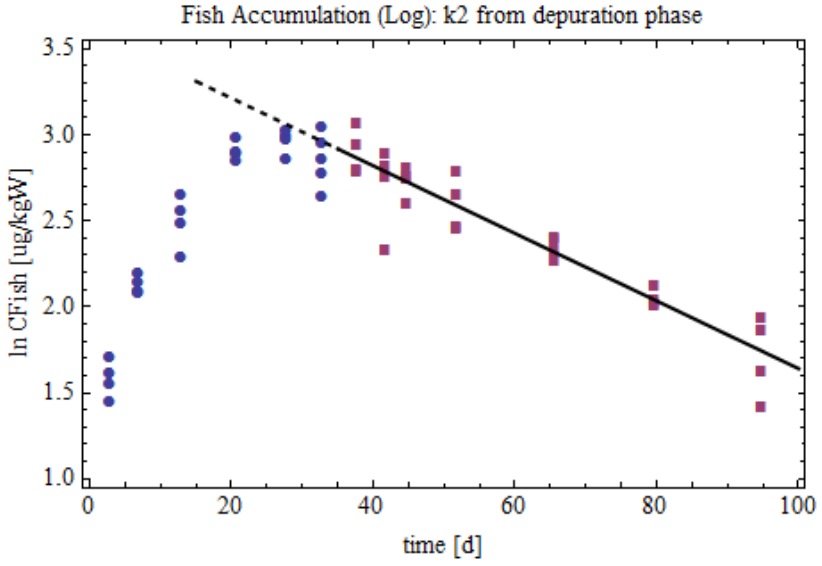


**Figure A – 5: Estimation of $k_2$ based on depuration phase only for *Example 2* (Annex 5, A5.1, Table A – 7)**

[21] Note that any uncertainty in the $k_2$ estimate is generally ignored when the point estimate is used to estimate $k_1$.

### *A3.1.2 Constrained estimated fish concentration at the end of the uptake phase*

The $k_2$ estimate on the depuration phase only can also be used for an estimate of $k_1$ where the curve is forced through the estimated $C_{fish}$ (based on the depuration phase only) at the beginning of the depuration phase. As shown in Figure A – 5, the same fit for the depuration phase is obtained (cf. Figure A – 4).

In Equation 3-11, the lower part describes the depuration phase only. Natural log-transform yields

$$\ln C_{\text{fish}}\left(t \geq t_{\text{dep}}\right) = \ln\left[C_{\text{w}} \cdot \frac{k_1}{k_2} \cdot \left(e^{k_2 \cdot t_{\text{dep}}} - 1\right)\right] - k_2 \cdot t \qquad \text{Equation A – 6}$$

which can be written in linear regression form

$$\ln C_{\text{fish}}\left(t \geq t_{\text{dep}}\right) = \hat{\alpha}_{\text{dep}} - \hat{\beta}_{\text{dep}} \cdot t \qquad \text{Equation A – 7}$$

The linear regression coefficient estimates are denoted as $\hat{\alpha}_{\text{dep}}$ and $\hat{\beta}_{\text{dep}}$, indicating their dependence on the depuration data. One can directly estimate $k_2$ from $\beta$:

$$\hat{k}_2^{\text{dep}} = \hat{\beta}_{\text{dep}} \qquad \text{Equation A – 8}$$

An estimate of the BCF and its uncertainty can be obtained from fitting the uptake phase and calculating $k_1/k_2$. However, the latter quotient is already present in the expression for the intercept coefficient in the regression:

$$\alpha = \ln\left[C_{\text{w}} \cdot \frac{k_1}{k_2} \cdot \left(e^{k_2 \cdot t_{\text{dep}}} - 1\right)\right] = \ln\left[C_{\text{w}} \cdot \text{BCF} \cdot \left(e^{k_2 \cdot t_{\text{dep}}} - 1\right)\right] \qquad \text{Equation A – 9}$$

Solving for BCF, while substituting the regression coefficient estimates, yields

$$\text{BCF}_{\text{dep}} = \frac{\exp\left(\hat{\alpha}_{\text{dep}}\right)}{C_{\text{w}} \cdot \left[\exp\left(-\hat{\beta}_{\text{dep}} \cdot t_{\text{dep}}\right) - 1\right]} \qquad \text{Equation A – 10}$$

Note that $\text{BCF}_{\text{dep}}$ is not a dynamic value of BCF at the end of the uptake phase, but it estimates the kinetic BCF-value based on the linear regression coefficients *derived from the depuration phase only*[22].

Standard linear regression estimates $\alpha$ and $\beta$ (using procedure *lm* from the statistical software package *R*), as well as their standard errors, as follows[23]

[22] Please note that this method is used to generate initial estimates for simultaneous fitting of uptake and depuration phase as shown in Section 3.3.

```
        Estimate  Std.Error
alpha    3.60681    0.08933
beta    -0.01965    0.00142
```

Substitution of the coefficient estimates in Equation A – 10 yields a BCF estimate of 2327 (L/kgW).

The algorithm to calculate the standard error from Equation A – 10 is known as the 'delta method'[24] and is not easy to evaluate manually. Therefore, the *R*-package *car* (26) is used which takes the standard regression output of *lm* and Equation A – 10 (25). From these values, the 95% confidence interval of the BCF is calculated (1.96 times the SE):

```
BCF   2.5%  97.5%
2327  2181  2473
```

Ln($C_{fish}$) can now be plotted over time with coefficients derived from the depuration phase. Because the uptake part of the model is unrelated to the uptake data, the model curve is shown dashed. It is clear that the modelled uptake underestimates the uptake data (Figure A – 6).

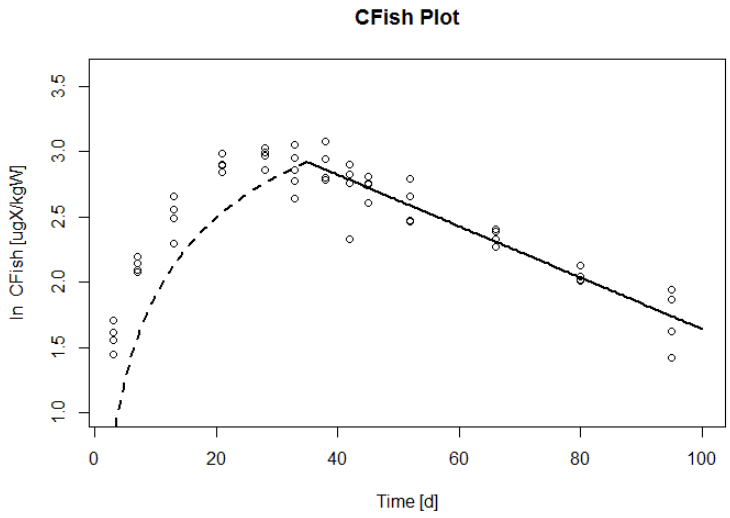


**Figure A – 6: Constrained sequential fit of $k_2$ and $k_1$ for *Example 2* (Annex 5, A5.1, Table A – 7).**

### *A3.1.3 Comparing different fitting procedures*

Both options for sequential fitting have some disadvantages. The non-constrained fit may lead to a better fit in the uptake phase, but may lead to a discontinuous uptake and depuration phase, indicating that the model interpretation does not match reality (although this discontinuity might indicate other issues with the data, e.g. kinetics that are non-first order). The constrained fit may lead to a suboptimal fit due to the fixed $C_{fish}$ at the end of the uptake phase. In both cases the estimation of parameter uncertainty for $k_1$ and $k_2$ now suffers from two problems:

---

23 Note that the estimate of alpha is the intercept at time equal to zero, not the value of ln($C_{fish}$) at the onset of the depuration phase: $t_{dep}$ = 35 (d) used in Equation A – 10.

24 http://en.wikipedia.org/wiki/Delta_method, and references therein.

- Data for the uptake phase cannot only be used to estimate the uptake rate $k_1$, but also the overall depuration rate $k_2$. The data for the uptake phase are therefore ignored when $k_2$ is estimated from the depuration phase only.
- Parameters $k_1$ and $k_2$ are correlated, as given by the covariance matrix generated in the simultaneous (joint) non-linear fitting procedure (see Sections 3.3 and 3.4). By fitting $k_2$ on the depuration phase only, we cannot establish the correlation between $k_1$ and $k_2$, i.e. they are uncorrelated.

It can be concluded that the unconstrained model (Section A3.1.1) is straightforward. Conceptually, the two models are decoupled for the two phases of the bioaccumulation experiment. This may have the advantage that if first order kinetics are not followed during the test, this can be more easily detected visualy from straight-line fit through the depuration data (assuming that ln-transformation is the optimal transformation). The point estimate of the depuration rate constant estimated for the depuration phase carries over to the uptake phase, however, without any possibility for recalibration. Only the uptake rate constant may adjust to the accumulation data in the uptake phase. This seems too rigid and there is no reason, a priori, why the depuration rate constant would not help in fitting the uptake phase.

The constrained sequential method (Section A3.1.2) completely ignores the uptake data, while fitting $k_1$. There may however be some datasets where the method works well (by serendipity). The practical advantage is that simple linear regression suffices and readily yields a BCF estimate and its uncertainty. This may be useful where the main parameter of interest from the test is $k_2$, or situations where $k_1$ might be mis-estimated by the simultaneous method (e.g. where uptake is not following the expected kinetics).

But the sequential methods suffer from the occurrence of discontinuity at the onset of depuration, without any rationale by itself, or from the possibility of a suboptimal fit in the uptake phase. Hence, both the parameter estimates and the confidence intervals will be different for the two sequential procedures when compared to the simultaneous procedure. Furthermore, $k_1$ estimates and confidence intervals will generally differ between the two sequential procedures.

If fish grow during the test duration, this might affect kinetics of the bioaccumulation process. Determining $k_2$ for bigger fish from the depuration phase (end of the test) and applying that value to the smaller fish in the uptake phase (beginning of the test) introduces a possible systematic mismatch between $k_1$ and $k_2$. Applying the simultaneous method would largely circumvent this by forcing the data to the same median kinetics, although in extreme cases this could lead to observed deviations from first-order kinetics.

In general, it is recommended to rely on simultaneous BCF estimation as this will in most cases produce more realistic $BCF_K$ estimates (due to a lack of discontinuity at the onset of depuration), including a single direct measure of uncertainty (confidence limits for the model's fit) (cf. Section 3.3).

## A3.2 Model tests

### *A3.2.1 Runs test*

The runs test (33) can be used to decide if a data set is from a random process.

A run is defined as a series of increasing values or a series of decreasing values. The number of increasing, or decreasing, values is the length of the run. In a random data set, the probability that the $(I+1)^{th}$ value is larger or smaller than the $I^{th}$ value follows a binomial distribution, which forms the basis of the runs test.

### *A3.2.2 Shapiro-Wilk test*

The Shapiro-Wilk test calculates a W statistic that tests whether a random sample, $x_1$, $x_2$, …, $x_n$ comes from a normal distribution. Small values of W are evidence of departure from normality. The test rejects the hypothesis of normality when the *p*-value is less than or equal to e.g. 0.05. Failing the normality test allows you to state with 95% confidence the data does not fit the normal distribution. Passing the normality test only allows for the conclusion that no significant departure from normality was found.

## A3.3 BCF dynamics for General Fish growth

### *A3.3.1 BCF dynamics for General Fish Growth*

A mass-based dynamical system is defined for both fish wet weight, unit of kgW (kilograms of fish in wet weight), and chemical mass (X) in the fish, expressed in mgX. Then, the dynamics of internal chemical concentration in units of mgX/kgW are derived from these mass-oriented equations. This will work also when the mass-related equations are adapted to include growth in different ways.

The equations of rate of change of toxicant mass in fish and fish biomass are written as

$$\begin{cases} \dfrac{dX_{\text{fish}}}{dt} = \lambda_1 \cdot C_{\text{water}} \cdot W_{\text{fish}} - \lambda_2 \cdot X_{\text{fish}} \\ \dfrac{dW_{\text{fish}}}{dt} = 0 \end{cases} \qquad \text{Equation A – 11}$$

Where $\frac{dX_{\text{fish}}}{dt}$: rate of change of fish chemical mass ($\text{mgX}\cdot\text{d}^{-1}$)
$X_{\text{fish}}(t)$: fish chemical mass over time (mgX)
$\frac{dW_{\text{fish}}}{dt}$: rate of change of fish biomass ($\text{kgW}\cdot\text{d}^{-1}$)
$W_{\text{fish}}(t)$: fish biomass over time ($\text{kgW}\cdot\text{d}^{-1}$)
$C_{\text{water}}$: exposure concentration constant ($\text{mgX}\cdot\text{L}^{-1}$)
$\lambda_1$: uptake rate constant ($\text{L}\cdot\text{kgW}^{-1}\cdot\text{d}^{-1}$)
$\lambda_1$: depuration rate constant ($\text{d}^{-1}$)

The same constants from the basic BCF model (Equation 3-5) are used, but renamed as $\lambda_1$ and $\lambda_2$ to allow for their proper interpretation as being distinct from $k_1$ and $k_2$.

The first dynamical equation for chemical mass in the fish contains two subtleties. The uptake term $\lambda_1 \cdot C_{\text{water}} \cdot W_{\text{fish}}$ involves the *product* of exposure concentration and fish mass, i.e. a law of mass action. This way, the uptake is linear in both exposure, *as well as* fish mass. The second subtlety is that the depuration rate term: $-\lambda_2 \cdot X_{\text{fish}}$ is linear in the fish chemical *mass*, not in fish chemical concentration. Thus, twice as much fish chemical mass leaks out twice as fast. The dynamic equation for fish biomass just states that the fish does not grow here, as it has derivative equal to 0.

The dynamics of the fish chemical concentration can now be derived from the two mass-based equations

$$C_{\text{fish}}(t) = \frac{X_{\text{fish}}(t)}{W_{\text{fish}}(t)} \qquad \text{Equation A – 12}$$

Here, $C_{\text{fish}}(t)$ is the chemical concentration in fish over time (mgX·kgW$^{-1}$), as in the basic equation in paragraph 83.

It follows from the derivative of a product that:

$$\frac{dX_{\text{fish}}}{dt} = \frac{dC_{\text{fish}}}{dt} \cdot W_{\text{fish}}(t) + C_{\text{fish}}(t) \cdot \frac{dW_{\text{fish}}}{dt} \qquad \text{Equation A – 13}$$

Substituting the mass dynamics of both fish chemical mass and fish biomass, and after rearranging, it follows that:

$$\frac{dC_{\text{fish}}}{dt} = \lambda_1 \cdot C_{\text{water}} - \lambda_2 \cdot C_{\text{fish}} \qquad \text{Equation A – 14}$$

With the same rates of change as in the basic BCF equation:

$\frac{dC_{\text{fish}}}{dt}$: rate of change of fish chemical concentration (mgX·kgW$^{-1}$·d$^{-1}$)

To extend the model with fish growth the dynamics at the mass balance level can be determined first, and only then derive the rate of change of the internal fish chemical concentration. This allows for the use of other (more complex) growth models if the usual exponential growth model does not fit properly to the data (see Section A3.3.2).

The dynamical equations on the basis of accumulated chemical mass in fish and fish biomass can be written for a general fish growth function $G(W_{\text{fish}})$:

$$\begin{cases} \dfrac{dX_{\text{fish}}}{dt} = \lambda_1 \cdot C_{\text{water}} \cdot W_{\text{fish}} - \lambda_2 \cdot X_{\text{fish}} \\ \dfrac{dW_{\text{fish}}}{dt} = G(W_{\text{fish}}) \end{cases} \qquad \text{Equation A – 15}$$

Growth function $G$ may contain additional parameters.

Employing the relationship between chemical mass in fish, the chemical concentration in the fish, and the fish biomass, $X_{\text{fish}}(t) = C_{\text{fish}}(t) \cdot W_{\text{fish}}(t)$, obtains the constraint on the dynamics of each:

$$\frac{dX_{\text{fish}}}{dt} = \frac{dC_{\text{fish}}}{dt} \cdot W_{\text{fish}}(t) + C_{\text{fish}}(t) \cdot \frac{dW_{\text{fish}}}{dt} \qquad \text{Equation A – 16}$$

Substituting and rearranging yields

$$\frac{dC_{\text{fish}}}{dt} = \lambda_1 \cdot C_{\text{water}} - \left( \lambda_2 + \frac{G\big(W_{\text{fish}}(t)\big)}{W_{\text{fish}}(t)} \right) \cdot C_{\text{fish}} \qquad \text{Equation A – 17}$$

Note that the relative growth rate term adding to the proper depuration rate constant can be written as follows

$$\frac{G\left(W_{\text{fish}}(t)\right)}{W_{\text{fish}}(t)} = \frac{dW_{\text{fish}}/dt}{W_{\text{fish}}(t)} = \frac{d\ln(W_{\text{fish}})}{dt} \qquad (\text{d}^{-1}) \qquad \text{Equation A – 18}$$

For exponential growth this becomes the former: $\lambda_G$.

For linear growth: $W_{\text{fish}}(t) = \alpha_G + \beta_G \cdot t$, the relative growth rate turns out to be

$$\frac{\beta_G}{\alpha_G + \beta_G \cdot t} \qquad \text{Equation A – 19}$$

The latter case of linear growth yields a preview of what may happen quite often. At $t = 0$ the growth rate correction equals $\alpha_G / \beta_G$, i.e. some number with unit ($d^{-1}$), while at 'infinite' time, $t \to \infty$, the growth correction term becomes 0 (zero).

In general, any fish biomass levelling off over time, will finally lead to $G = 0$, causing a zero growth rate correction of the depuration rate. In this case, we may expect Equation A – 9 to reduce to Equation A – 14

$$\frac{dC_{\text{fish}}}{dt} = \lambda_1 \cdot C_{\text{water}} - \lambda_2 \cdot C_{\text{fish}} \qquad \text{Equation A – 20}$$

at large $t$.

### *A3.3.2 Von Bertalanffy Growth Equation for Fish*

The Von Bertalanffy growth equation is almost universally applied to fish growth (97) (98). The three-parameter equation reads

$$W_{\text{fish}}(t) = \left({w_{\max}}^{1/3} - \left({w_{\max}}^{1/3} - {w_0}^{1/3}\right) \cdot e^{-\gamma \cdot t}\right)^3 \qquad \text{Equation A – 21}$$

Where: $w_{\max}$: maximum weight
$w_0$: weight at the start of the experiment
$\gamma$: Von Bertalanffy growth rate

The cube root powers arise from the original equation being defined for *length* of fish.

The growth rate can be written as (98):

$$\frac{dW_{\text{fish}}}{dt} = 3 \cdot \gamma \cdot \left({W_{\text{fish}}}^{2/3} \cdot {w_{\max}}^{1/3} - W_{\text{fish}}\right) \qquad \text{Equation A – 22}$$

At maximum weight, i.e. $W_{\text{fish}} = w_{\max}$, growth rate becomes zero.

The depuration correction term, is the relative growth rate:

$$\frac{1}{W_{\text{fish}}} \cdot \frac{dW_{\text{fish}}}{dt} = 3 \cdot \gamma \cdot \left( \left( \frac{w_{\max}}{W_{\text{fish}}(t)} \right)^{1/3} - 1 \right) \qquad \text{Equation A – 23}$$

which is positive for fish biomass below its maximum weight, and becoming zero, when growth saturates.

Kooijman and Bedaux (98) present von Bertalanffy parameters for rainbow trout at 12° Celsius:

$\gamma$: 0.00236 ($d^{-1}$)
$w_0$: 0.00123 (kgW),
$w_{\max}$: 3.5 (kgW).

We can adapt the parameters somewhat to fit the biomass data in *Example 2* (Annex 5,A5.1, Table A – 7):

$\gamma$: 0.0013 ($d^{-1}$)
$w_0$: 0.0015 (kgW),
$w_{\max}$: 3.5 (kgW).

This fish weight curve is plotted in Figure A – 7.

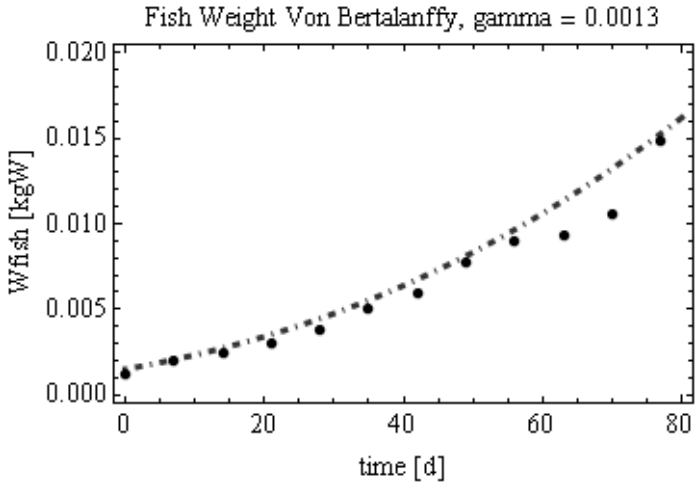


**Figure A – 7: Growth of fish from *Example 2* (Annex 5, A5.1, Table A – 7). Growth curve according to the Von Bertalanffy equation.**

In Figure A – 8, the time horizon is prolonged to 3000 days, somewhat over 8 years, to show the full von Bertalanffy growth curve. The maximum lifespan for a rainbow trout is about 11 years.

This should illustrate our point that we have only exponential growth data in the lower left corner of the growth curve in the first 80 days (Figure A – 7). The BCF is calculated from a steady state argument, assuming indefinite exponential growth, using the 'overall' depuration rate constant. This leads to 9100 ($L \cdot kgW^{-1}$).

The overall depuration rate constant overestimates a better (lower) depuration rate constant that results when correcting for growth dilution. But, when growth levels off, the growth rate correction to the

depuration rate constant would diminish and finally become zero. This implies that the ultimate BCF (in a no growth situation) would settle at 29,500 ($L \cdot kgW^{-1}$), roughly three times higher.

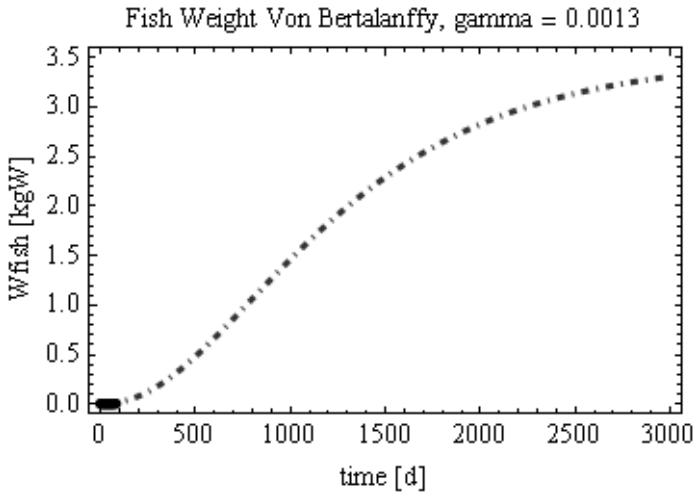


**Figure A – 8: Prolonged Von Bertalanffy growth curve for fish from *Example 2* (Annex 5, A5.1).**

Correction for growth is usually done using the subtraction method, described in Section 3.5 and 4.5.1 of this guidance. This is preferred as it is straightforward to determine $k_g$, and subsequently calculate $k_{2g}$. However, for this form of correction, both the overall depuration and rate of growth dilution must follow first order kinetics. Therefore if other growth models indicate that growth is not following first order kinetics, simple subtraction to obtain $k_{2g}$ is not possible.

Annex 5 of the OECD TG 305 (1) summarises an alternative method where $k_{2g}$ can be directly determined from the raw test data without requiring or assuming that growth dilution is a first order process. This is further discussed in Brooke and Crookes (51). The approach may also be useful for studies using rapidly growing fish and a chemical that is only slowly eliminated by processes other than growth (i.e. growth dominates the overall depuration rate constant). In this situation as $k_{2g}$ will be small in comparison to $k_2$ and $k_g$, there is potential for high uncertainty in $k_{2g}$, and in some instances the error in $k_2$ and $k_g$ could lead to a negative $k_{2g}$.

# ANNEX 4 DATA CORRECTIONS AND EXCEL SPREADSHEET[25] FOR BCF ESTIMATION FROM DIETARY STUDY DATA

## A4.1 Calculating the lipid normalised, growth corrected depuration rate constant

When estimating a BCF or comparing depuration between studies with similar experimental set ups, the depuration rate constant needs to be corrected both for growth and for lipid content. A method for correcting a depuration rate constant for fish lipid has been developed by Crookes and Brooke (51), which is derived from the principle commonly used to normalise a (kinetic) BCF to a set lipid content (5% is recommended in OECD TG 305 (1)).

The lipid normalised growth-corrected BCF is calculated by multiplying the growth-corrected BCF by the standard lipid fraction divided by the mean measured lipid fraction:

$$BCF_{gL} = \frac{BCF_g \times F_{L,std}}{F_{L,exp}} \qquad \text{Equation A – 24}$$

Where $BCF_{gL}$: lipid normalised, growth-corrected BCF
$BCF_g$: growth corrected BCF
$F_{L,std}$: standard fractional lipid content (i.e. 0.05)
$F_{L,exp}$: experimental fractional fish lipid content

Since the $BCF_g$ is derived from the uptake rate constant divided by the growth-corrected depuration rate constant, this can be written as:

$$BCF_{gL} = \frac{k_1}{k_{2g}} \times \frac{F_{L,std}}{F_{L,exp}} \qquad \text{Equation A – 25}$$

Where $k_1$: uptake rate constant
$k_{2g}$: growth corrected depuration rate constant

If it is assumed that $k_1$ has no dependence on lipid, it follows that $k_{2gL}$ can be derived as:

$$k_{2gL} = k_{2g} \times \frac{F_{L,exp}}{F_{L,std}} \qquad \text{Equation A – 26}$$

This equation is automated in the Excel Spreadsheet.

[25] The Excel Spreadsheet alluded to in Annex 4 is the one that the user can find together with the *R*-package on the OECD public site. Its specific name is "Dietary_BCF_estimation_tool.xls"

### A4.2 Estimating the mean experimental fractional fish lipid content ($F_{L,exp}$)

Fish lipid content is usually measured at three time points during a study. However, these time points can vary, as can the durations of the uptake and depuration phase of studies. This means that an arithmetic mean of the available measurements would not necessarily represent the true, time-weighted average, lipid content. Analysis by Brooke and Crookes (51) showed that there was little difference between several different ways of deriving average lipid content. For simplicity, it is suggested to use the mean lipid content over the depuration phase (i.e. the midpoint of the depuration phase calculated from the mean lipid values at the end of the uptake phase/start of depuration and the end of the depuration phase). This calculation is automated in the Excel spreadsheet. If additional lipid measurements within the depuration phase are available, this value can be overwritten in the spreadsheet with the time-weighted mean lipid content over the depuration phase.

This approach to calculating $k_{2gL}$ has been tested for studies where lipid content increased during the course of the study, but not for situations where lipid contents decreased.

### A4.3 Estimating a time-weighted mean fish weight (for the uptake rate constant $k_1$ and BCF estimation method)

This data transformation must be carried out when estimating a $k_1$ and BCF according to BCF estimation method 1 (cf. Section 4.6.3.1) for those models that use fish weight as an input, in cases where fish growth has been significant during a study. Linear regression of the natural log transformed mean fish weight data (measured before the start of the test and at the end of the uptake phase) can be used to estimate the mean fish weight at the midpoint of the uptake phase.

This calculation is automated in the Excel Spreadsheet.

### A4.4 Excel Spreadsheet for the estimation of $k_1$ and BCF according to Methods 1, 2 and 3

The Excel Spreadsheet is made available via OECD[26].

Notes:

- This spreadsheet must be used in conjunction with the guidance given above (cf. A4.1 to A4.3).
- BCF estimates are calculated for the 3 methods presented in the Guidance Document (Section 4.6.3); how to judge the relevance of these estimates is described in Section 4.6.4.
- All estimates are based on a fish of 5% lipid content (for methods 1 and 2 the depuration rate constant is normalised to 5% lipid; for method 3 normalisation to 5% lipid is implicit as the equation was derived using BCF data normalised to 5%).
- Normalisation of the depuration rate constant is done using the estimated mean lipid content at the midpoint of the depuration phase (based on mean lipid content at the end of uptake/start of depuration and mean lipid content at the end of the depuration phase assuming a linear relationship with time, cf. Section 4.5.2.5). If additional lipid contents were measured during the

[26] The Excel Spreadsheet alluded to in Annex 4 is the one that the user can find together with the *R*-package on the OECD public site. Its specific name is "Dietary_BCF_estimation_tool.xls"

depuration phase then the mean lipid content midpoint depuration phase value can be overwritten with a separate value derived using all data points (a time-weighted mean).

- Method 1 consists of a number of models to estimate $k_1$ from which the BCF is estimated (cf. Section 4.6.3.1). Most models use fish weight, estimated for the midpoint of the uptake phase, which is estimated using the mean fish starting weight, growth rate (calculated for the entire study according to OECD TG 305 (1)) and duration of the uptake phase.
- For methods 2 and 3, which do not include a step in which a $k_1$ value is calculated, $k_1$ estimates are presented here for comparative purposes based on the estimated BCF multiplied by the $k_{2gL}$ value.

# ANNEX 5 DATASETS USED IN THE EVALUATION OF METHODS

## A5.1 Datasets used in BCF estimations (Sections 3.4 and 3.5, and Annex 3)

**Table A – 6: Data set for *Example 1***

| Day | $C_{water}$ (µg/L) |
|---|---|
| 0.04 | 2.21 |
| 0.08 | 2.09 |
| 0.17 | 0.77 |
| 0.33 | 0.84 |
| 1 | 0.71 |
| 2 | 1.30 |
| 3 | 1.08 |
| 4 | 1.73 |
| 5 | 1.82 |
| 6 | 1.85 |
| 7 | 1.40 |
| 8 | 1.90 |
| 9 | 2.01 |
| 10 | 2.00 |
| 11 | 2.09 |
| 12 | 2.00 |
| 13 | 1.81 |
| 14 | 1.78 |

| Day | $C_{fish}$(µg/kgWW) | $W_{fish}$ (gWW) |
|---|---|---|
| 0.04 | 10.50 | 359.25 |
| 0.08 | 7.73 | 356.50 |
| 0.17 | 24.12 | 435.25 |
| 0.33 | 128.80 | 396.00 |
| 1 | 553.70 | 268.25 |
| 2 | 1105.47 | 279.25 |
| 4 | 2464.88 | 331.50 |
| 7 | 3025.53 | 359.00 |
| 9 | 3195.05 | 372.17 |
| 11 | 4485.04 | 385.00 |
| 14 | 4652.28 | 276.83 |
| 14.04 | 4167.07 | 352.00 |
| 14.08 | 5385.64 | 302.50 |
| 14.17 | 6692.33 | 367.75 |
| 14.33 | 4674.34 | 317.75 |
| 15 | 2329.99 | 276.25 |
| 16 | 3797.43 | 249.00 |
| 18 | 1328.29 | 286.00 |
| 21 | 1080.29 | 300.25 |
| 29 | 438.57 | 314.50 |
| 35 | 128.83 | 370.25 |

Lipid measured in pooled samples of control fish. Measurements for every sampling point in the uptake phase, and the first four sampling points in depuration phase.
Mean lipid content in the uptake phase: 12.3 % (standard deviation: 3.3). Mean overall lipid content: 13.76 %.
Fish were not fed during uptake.

**Table A – 7: Data set for *Example 2***

| Day | $C_{water}$ (µg/L) |
|---|---|
| 0 | 0.0185 |
| 0 | 0.0179 |
| 3 | 0.0160 |
| 3 | 0.0158 |
| 7 | 0.0166 |
| 7 | 0.0167 |
| 13 | 0.0142 |
| 13 | 0.0146 |
| 21 | 0.0161 |
| 21 | 0.0165 |
| 28 | 0.0178 |
| 28 | 0.0168 |
| 33 | 0.0134 |
| 33 | 0.0131 |

| Day | $C_{fish}$(µg/kgWW) | $W_{fish}$ (gWW) |
|---|---|---|
| 3 | 4.72 | 3.232 |
| 3 | 5.50 | 4.501 |
| 3 | 4.24 | 3.475 |
| 3 | 5.04 | 2.901 |
| 7 | 8.55 | 3.643 |
| 7 | 9.01 | 3.392 |
| 7 | 8.14 | 3.155 |
| 7 | 8.01 | 4.029 |
| 13 | 9.89 | 4.440 |
| 13 | 12.90 | 3.702 |
| 13 | 14.20 | 3.610 |
| 13 | 12.00 | 4.232 |
| 21 | 17.20 | 5.274 |
| 21 | 18.20 | 3.106 |
| 21 | 19.80 | 4.999 |
| 21 | 18.00 | 3.625 |
| 28 | 17.40 | 6.296 |
| 28 | 19.50 | 6.038 |
| 28 | 20.60 | 5.312 |
| 28 | 20.00 | 4.317 |
| 33 | 16.00 | 6.398 |
| 33 | 19.10 | 5.075 |
| 33 | 14.00 | 5.310 |
| 33 | 17.40 | 5.290 |
| 33 | 21.10 | 4.402 |
| 38 | 16.20 | 6.436 |
| 38 | 19.00 | 5.525 |
| 38 | 16.40 | 5.724 |
| 38 | 21.60 | 5.820 |

| Day | $C_{fish}$(µg/kgWW) | $W_{fish}$ (gWW) |
|---|---|---|
| 42 | 15.70 | 7.143 |
| 42 | 10.30 | 9.966 |
| 42 | 16.80 | 6.824 |
| 42 | 18.10 | 7.337 |
| 45 | 15.80 | 8.742 |
| 45 | 16.50 | 9.398 |
| 45 | 13.50 | 6.520 |
| 45 | 15.60 | 7.304 |
| 52 | 11.70 | 9.967 |
| 52 | 14.20 | 6.337 |
| 52 | 16.30 | 6.291 |
| 52 | 11.80 | 8.158 |
| 66 | 10.90 | 7.992 |
| 66 | 9.64 | 13.432 |
| 66 | 11.10 | 11.822 |
| 66 | 10.30 | 11.431 |
| 80 | 8.38 | 10.430 |
| 80 | 7.70 | 12.330 |
| 80 | 7.55 | 13.496 |
| 80 | 7.44 | 9.744 |
| 95 | 4.13 | 18.349 |
| 95 | 5.09 | 20.310 |
| 95 | 6.47 | 13.030 |
| 95 | 6.95 | 12.406 |

## A5.2 Datasets used in the evaluation of methods to estimate uptake rate constants and BCF from depuration rate constants (reproduced from (36))

**Table A – 8: Arnot data set – substance identities**

| Ref#[1)] | CAS No | Name | Log $K_{OW}$ | Molecular weight | Smiles | Initial evaluation of data quality |
|---|---|---|---|---|---|---|
| 432 | 120-12-7 | Anthracene | 4.45 | 178.24 | c(c(ccc1)cc(c2ccc3)c3)(c1)c2 | Acceptable |
| 996 | 69806-40-2 | Haloxyfop-methyl | 4.05 | 375.73 | Clc1cc(C(F)(F)F)cnc1Oc2ccc(OC(C)C(=O)OC)cc2 | Acceptable with restrictions |
| 16 | 50-32-8 | Benzo[a]pyrene | 6.13 | 252.32 | c(c(c(cc1)ccc2)c2cc3)(c3cc(c4ccc5)c5)c14 | Acceptable |
| 710 | 1582-09-8 | Benzenamine, 2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)- | 5.34 | 335.29 | CCCN(CCC)c1c(cc(cc1N(=O)(=O))C(F)(F)F)N(=O)(=O) | Acceptable |
| 711 | 1582-09-8 | Benzenamine, 2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)- | 5.34 | 335.29 | CCCN(CCC)c1c(cc(cc1N(=O)(=O))C(F)(F)F)N(=O)(=O) | Acceptable |
| 458 | 121-82-4 | 1,3,5-Triazine, hexahydro-1,3,5-trinitro- (RDX) | 0.87 | 222.12 | N(=O)(=O)N(CN(N(=O)(=O))CN1N(=O)(=O))C1 | Acceptable |
| 726 | 1746-01-6 | 2,3,7,8-Tetrachlorodibenzo[b,e][1,4]dioxin | 6.80 | 321.98 | Clc3cc2Oc1cc(Cl)c(Cl)cc1Oc2cc3Cl | Acceptable |
| 727 | 1746-01-6 | 2,3,7,8-Tetrachlorodibenzo[b,e][1,4]dioxin | 6.80 | 321.98 | Clc3cc2Oc1cc(Cl)c(Cl)cc1Oc2cc3Cl | Acceptable |
| 725 | 1746-01-6 | 2,3,7,8-Tetrachlorodibenzo[b,e][1,4]dioxin | 6.80 | 321.98 | Clc3cc2Oc1cc(Cl)c(Cl)cc1Oc2cc3Cl | Acceptable |
| 729 | 1746-01-6 | 2,3,7,8-Tetrachlorodibenzo[b,e][1,4]dioxin | 6.80 | 321.98 | Clc3cc2Oc1cc(Cl)c(Cl)cc1Oc2cc3Cl | Acceptable |
| 728 | 1746-01-6 | 2,3,7,8-Tetrachlorodibenzo[b,e][1,4]dioxin | 6.80 | 321.98 | Clc3cc2Oc1cc(Cl)c(Cl)cc1Oc2cc3Cl | Acceptable |
| 712 | 1582-09-8 | Benzenamine, 2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)- | 5.34 | 335.29 | CCCN(CCC)c1c(cc(cc1N(=O)(=O))C(F)(F)F)N(=O)(=O) | Acceptable |
| 29 | 56-55-3 | Benzo[a]anthracene | 5.76 | 228.30 | c(c(c(c(c1)ccc2)c2)cc(c3ccc4)c4)(c1)c3 | Acceptable |
| 2 | | Octaethylene glycol monotridecyl ether | 3.07 | 552.80 | OCCOCCOCCOCCOCCOCCOCCOCCOCCCCCCCCCCCCCC | Acceptable |
| 3 | | Octaethylene glycol monotridecyl ether | 5.11 | 552.80 | OCCOCCOCCOCCOCCOCCOCCOCCOCCCCCCCCCCCCCC | Acceptable |
| 531 | 226-36-8 | Dibenz(a,h)acridine | 5.67 | 279.34 | c1ccc4c(c1)ccc5nc2c(ccc3ccccc23)cc45 | Acceptable |
| 1 | | C-12-2-LAS | 4.71 | 326.50 | S(=O)(=O)(O)c1ccc(C(CCCCCCCCCC)C)cc1 | Acceptable with restrictions |
| 4 | | C-12-5-LAS | 4.71 | 326.50 | S(=O)(=O)(O)c1ccc(C(CCCCCCC)CCCC)cc1 | Acceptable with restrictions |
| 236 | 95-94-3 | Benzene, 1,2,4,5-tetrachloro- | 4.64 | 215.89 | c(c(cc(c1Cl)Cl)Cl)(c1)Cl | Acceptable |
| 441 | 120-82-1 | Benzene, 1,2,4-trichloro- | 4.02 | 181.45 | c(ccc(c1Cl)Cl)(c1)Cl | Acceptable |
| 164 | 88-06-2 | 2,4,6-Trichlorophenol | 3.69 | 197.45 | Oc(c(cc(c1)Cl)Cl)c1Cl | Acceptable with restrictions |
| 321 | 106-46-7 | Benzene, 1,4-dichloro- | 3.44 | 147.00 | c(ccc(c1)Cl)(c1)Cl | Acceptable |

| Ref#[1)] | CAS No | Name | Log $K_{OW}$ | Molecular weight | Smiles | Initial evaluation of data quality |
|---|---|---|---|---|---|---|
| 680 | 935-95-5 | 2,3,5,6-Tetrachlorophenol | 3.88 | 231.89 | Oc1c(Cl)c(Cl)cc(Cl)c1Cl | Acceptable with restrictions |
| 160 | 87-86-5 | Phenol, pentachloro- | 5.12 | 266.34 | Oc(c(c(c(c1Cl)Cl)Cl)Cl)c1Cl | Acceptable with restrictions |
| 163 | 87-86-5 | Phenol, pentachloro- | 5.12 | 266.34 | Oc(c(c(c(c1Cl)Cl)Cl)Cl)c1Cl | Acceptable with restrictions |
| 162 | 87-86-5 | Phenol, pentachloro- | 5.12 | 266.34 | Oc(c(c(c(c1Cl)Cl)Cl)Cl)c1Cl | Acceptable with restrictions |
| 200 | 92-86-4 | 4,4'-dibromobiphenyl | 5.72 | 312.01 | c(ccc(c(ccc(c1)Br)c1)c2)(c2)Br | Acceptable |
| 653 | 634-91-3 | 3,4,5-Trichloroaniline | 3.32 | 196.46 | Nc1cc(Cl)c(Cl)c(Cl)c1 | Acceptable with restrictions |
| 659 | 636-30-6 | 2,4,5-Trichloroaniline | 3.45 | 196.46 | Nc(c(cc(c1Cl)Cl)Cl)c1 | Acceptable with restrictions |
| 145 | 87-61-6 | Benzene, 1,2,3-trichloro- | 4.05 | 181.45 | c(c(c(cc1)Cl)Cl)(c1)Cl | Acceptable |
| 654 | 634-93-5 | 2,4,6-Trichloroaniline | 3.52 | 196.46 | Nc(c(cc(c1)Cl)Cl)c1Cl | Acceptable with restrictions |
| 652 | 634-67-3 | 2,3,4-Trichloroaniline | 3.33 | 196.46 | Nc1ccc(Cl)c(Cl)c1Cl | Acceptable with restrictions |
| 984 | 57117-44-9 | 1,2,3,6,7,8-Hexachlorodibenzofuran | 7.30 | 374.87 | Clc1c(Cl)c2c3cc(Cl)c(Cl)c(Cl)c3Oc2cc1Cl | Acceptable |
| 940 | 35693-99-3 | 2,2',5,5'-Tetrachloro-1,1'-biphenyl | 5.84 | 291.99 | c1c(Cl)ccc(Cl)c1c2c(Cl)ccc(Cl)c2 | Acceptable |
| 986 | 59080-33-0 | 2,4,6-Tribromobiphenyl | 6.03 | 390.90 | Brc2c(c(cc(c2)Br)Br)c1ccccc1 | Acceptable |
| 991 | 60851-34-5 | 2,3,4,6,7,8-Hexachlorodibenzofuran | 7.30 | 374.87 | Clc1cc2c3cc(Cl)c(Cl)c(Cl)c3Oc2c(Cl)c1Cl | Acceptable |
| 982 | 57117-31-4 | 2,3,4,7,8-Pentachlorodibenzofuran | 6.64 | 340.42 | c1(Cl)cc2c3cc(Cl)c(Cl)cc3oc2c(Cl)c1Cl | Acceptable |
| 943 | 35693-99-3 | 2,2',5,5'-Tetrachloro-1,1'-biphenyl | 5.84 | 291.99 | c1c(Cl)ccc(Cl)c1c2c(Cl)ccc(Cl)c2 | Acceptable |
| 929 | 30746-58-8 | 1,2,3,4-Tetrachlorodibenzo-*p*-dioxin | 6.60 | 321.98 | Clc1c(Cl)c(Cl)c2Oc3ccccc3Oc2c1Cl | Uncertain |
| 961 | 40321-76-4 | 1,2,3,7,8-Pentachlorodibenzo-*p*-dioxin | 6.64 | 356.42 | ClC(C1=C2OC(C=C3Cl)=C(C=C3Cl)O1)=C(C(Cl)=C2)Cl | Acceptable |
| 987 | 59080-37-4 | 2,2',5,5'-Tetrabromobiphenyl | 6.50 | 469.80 | Brc2c(c(ccc2)Br)c1c(cccc1Br)Br | Acceptable |
| 941 | 35693-99-3 | 2,2',5,5'-Tetrachloro-1,1'-biphenyl | 5.84 | 291.99 | c1c(Cl)ccc(Cl)c1c2c(Cl)ccc(Cl)c2 | Acceptable |
| 876 | 16606-02-3 | 2,4',5-Trichloro-1,1'-biphenyl | 5.67 | 257.55 | Clc1ccc(cc1)c2cc(Cl)ccc2Cl | Acceptable |
| 939 | 35065-27-1 | 2,2',4,4',5,5'-Hexachloro-1,1'-biphenyl | 6.92 | 360.88 | Clc1cc(Cl)c(cc1Cl)c2cc(Cl)c(Cl)cc2Cl | Acceptable |
| 959 | 39227-28-6 | 1,2,3,4,7,8-Hexachlorodibenzo-*p*-dioxin | 7.30 | 390.87 | Clc1c(Cl)c(Cl)c2Oc3ccc(Cl)c(Cl)c3Oc2c1Cl | Acceptable |
| 942 | 35693-99-3 | 2,2',5,5'-Tetrachloro-1,1'-biphenyl | 5.84 | 291.99 | c1c(Cl)ccc(Cl)c1c2c(Cl)ccc(Cl)c2 | Acceptable |
| 985 | 57653-85-7 | 1,2,3,6,7,8-Hexachlorodibenzo-*p*-dioxin | 7.30 | 390.87 | Clc2cc1Oc3c(Oc1c(Cl)c2Cl)cc(Cl)c(Cl)c3Cl | Acceptable |

| Ref#[1)] | CAS No | Name | Log $K_{OW}$ | Molecular weight | Smiles | Initial evaluation of data quality |
|---|---|---|---|---|---|---|
| 938 | 35065-27-1 | 2,2',4,4',5,5'-Hexachloro-1,1'-biphenyl | 6.92 | 360.88 | Clc1cc(Cl)c(cc1Cl)c2cc(Cl)c(Cl)cc2Cl | Acceptable |
| 641 | 626-39-1 | Benzene, 1,3,5-tribromo- | 4.51 | 314.80 | c(cc(cc1Br)Br)(c1)Br | Acceptable |
| 888 | 19408-74-3 | 1,2,3,7,8,9-Hexachlorodibenzo-*p*-dioxin | 7.30 | 390.87 | Clc(c1c3Oc2c(c(Cl)c(c(Cl)c2)Cl)O1)c(c(Cl)c3)Cl | Acceptable |
| 790 | 2921-88-2 | Phosphorothioic acid, O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) ester | 4.96 | 350.59 | CCOP(=S)(OCC)Oc1nc(Cl)c(Cl)cc1Cl | Acceptable |
| 968 | 51207-31-9 | 2,3,7,8-Tetrachlorodibenzofuran | 6.53 | 305.98 | Clc3cc2oc1cc(Cl)c(Cl)cc1c2cc3Cl | Acceptable |
| 960 | 39227-58-2 | 1,2,4-Trichlorodibenzo[b,e][1,4]dioxin | 6.35 | 287.53 | Clc3cc(Cl)c2Oc1ccccc1Oc2c3Cl | Acceptable |
| 934 | 33857-26-0 | 2,7-Dichlorodibenzo[b,e][1,4]dioxin | 5.75 | 253.09 | Clc3ccc2Oc1cc(Cl)ccc1Oc2c3 | Acceptable |
| 937 | 35065-27-1 | 2,2',4,4',5,5'-Hexachloro-1,1'-biphenyl | 6.92 | 360.88 | Clc1cc(Cl)c(cc1Cl)c2cc(Cl)c(Cl)cc2Cl | Acceptable |
| 995 | 67562-39-4 | 1,2,3,4,6,7,8-Heptachlorodibenzofuran | 7.40 | 409.31 | c1(Cl)c(Cl)c2c3cc(Cl)c(Cl)c(Cl)c3oc2c(Cl)c1Cl | Acceptable |
| 730 | 1746-01-6 | 2,3,7,8-Tetrachlorodibenzo[b,e][1,4]dioxin | 6.80 | 321.98 | Clc3cc2Oc1cc(Cl)c(Cl)cc1Oc2cc3Cl | Acceptable |
| 945 | 35822-46-9 | 1,2,3,4,6,7,8-Heptachlorodibenzo-*p*-dioxin | 7.80 | 425.31 | Clc1c(Cl)c(Cl)c2Oc3cc(Cl)c(Cl)c(Cl)c3Oc2c1Cl | Acceptable |
| 871 | 15862-07-4 | 2,4,5-Trichloro-1,1'-biphenyl | 5.60 | 257.55 | Clc1cc(Cl)c(cc1Cl)c2ccccc2 | Acceptable |
| 988 | 59261-08-4 | 2,2',4,4',6,6'-Hexabromobiphenyl | 7.20 | 627.59 | Brc2c(c(cc(c2)Br)Br)c1c(cc(cc1Br)Br)Br | Acceptable |
| 808 | 3268-87-9 | 1,2,3,4,5,6,7,8-Octachlorodibenzo-*p*-dioxin | 8.20 | 459.76 | Clc3c(Cl)c(Cl)c2Oc1c(Cl)c(Cl)c(Cl)c(Cl)c1Oc2c3Cl | Uncertain |
| 958 | 39001-02-0 | 1,2,3,4,5,6,7,8-Octachlorodibenzofuran | 8.20 | 443.76 | Clc3c(Cl)c(Cl)c1c(oc2c(Cl)c(Cl)c(Cl)c(Cl)c12)c3Cl | Acceptable |
| 316 | 106-37-6 | Benzene, 1,4-dibromo- | 3.79 | 235.91 | c(ccc(c1)Br)(c1)Br | Acceptable |
| 765 | 2385-85-5 | Mirex | 6.89 | 545.55 | ClC2(Cl)C4(Cl)C1(Cl)C5(Cl)C(Cl)(Cl)C3(Cl)C1(Cl)C2(Cl)C3(Cl)C45Cl | Acceptable |
| 746 | 2051-24-3 | Decachlorobiphenyl | 8.18 | 498.66 | Clc1c(Cl)c(Cl)c(c(Cl)c1Cl)c2c(Cl)c(Cl)c(Cl)c(Cl)c2Cl | Acceptable |
| 326 | 106-47-8 | Benzenamine, 4-chloro- | 1.83 | 127.57 | Nc(ccc(c1)Cl)c1 | Acceptable with restrictions |
| 54 | 62-53-3 | Benzenamine | 0.90 | 93.13 | Nc(cccc1)c1 | Acceptable with restrictions |
| 706 | 1582-09-8 | Benzenamine, 2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)- | 5.34 | 335.29 | CCCN(CCC)c1c(cc(cc1N(=O)(=O))C(F)(F)F)N(=O)(=O) | Acceptable |

| Ref#[1)] | CAS No | Name | Log $K_{OW}$ | Molecular weight | Smiles | Initial evaluation of data quality |
|---|---|---|---|---|---|---|
| 731 | 1746-01-6 | 2,3,7,8-Tetrachlorodibenzo[b,e][1,4]dioxin | 6.80 | 321.98 | Clc3cc2Oc1cc(Cl)c(Cl)cc1Oc2cc3Cl | Uncertain |
| 707 | 1582-09-8 | Benzenamine, 2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)- | 5.34 | 335.29 | CCCN(CCC)c1c(cc(cc1N(=O)(=O))C(F)(F)F)N(=O)(=O) | Acceptable |
| 399 | 117-81-7 | 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester | 7.73 | 390.57 | O=C(OCC(CCCC)CC)c(c(ccc1)C(=O)OCC(CCCC)CC)c1 | Uncertain |
| 708 | 1582-09-8 | Benzenamine, 2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)- | 5.34 | 335.29 | CCCN(CCC)c1c(cc(cc1N(=O)(=O))C(F)(F)F)N(=O)(=O) | Acceptable |
| 398 | 117-81-7 | 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester | 7.73 | 390.57 | O=C(OCC(CCCC)CC)c(c(ccc1)C(=O)OCC(CCCC)CC)c1 | Uncertain |
| 161 | 87-86-5 | Phenol, pentachloro- | 5.12 | 266.34 | Oc(c(c(c(c1Cl)Cl)Cl)Cl)c1Cl | Uncertain |
| 992 | 61949-76-6 | cis-Permethrin | 7.43 | 391.30 | O=C(OCC2=CC=CC(OC3=CC=CC=C3)=C2)C1C(C)(C)C1C=C(Cl)Cl | Acceptable |
| 970 | 51630-58-1 | Cyano(3-phenoxyphenyl)methyl ester, 4-Chloro-alpha-(1-methylethyl)benzeneacetic acid | 6.20 | 419.91 | CC(C)C(C(=O)OC(C#N)c2cccc(Oc1ccccc1)c2)c3ccc(Cl)cc3 | Acceptable |
| 158 | 87-86-5 | Phenol, pentachloro- | 5.12 | 266.34 | Oc(c(c(c(c1Cl)Cl)Cl)Cl)c1Cl | Acceptable with restrictions |
| 159 | 87-86-5 | Phenol, pentachloro- | 5.12 | 266.34 | Oc(c(c(c(c1Cl)Cl)Cl)Cl)c1Cl | Acceptable with restrictions |
| 971 | 52918-63-5 | [1R-[1 alpha(S*),3 alpha]]Cyano(3-phenoxyphenyl)methyl ester 3-(2,2-dibromoethenyl)-2,2-dimethyl cyclopropane carboxylic acid | 6.20 | 505.21 | CC1(C)C(C=C(Br)Br)C1C(=O)OC(C#N)c3cccc(Oc2ccccc2)c3 | Acceptable |
| 994 | 67375-30-8 | [1 alpha(S*), 3 alpha]-(+-)-3-(2,2-Dichloroethenyl)-2,2-dimethylcyclopropane carboxylic acid cyano (3-phenoxyphenyl)methyl ester | 6.38 | 416.31 | ClC(Cl)=CC1C(C)(C)C1C(=O)OC(C#N)c2cccc(Oc3ccccc3)c2 | Acceptable |
| 393 | 117-81-7 | 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester | 7.73 | 390.57 | O=C(OCC(CCCC)CC)c(c(ccc1)C(=O)OCC(CCCC)CC)c1 | Acceptable |
| 392 | 117-81-7 | 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester | 7.73 | 390.57 | O=C(OCC(CCCC)CC)c(c(ccc1)C(=O)OCC(CCCC)CC)c1 | Acceptable |
| 418 | 118-96-7 | Benzene, 2-methyl-1,3,5-trinitro- | 1.60 | 227.13 | O=N(=O)c(cc(N(=O)=O)c(c1N(=O)=O)C)c1 | Acceptable |
| 459 | 121-82-4 | 1,3,5-Triazine, hexahydro-1,3,5-trinitro- (RDX) | 0.87 | 222.12 | N(=O)(=O)N(CN(N(=O)(=O))CN1N(=O)(=O))C1 | Acceptable |

| Ref#[1)] | CAS No | Name | Log $K_{OW}$ | Molecular weight | Smiles | Initial evaluation of data quality |
|---|---|---|---|---|---|---|
| 788 | 2691-41-0 | Octahydro-1,3,5,7-Tetranitro-1,3,5,7-Tetrazocine (HMX) | 0.19 | 296.16 | O=N(=O)N(CN(N(=O)=O)CN(N(=O)=O)CN1N(=O)=O)C1 | Acceptable |
| 795 | 2921-88-2 | Phosphorothioic acid, O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) ester | 4.96 | 350.59 | CCOP(=S)(OCC)Oc1nc(Cl)c(Cl)cc1Cl | Acceptable |
| 111 | 80-05-7 | Phenol, 4,4 -(1-methylethylidene)bis- | 3.32 | 228.29 | Oc(ccc(c1)C(c(ccc(O)c2)c2)(C)C)c1 | Acceptable with restrictions |

1) Reference number from the original data set

**Table A – 9: Arnot data set – bioconcentration data**

| | | | | Experimental data | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ref#[1)] | Substance | Common name | Scientific name | $k_1$ (l $kg^{-1}$ $day^{-1}$) | Fish weight (kg)[2)] | Lipid (kg $kg^{-1}$) | Temperature (°C) | Dissolved oxygen (mg $l^{-1}$) | Reference[3)] |
| 432 | Anthracene | Bluegill sunfish | *Lepomis macrochirus* | 900 | $5\times10^{-4}$ | 0.048 | 23.5 | 7.4 | Spacie *et al*., 1983 |
| 996 | Haloxyfop-methyl | Bluegill sunfish | *Lepomis macrochirus* | 720 | $6\times10^{-4}$ | 0.048 | 17 | 8.6 | Murphy and Lutenske, 1990 |
| 16 | Benzo[a]pyrene | Bluegill sunfish | *Lepomis macrochirus* | 416 | $5\times10^{-4}$ | 0.048 | 23.5 | 7.4 | Spacie *et al*., 1983 |
| 710 | Benzenamine, 2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)- | Channel catfish | *Ictalurus punctatus* | 3,480 | $6.2\times10^{-3}$ | 0.040 | 23 | 7.5 | Schultz and Hayton, 1999 |
| 711 | Benzenamine, 2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)- | Channel catfish | *Ictalurus punctatus* | 3,480 | $6.89\times10^{-3}$ | 0.070 | 15 | 8.8 | Schultz and Hayton, 1999 |
| 458 | 1,3,5-Triazine, hexahydro-1,3,5-trinitro- (RDX) | Channel catfish | *Ictalurus punctatus* | 30.7 | $8.4\times10^{-5}$ | 0.048 | 25 | 8.0 | Belden *et al*., 2005 |
| 726 | 2,3,7,8-Tetrachlorodibenzo[b,e][1,4]dioxin | Common carp | *Cyprinus carpio* | 765 | 0.015 | 0.085 | 25 | 7.2 | Cook *et al*., 1991 |
| 727 | 2,3,7,8-Tetrachlorodibenzo[b,e][1,4]dioxin | Common carp | *Cyprinus carpio* | 736 | 0.015 | 0.055 | 25 | 7.2 | Cook *et al*., 1991 |
| 725 | 2,3,7,8-Tetrachlorodibenzo[b,e][1,4]dioxin | Common carp | *Cyprinus carpio* | 712 | 0.015 | 0.096 | 25 | 7.2 | Cook *et al*., 1991 |

| | | | | | | Experimental data | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ref#[1)] | Substance | Common name | Scientific name | $k_1$ (l $kg^{-1}$ $day^{-1}$) | Fish weight (kg)[2)] | Lipid (kg $kg^{-1}$) | Temperature (°C) | Dissolved oxygen (mg $l^{-1}$) | Reference[3)] |
| 729 | 2,3,7,8-Tetrachlorodibenzo[b,e][1,4]dioxin | Fathead minnow | *Pimephales promelas* | 1,870 | $1\times10^{-3}$ | 0.190 | 25 | 7.2 | Cook *et al*., 1991 |
| 728 | 2,3,7,8-Tetrachlorodibenzo[b,e][1,4]dioxin | Fathead minnow | *Pimephales promelas* | 1,280 | $1\times10^{-3}$ | 0.190 | 25 | 7.2 | Cook *et al*., 1991 |
| 712 | Benzenamine, 2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)- | Fathead minnow | *Pimephales promelas* | 756 | $8.5\times10^{-4}$ | 0.048 | 20 | 8.0 | Spacie and Hamelink, 1979 |
| 29 | Benzo[a]anthracene | Fathead minnow | *Pimephales promelas* | 405 | $4.2\times10^{-4}$ | 0.048 | 20.5 | 7.9 | de Maagd *et al*., 1998 |
| 2 | Octaethylene glycol monotridecyl ether | Fathead minnow | *Pimephales promelas* | 317 | $6.6\times10^{-4}$ | 0.033 | 22 | 7.7 | Tolls and Sijm, 1999 |
| 3 | Octaethylene glycol monotridecyl ether | Fathead minnow | *Pimephales promelas* | 317 | $6.6\times10^{-4}$ | 0.033 | 22 | 7.7 | Tolls and Sijm, 1999 |
| 531 | Dibenz(a,h)acridine | Fathead minnow | *Pimephales promelas* | 276 | $7.5\times10^{-5}$ | 0.048 | 22 | 7.7 | Southworth *et al*., 1980 |
| 1 | C-12-2-LAS | Fathead minnow | *Pimephales promelas* | 130 | $7.2\times10^{-4}$ | 0.050 | 21 | 7.8 | Tolls and Sijm, 1999 |
| 4 | C-12-5-LAS | Fathead minnow | *Pimephales promelas* | 11.1 | $7.2\times10^{-4}$ | 0.050 | 21 | 7.8 | Tolls, 1998 |
| 236 | Benzene, 1,2,4,5-tetrachloro- | Flagfish | *Jordanella floridae* | 1,630 | $2.25\times10^{-3}$ | 0.085 | 25 | 7.2 | Smith *et al*., 1990 |
| 441 | Benzene, 1,2,4-trichloro- | Flagfish | *Jordanella floridae* | 1,160 | $2.25\times10^{-3}$ | 0.114 | 25 | 7.2 | Smith *et al*., 1990 |
| 164 | 2,4,6-Trichlorophenol | Flagfish | *Jordanella floridae* | 421 | $2.25\times10^{-3}$ | 0.124 | 25 | 7.2 | Smith *et al*., 1990 |
| 321 | Benzene, 1,4-dichloro- | Flagfish | *Jordanella floridae* | 291 | $2.25\times10^{-3}$ | 0.085 | 25 | 7.2 | Smith *et al*., 1990 |
| 680 | 2,3,5,6-Tetrachlorophenol | Flagfish | *Jordanella floridae* | 243 | $2.25\times10^{-3}$ | 0.098 | 25 | 7.2 | Smith *et al*., 1990 |
| 160 | Phenol, pentachloro- | Flagfish | *Jordanella floridae* | 222 | $2.25\times10^{-3}$ | 0.133 | 25 | 7.2 | Smith *et al*., 1990 |
| 163 | Phenol, pentachloro- | Goldfish | *Carassius auratus* | 948 | $1\times10^{-3}$ | 0.048 | 20 | 8.0 | Stehly and Hayton, 1990 |
| 162 | Phenol, pentachloro- | Goldfish | *Carassius auratus* | 509 | $1.75\times10^{-3}$ | 0.048 | 20 | 8.0 | Stehly and Hayton, 1990 |

| | | | | Experimental data | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ref#[1)] | Substance | Common name | Scientific name | $k_1$ (l $kg^{-1}$ $day^{-1}$) | Fish weight (kg)[2)] | Lipid (kg $kg^{-1}$) | Temperature (°C) | Dissolved oxygen (mg $l^{-1}$) | Reference[3)] |
| 200 | 4,4'-Dibromobiphenyl | Guppy | *Poecilia reticulata* | 2,140 | $9.8\times10^{-5}$ | 0.065 | 22 | 8.0 | Gobas *et al*., 1989 |
| 653 | 3,4,5-Trichloroaniline | Guppy | *Poecilia reticulata* | 1,970 | $3.37\times10^{-4}$ | 0.137 | 23.5 | 7.4 | de Wolf *et al*., 1993 |
| 659 | 2,4,5-Trichloroaniline | Guppy | *Poecilia reticulata* | 1,630 | $3.37\times10^{-4}$ | 0.137 | 23.5 | 7.4 | de Wolf *et al*., 1993 |
| 145 | Benzene, 1,2,3-trichloro- | Guppy | *Poecilia reticulata* | 1,580 | $3.37\times10^{-4}$ | 0.137 | 23.5 | 7.4 | de Wolf *et al*., 1993 |
| 654 | 2,4,6-Trichloroaniline | Guppy | *Poecilia reticulata* | 1,580 | $3.37\times10^{-4}$ | 0.137 | 23.5 | 7.4 | de Wolf *et al*., 1993 |
| 652 | 2,3,4-Trichloroaniline | Guppy | *Poecilia reticulata* | 1,460 | $3.37\times10^{-4}$ | 0.137 | 23.5 | 7.4 | de Wolf *et al*., 1993 |
| 984 | 1,2,3,6,7,8-Hexachlorodibenzofuran | Guppy | *Poecilia reticulata* | 1,310 | $9.1\times10^{-4}$ | 0.097 | 25 | 7.2 | Loonen *et al*., 1994 |
| 940 | 2,2',5,5'-Tetrachloro-1,1'-biphenyl | Guppy | *Poecilia reticulata* | 1,120 | $9.8\times10^{-5}$ | 0.065 | 22 | 8.0 | Gobas *et al*., 1989 |
| 986 | 2,4,6-Tribromobiphenyl | Guppy | *Poecilia reticulata* | 1,120 | $9.8\times10^{-5}$ | 0.065 | 22 | 8.0 | Gobas *et al*., 1989 |
| 991 | 2,3,4,6,7,8-Hexachlorodibenzo furan | Guppy | *Poecilia reticulata* | 1,100 | $9.1\times10^{-4}$ | 0.097 | 25 | 7.2 | Loonen *et al*., 1994 |
| 982 | 2,3,4,7,8-Pentachlorodibenzo furan | Guppy | *Poecilia reticulata* | 1,010 | $9.1\times10^{-4}$ | 0.097 | 25 | 7.2 | Loonen *et al*., 1994 |
| 943 | 2,2',5,5'-Tetrachloro-1,1'-biphenyl | Guppy | *Poecilia reticulata* | 1,000 | $1\times10^{-4}$ | 0.048 | 18 | 3.0 | Opperhuizen and Schrap, 1987 |
| 929 | 1,2,3,4-Tetrachlorodibenzo-*p*-dioxin | Guppy | *Poecilia reticulata* | 953 | $7.9\times10^{-5}$ | 0.075 | 22 | 8.0 | Gobas and Schrap, 1990 |
| 961 | 1,2,3,7,8-Pentachlorodibenzo-*p*-dioxin | Guppy | *Poecilia reticulata* | 952 | $9.1\times10^{-4}$ | 0.097 | 25 | 7.2 | Loonen *et al*., 1994 |
| 987 | 2,2',5,5'-Tetrabromobiphenyl | Guppy | *Poecilia reticulata* | 912 | $9.8\times10^{-5}$ | 0.065 | 22 | 8.0 | Gobas *et al*., 1989 |
| 941 | 2,2',5,5'-Tetrachloro-1,1'-biphenyl | Guppy | *Poecilia reticulata* | 910 | $1\times10^{-4}$ | 0.048 | 18 | 7.0 | Opperhuizen and Schrap, 1987 |
| 876 | 2,4',5-Trichloro-1,1'-biphenyl | Guppy | *Poecilia reticulata* | 890 | $7.9\times10^{-5}$ | 0.075 | 22 | 8.0 | Gobas and Schrap, 1990 |

| | | | | Experimental data | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ref#[1)] | Substance | Common name | Scientific name | $k_1$ (l $kg^{-1}$ $day^{-1}$) | Fish weight (kg)[2)] | Lipid (kg $kg^{-1}$) | Temperature (°C) | Dissolved oxygen (mg $l^{-1}$) | Reference[3)] |
| 939 | 2,2',4,4',5,5'-Hexachloro-1,1'-biphenyl | Guppy | *Poecilia reticulata* | 880 | $1\times10^{-4}$ | 0.048 | 18 | 3.0 | Opperhuizen and Schrap, 1987 |
| 959 | 1,2,3,4,7,8-Hexachlorodibenzo-*p*-dioxin | Guppy | *Poecilia reticulata* | 868 | $9.1\times10^{-4}$ | 0.097 | 25 | 7.2 | Loonen *et al*., 1994 |
| 942 | 2,2',5,5'-Tetrachloro-1,1'-biphenyl | Guppy | *Poecilia reticulata* | 860 | $1\times10^{-4}$ | 0.048 | 18 | 5.0 | Opperhuizen and Schrap, 1987 |
| 985 | 1,2,3,6,7,8-Hexachlorodibenzo-*p*-dioxin | Guppy | *Poecilia reticulata* | 844 | $9.1\times10^{-4}$ | 0.097 | 25 | 7.2 | Loonen *et al*., 1994 |
| 938 | 2,2',4,4',5,5'-Hexachloro-1,1'-biphenyl | Guppy | *Poecilia reticulata* | 840 | $1\times10^{-4}$ | 0.048 | 18 | 7.0 | Opperhuizen and Schrap, 1987 |
| 641 | Benzene, 1,3,5-tribromo- | Guppy | *Poecilia reticulata* | 708 | $9.8\times10^{-5}$ | 0.065 | 22 | 8.0 | Gobas *et al*., 1989 |
| 888 | 1,2,3,7,8,9-Hexachlorodibenzo-*p*-dioxin | Guppy | *Poecilia reticulata* | 687 | $9.1\times10^{-4}$ | 0.097 | 25 | 7.2 | Loonen *et al*., 1994 |
| 790 | Phosphorothioic acid, O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) ester | Guppy | *Poecilia reticulata* | 630 | $9.4\times10^{-5}$ | 0.090 | 22 | 7.7 | Deneer, 1993 |
| 968 | 2,3,7,8-Tetrachlorodibenzofuran | Guppy | *Poecilia reticulata* | 603 | $9.1\times10^{-4}$ | 0.097 | 25 | 7.2 | Loonen *et al*., 1994 |
| 960 | 1,2,4-Trichlorodibenzo[b,e][1,4]dioxin | Guppy | *Poecilia reticulata* | 601 | $7.9\times10^{-5}$ | 0.075 | 22 | 8.0 | Gobas and Schrap, 1990 |
| 934 | 2,7-Dichlorodibenzo[b,e][1,4]dioxin | Guppy | *Poecilia reticulata* | 543 | $7.9\times10^{-5}$ | 0.075 | 22 | 8.0 | Gobas and Schrap, 1990 |
| 937 | 2,2',4,4',5,5'-Hexachloro-1,1'-biphenyl | Guppy | *Poecilia reticulata* | 540 | $1\times10^{-4}$ | 0.048 | 18 | 5.0 | Opperhuizen and Schrap, 1987 |
| 995 | 1,2,3,4,6,7,8-Hepta chlorodibenzofuran | Guppy | *Poecilia reticulata* | 524 | $9.1\times10^{-4}$ | 0.097 | 25 | 7.2 | Loonen *et al*., 1994 |
| 730 | 2,3,7,8-Tetrachlorodibenzo[b,e][1,4]dioxin | Guppy | *Poecilia reticulata* | 500 | $9.1\times10^{-4}$ | 0.097 | 25 | 7.2 | Loonen *et al*., 1994 |

| | | | | | | Experimental data | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ref#[1)] | Substance | Common name | Scientific name | $k_1$ (l $kg^{-1}$ $day^{-1}$) | Fish weight (kg)[2)] | Lipid (kg $kg^{-1}$) | Temperature (°C) | Dissolved oxygen (mg $l^{-1}$) | Reference[3)] |
| 945 | 1,2,3,4,6,7,8-Heptachlorodibenzo-*p*-dioxin | Guppy | *Poecilia reticulata* | 456 | $9.1\times10^{-4}$ | 0.097 | 25 | 7.2 | Loonen *et al*., 1994 |
| 871 | 2,4,5-Trichloro-1,1'-biphenyl | Guppy | *Poecilia reticulata* | 380 | $9.8\times10^{-5}$ | 0.065 | 22 | 8.0 | Gobas *et al*., 1989 |
| 988 | 2,2',4,4',6,6'-Hexabromobiphenyl | Guppy | *Poecilia reticulata* | 324 | $9.8\times10^{-5}$ | 0.065 | 22 | 8.0 | Gobas *et al*., 1989 |
| 808 | 1,2,3,4,5,6,7,8-Octachlorodibenzo-*p*-dioxin | Guppy | *Poecilia reticulata* | 275 | $9.1\times10^{-4}$ | 0.097 | 25 | 7.2 | Loonen *et al*., 1994 |
| 958 | 1,2,3,4,5,6,7,8-Octachlorodibenzofuran | Guppy | *Poecilia reticulata* | 217 | $9.1\times10^{-4}$ | 0.097 | 25 | 7.2 | Loonen *et al*., 1994 |
| 316 | Benzene, 1,4-dibromo- | Guppy | *Poecilia reticulata* | 129 | $9.8\times10^{-5}$ | 0.065 | 22 | 8.0 | Gobas *et al*., 1989 |
| 765 | Mirex | Guppy | *Poecilia reticulata* | 93.3 | $9.8\times10^{-5}$ | 0.065 | 22 | 8.0 | Gobas *et al*., 1989 |
| 746 | Decachlorobiphenyl | Guppy | *Poecilia reticulata* | 41.7 | $9.8\times10^{-5}$ | 0.065 | 22 | 8.0 | Gobas *et al*., 1989 |
| 326 | Benzenamine, 4-chloro- | Medaka, high-eyes | *Oryzias latipes* | 689 | $2.6\times10^{-4}$ | 0.048 | 25 | 7.2 | Bradbury *et al*., 1993 |
| 54 | Benzenamine | Medaka, high-eyes | *Oryzias latipes* | 250 | $2.9\times10^{-4}$ | 0.048 | 25 | 7.2 | Bradbury *et al*., 1993 |
| 706 | Benzenamine, 2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)- | Rainbow trout | *Oncorhynchus mykiss* | 3,140 | $1.8\times10^{-4}$ | 0.048 | 12 | 9.8 | Schultz and Hayton, 1994 |
| 731 | 2,3,7,8-Tetrachlorodibenzo[b,e][1,4]dioxin | Rainbow trout | *Oncorhynchus mykiss* | 1,850 | $3.8\times10^{-4}$ | 0.048 | 12 | 8.0 | Mehrle *et al*., 1988 |
| 707 | Benzenamine, 2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)- | Rainbow trout | *Oncorhynchus mykiss* | 1,630 | $4.07\times10^{-3}$ | 0.074 | 12 | 9.8 | Schultz and Hayton, 1994 |
| 399 | 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester | Rainbow trout | *Oncorhynchus mykiss* | 1,550 | $2.89\times10^{-3}$ | 0.048 | 12 | 9.8 | Tarr *et al*., 1990 |
| 708 | Benzenamine, 2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)- | Rainbow trout | *Oncorhynchus mykiss* | 538 | 0.0836 | 0.076 | 12 | 9.8 | Schultz and Hayton, 1994 |

| | | | | | Experimental data | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ref#[1)] | Substance | Common name | Scientific name | $k_1$ (l $kg^{-1}$ $day^{-1}$) | Fish weight (kg)[2)] | Lipid (kg $kg^{-1}$) | Temperature (°C) | Dissolved oxygen (mg $l^{-1}$) | Reference[3)] |
| 398 | 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester | Rainbow trout | *Oncorhynchus mykiss* | 386 | 0.0613 | 0.048 | 12 | 9.8 | Tarr *et al*., 1990 |
| 161 | Phenol, pentachloro- | Rainbow trout | *Oncorhynchus mykiss* | 341 | $4.6\times10^{-3}$ | 0.048 | 12 | 9.8 | Stehly and Hayton, 1989 |
| 992 | cis-Permethrin | Rainbow trout | *Oncorhynchus mykiss* | 201 | $1.5\times10^{-3}$ | 0.080 | 10 | 10.3 | Muir *et al*., 1994 |
| 970 | Cyano(3-phenoxyphenyl)methyl ester, 4-Chloro-alpha-(1-methylethyl)benzeneacetic acid | Rainbow trout | *Oncorhynchus mykiss* | 157 | $1.5\times10^{-3}$ | 0.080 | 10 | 10.3 | Muir *et al*., 1994 |
| 158 | Phenol, pentachloro- | Rainbow trout | *Oncorhynchus mykiss* | 120 | 0.723 | 0.070 | 11 | 10.5 | McKim *et al*., 1986 |
| 159 | Phenol, pentachloro- | Rainbow trout | *Oncorhynchus mykiss* | 118 | 0.723 | 0.070 | 11 | 10.5 | McKim *et al*., 1986 |
| 971 | [1R-[1 alpha(S*),3 alpha]]Cyano(3-phenoxyphenyl)methyl ester 3-(2,2-dibromoethenyl)-2,2-dimethyl cyclopropane carboxylic acid | Rainbow trout | *Oncorhynchus mykiss* | 105 | $1.5\times10^{-3}$ | 0.080 | 10 | 10.3 | Muir *et al*., 1994 |
| 994 | [1 alpha(S*), 3 alpha]-(+-)-3-(2,2-Dichloroethenyl)-2,2-dimethylcyclopropane carboxylic acid cyano (3-phenoxyphenyl)methyl ester | Rainbow trout | *Oncorhynchus mykiss* | 59.3 | $1.5\times10^{-3}$ | 0.080 | 10 | 10.3 | Muir *et al*., 1994 |
| 393 | 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester | Sheepshead minnow | *Cyprinodon variegatus* | 672 | $2\times10^{-3}$ | 0.048 | 29 | 6.8 | Karara and Hayton, 1989 |
| 392 | 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester | Sheepshead minnow | *Cyprinodon variegatus* | 317 | $2\times10^{-3}$ | 0.048 | 23 | 7.5 | Karara and Hayton, 1989 |
| 418 | Benzene, 2-methyl-1,3,5-trinitro- | Sheepshead minnow | *Cyprinodon variegatus* | 200 | $2\times10^{-4}$ | 0.048 | 23 | 7.0 | Lotufo and Lydy, 2005 |

| | | | | Experimental data | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ref#[1)] | Substance | Common name | Scientific name | $k_1$ (l $kg^{-1}$ $day^{-1}$) | Fish weight (kg)[2)] | Lipid (kg $kg^{-1}$) | Temperature (°C) | Dissolved oxygen (mg $l^{-1}$) | Reference[3)] |
| 459 | 1,3,5-Triazine, hexahydro-1,3,5-trinitro- (RDX) | Sheepshead minnow | *Cyprinodon variegatus* | 3.6 | $1.58\times10^{-4}$ | 0.048 | 23 | 7.0 | Lotufo and Lydy, 2005 |
| 788 | Octahydro-1,3,5,7-Tetranitro-1,3,5,7-Tetrazocine (HMX) | Sheepshead minnow | *Cyprinodon variegatus* | 1.4 | $1.79\times10^{-4}$ | 0.048 | 23 | 7.0 | Lotufo and Lydy, 2005 |
| 795 | Phosphorothioic acid, O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) ester | Threespine stickleback | *Gasterosteus aculeatus* | 1,380 | $3.22\times10^{-4}$ | 0.053 | 21.5 | 8.4 | Deneer, 1994 |
| 111 | Phenol, 4,4 -(1-methylethylidene)bis- | Zebrafish | *Brachydanio rerio* | 5.5 | $5\times10^{-4}$ | 0.048 | 27 | 7.0 | Lindholst *et al*., 2003 |

1) Reference number from the original data set.
2) The initial fish weight (either reported or estimated where available/possible).
3) References for the bioconcentration data are as follows:


Belden J.B., Lotufo G.R. and Lydy M.J. (2005). Accumulation of hexahydro-1,3,5-trinitro-1,3,5-triazine in channel catfish (Ictalurus punctatus) and aquatic oligochaetes (*Lumbriculus variegatus*). Environ. Toxicol. Chem. 24: 1962-1967.

Bradbury S.P., Dady J.M., Fitzsimmons P.N., Voit M.M., Hammermeister D.E. and Erickson R.J. (1993). Toxicokinetics and metabolism of aniline and 4-chloroaniline in medaka (*Oryzias latipes*). Toxicol. Appl. Pharmacol. 118: 205-214.

Cook P.M., Walker M.K., Kuehl D.W. and Peterson R.E. (1991). Bioaccumulation and toxicity of TCDD and related compounds in aquatic ecosystems, in Banbury Report 35: Biological Basis for Risk Assessment of Dioxins and Related Compounds. Cold Spring Harbor Laboratory Press, Plainview, NY, USA: 143-167.

De Maagd P.G.-J., de Poorte J., Opperhuizen A. and Sijm D.T.H.M. (1998). No influence after various exposure times on the biotransformation rate constants of benzo(a)anthracene in fathead minnow (*Pimephales promelas*). Aquat. Toxicol. 40: 157-169.

De Wolf W., Seinen W. and Hermens J.L.M. (1993). Biotransformation and toxicokinetics of trichloroanilines in fish in relation to their hydrophobicity. Arch. Environ. Con. Tox. 25: 110-117.

Deneer J.W. (1993). Uptake and elimination of chlorpyrifos in the guppy at sublethal and lethal aqueous concentrations. Chemosphere. 26: 1607-1616.

Deneer J.W. (1994). Bioconcentration of chlorpyrifos by the three-spined stickleback under laboratory and field conditions. Chemosphere. 29: 1561-1575.

Gobas F.A.P.C., Clark K.E., Shiu W.Y. and Mackay D. (1989). Bioconcentration of polybrominated benzenes and biphenyls and related superhydrophobic chemicals in fish: Role of bioavailability and elimination into the feces. Environ. Toxicol. Chem. 8: 231-245.

Gobas F.A.P.C. and Schrap S.M. (1990). Bioaccumulation of some polychlorinated dibenzo-p-dioxins and octachlorodibenzofuran in the guppy (*Poecilia reticulata*). Chemosphere. 20: 495-512.

Karara A.H. and Hayton W.L. (1989). A pharmacokinetic analysis of the effect of temperature on the accumulation of di-2-ethylhexyl phthalate (DEHP) in sheepshead minnow. Aquat. Toxicol. 15: 27-36.

Lindholst C., Wynne P.M., Marriott P., Pedersen S.N. and Bjerregaard P. (2003). Metabolism of bisphenol A in zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*) in relation to estrogenic response. Comp. Biochem. Physiol. C. 135: 169-177.

Loonen H., Tonkes M., Parsons J.R. and Govers H.A.J. (1994). Bioconcentration of polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans in guppies after aqueous exposure to a complex PCDD/PCDF mixture: Relationship with molecular structure. Aquat. Toxicol. 30: 153-169.

Lotufo G.R. and Lydy M.J. (2005). Comparative toxicokinetics of explosive compounds in sheepshead minnows. Arch. Environ. Con. Tox. 49: 206-214.

McKim J.M., Schmieder P.K. and Erickson R.J. (1986). Toxicokinetic modeling of [$^{14}$C]pentachlorophenol in the rainbow trout (*Salmo gairdneri*). Aquat. Toxicol. 9: 59-80.

Mehrle P.M., Buckler D.R., Little E.E., Smith L.M., Petty J.D., Peterman P.H., Stalling D.L., De Graeve G.M., Coyle J.J. and Adams W.J. (1988). Toxicity and bioconcentration of 2,3,7,8-tetrachlorodibenzodioxin and 2,3,7,8-tetrachlorodibenzofuran in rainbow trout. Environ. Toxicol. Chem. 7: 47-62.

Muir D.C.G., Hobden B.R. and Servos M.R. (1994). Bioconcentration of pyrethroid insecticides and DDT by rainbow trout - Uptake, depuration, and effect of dissolved organic carbon. Aquat. Toxicol. 29: 223-240.

Murphy P.G. and Lutenske N.E. (1990). Bioconcentration of haloxyfop-methyl in bluegill (*Lepomis macrochirus* Rafinesque). Environ. Intern. 16: 219-230.

Opperhuizen A. and Schrap S.M. (1987). Relationships between aqueous oxygen concentration and uptake and elimination rates during bioconcentration of hydrophobic chemicals in fish. Environ. Toxicol. Chem. 6: 335-342.

Schultz I.R. and Hayton W.L. (1994). Body size and the toxicokinetics of trifluralin in rainbow trout. Toxicol. Appl. Pharmacol. 129: 138-145.

Schultz I.R. and Hayton W.L. (1999). Interspecies scaling of the bioaccumulation of hydrophobic xenobiotics in fish: an example using trifluralin. Environ. Toxicol. Chem. 18: 1440-1449.

Smith A.D., Bharath A., Mallard C., Orr D., McCarty L.S. and Ozbu rn G.W. (1990). Bioconcentration kinetics of some chlorinated benzenes and chlorinated phenols in American Flagfish, *Jordanella floridae* (Goode and Bean). Chemosphere. 20: 379-386.

Southworth G.R., Keffer C.C. and Beauchamp J.J. (1980). Potential and realized bioconcentration. A comparison of observed and predicted bioconcentration of azaarenes in the fathead minnow (*Pimephales promelas*). Environ. Sci. Technol. 14: 1529-1531.

Spacie A. and Hamelink J.L. (1979). Dynamics of trifluralin accumulation in river fishes. Environ. Sci. Technol. 13: 817-822.

Spacie A., Landrum P.F. and Leversee G.J. (1983). Uptake, depuration, and biotransformation of anthracene and benzo[a]pyrene in bluegill sunfish. Ecotox. Environ. Saf. 7: 330-341.

Stehly G.R. and Hayton W.L. (1989). Disposition of pentachlorophenol in rainbow trout (*Salmo gairdneri*): effect of inhibition of metabolism. Aquat. Toxicol. 14: 131-147.

Stehly G.R. and Hayton W.L. (1990). Effect of pH on the accumulation kinetics of pentachlorophenol in goldfish. Arch. Environ. Con. Tox. 19: 464-470.

Tarr B.D., Barron M.G. and Hayton W.L. (1990). Effect of body size on the uptake and bioconcentration of di-2-ethylhexyl phthalate in rainbow trout. Environ. Toxicol. Chem. 9: 989-995.

Tolls J. (1998). Bioconcentration of surfactants. PhD Thesis, Utrecht University, Utrecht, The Netherlands.

Tolls J. and Sijm D.T.H.M. (1999). Bioconcentration and biotransformation of the nonionic surfactant octaethylene glycol monotridecyl ether $^{14}$C-C13EO8. Environ. Toxicol. Chem. 18: 2689-2695.

**Table A – 10: UBA set – substance identities**

| Ref#[1)] | CAS No | Name | Log $K_{OW}$ | Molecular weight | Smiles |
|---|---|---|---|---|---|
| UBA 11-1 | Confidential | Confidential | 3.8 | Confidential | Confidential |
| UBA 11-2 | Confidential | Confidential | 3.8 | Confidential | Confidential |
| UBA 4-1 | Confidential | Confidential | 2.9 | Confidential | Confidential |
| UBA 4-2 | Confidential | Confidential | 2.9 | Confidential | Confidential |
| UBA 6-1 | Confidential | Confidential | 2.86 | Confidential | Confidential |
| UBA 6-2 | Confidential | Confidential | 2.86 | Confidential | Confidential |
| UBA 10-1 | Confidential | Confidential | 3.2 | Confidential | Confidential |
| UBA 10-2 | Confidential | Confidential | 3.2 | Confidential | Confidential |
| UBA 5-1 | Confidential | Confidential | 4.9 | Confidential | Confidential |
| UBA 5-2 | Confidential | Confidential | 4.9 | Confidential | Confidential |
| UBA 13-1 | Confidential | Confidential | 3.4 | Confidential | Confidential |
| UBA 14-1 | Confidential | Confidential | 3.4 | Confidential | Confidential |
| UBA 14-2 | Confidential | Confidential | 3.4 | Confidential | Confidential |
| UBA 13-2 | Confidential | Confidential | 3.4 | Confidential | Confidential |
| UBA 9-1 | Confidential | Confidential | 2.59 | Confidential | Confidential |
| UBA 3-1 | Confidential | Confidential | 5.1 | Confidential | Confidential |
| UBA 3-2 | Confidential | Confidential | 5.1 | Confidential | Confidential |
| UBA 9-2 | Confidential | Confidential | 2.59 | Confidential | Confidential |

[1)] Reference number from the original data set, expanded by an additional number (1 or 2 behind the hyphen) to indicate the different concentrations tested.

**Table A – 11: UBA data set – bioconcentration data**

| | | | | Experimental data | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ref#[1)] | Substance | Common name | Scientific name | $k_1$ (l kg$^{-1}$ day$^{-1}$) | Fish weight (kg)[2)] | Lipid (kg kg$^{-1}$) | Temperature (°C)[3)] | Dissolved oxygen (mg l$^{-1}$)[3)] | Reference[4)] |
| UBA 11-1 | Confidential | Bluegill sunfish | *Lepomis macrochirus* | 40.9 | $7.03\times10^{-3}$ | 0.061 | no data | no data | UBA |
| UBA 11-2 | Confidential | Bluegill sunfish | *Lepomis macrochirus* | 28.3 | $6.32\times10^{-3}$ | 0.061 | no data | no data | UBA |
| UBA 4-1 | Confidential | Bluegill sunfish | *Lepomis macrochirus* | 15.5 | $2.58\times10^{-3}$ | 0.129 | no data | no data | UBA |
| UBA 4-2 | Confidential | Bluegill sunfish | *Lepomis macrochirus* | 11.1 | $2.58\times10^{-3}$ | 0.13 | no data | no data | UBA |
| UBA 6-1 | Confidential | Bluegill sunfish | *Lepomis macrochirus* | 7.73 | $3.40\times10^{-3}$ | 0.0629 | no data | no data | UBA |
| UBA 6-2 | Confidential | Bluegill sunfish | *Lepomis macrochirus* | 6.88 | $3.40\times10^{-3}$ | 0.0629 | no data | no data | UBA |
| UBA 10-1 | Confidential | Fathead minnow | *Pimephales promelas* | 76.7 | $3.90\times10^{-3}$ | 0.11 | no data | no data | UBA |
| UBA 10-2 | Confidential | Fathead minnow | *Pimephales promelas* | 28.9 | $3.90\times10^{-3}$ | 0.11 | no data | no data | UBA |
| UBA 5-1 | Confidential | Rainbow trout | *Oncorhynchus mykiss* | 411 | $1.35\times10^{-3}$ | 0.049 | no data | no data | UBA |
| UBA 5-2 | Confidential | Rainbow trout | *Oncorhynchus mykiss* | 339 | $1.35\times10^{-3}$ | 0.049 | no data | no data | UBA |

| | | | | | Experimental data | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ref# [1)] | Substance | Common name | Scientific name | $k_1$ (l kg$^{-1}$ day$^{-1}$) | Fish weight (kg)[2)] | Lipid (kg kg$^{-1}$) | Temperature (°C)[3)] | Dissolved oxygen (mg l$^{-1}$)[3)] | Reference[4)] |
| UBA 13-1 | Confidential | Rainbow trout | *Oncorhynchus mykiss* | 37.9 | $8.50\times10^{-4}$ | no data | no data | no data | UBA |
| UBA 14-1 | Confidential | Rainbow trout | *Oncorhynchus mykiss* | 11.2 | $7.87\times10^{-4}$ | 0.0323 | no data | no data | UBA |
| UBA 14-2 | Confidential | Rainbow trout | *Oncorhynchus mykiss* | 11.1 | $7.87\times10^{-4}$ | 0.0323 | no data | no data | UBA |
| UBA 13-2 | Confidential | Rainbow trout | *Oncorhynchus mykiss* | 8.66 | $8.70\times10^{-4}$ | no data | no data | no data | UBA |
| UBA 9-1 | Confidential | Zebrafish | *Brachydanio rerio* | 1,543 | $2.25\times10^{-4}$ | 0.049 | no data | no data | UBA |
| UBA 3-1 | Confidential | Zebrafish | *Brachydanio rerio* | 516 | $3.47\times10^{-4}$ | 0.123 | no data | no data | UBA |
| UBA 3-2 | Confidential | Zebrafish | *Brachydanio rerio* | 492 | $3.47\times10^{-4}$ | 0.123 | no data | no data | UBA |
| UBA 9-2 | Confidential | Zebrafish | *Brachydanio rerio* | 336 | $2.25\times10^{-4}$ | 0.049 | no data | no data | UBA |

1) Reference number from the original data set, expanded by an additional number (1 or 2 behind the hyphen) to indicate the different concentrations tested.
2) The initial fish weight (either reported or estimated where available/possible).
3) Data on the temperature and dissolved oxygen concentration were not supplied but should be available in the confidential test report.
4) All data provided by UBA. The test reports are confidential.

**Table A – 12: Gold standard data set – substance identities**

| Ref#[1)] | CAS No | Name | Log $K_{OW}$ | Molecular weight | Smiles |
|---|---|---|---|---|---|
| GS32 | 120-82-1 | 1,2,4-Trichlorobenzene | 4.02 | 181.45 | c(ccc(c1Cl)Cl)(c1)Cl |
| GS45 | 118-74-1 | Hexachlorobenzene | 5.73 | 284.78 | c(c(c(c(c1Cl)Cl)Cl)Cl)(c1Cl)Cl |
| GS44 | 615-54-3 | 1,2,4-Tribromobenzene | 4.66 | 314.80 | c(ccc(c1Br)Br)(c1)Br |
| GS43 | 634-90-2 | 1,2,3,5-Tetrachlorobenzene | 4.56 | 215.89 | c(cc(c(c1Cl)Cl)Cl)(c1)Cl |
| GS42 | 87-61-6 | 1,2,3-Trichlorobenzene | 4.05 | 181.45 | c(c(c(cc1)Cl)Cl)(c1)Cl |
| GS41 | 106-37-6 | 1,4-Dibromobenzene | 3.79 | 235.91 | c(ccc(c1)Br)(c1)Br |
| GS40 | 106-46-7 | 1,4-Dichlorobenzene | 3.44 | 147.00 | c(ccc(c1)Cl)(c1)Cl |
| GS7 | 2027-17-0 | 2-Isopropylnaphthalene | 4.63 | 170.26 | c(c(ccc1C(C)C)ccc2)(c2)c1 |
| GS8 | 2027-17-0 | 2-Isopropylnaphthalene | 4.63 | 170.26 | c(c(ccc1C(C)C)ccc2)(c2)c1 |
| GS5 | 575-41-7 | 1,3-Dimethylnaphthalene | 4.42 | 156.23 | Cc2cc(C)c1ccccc1c2 |
| GS3 | 91-57-6 | 2-Methylnaphthalene | 3.86 | 142.20 | c(c(ccc1C)ccc2)(c2)c1 |
| GS4 | 91-57-6 | 2-Methylnaphthalene | 3.86 | 142.20 | c(c(ccc1C)ccc2)(c2)c1 |
| GS6 | 575-41-7 | 1,3-Dimethylnaphthalene | 4.42 | 156.23 | Cc2cc(C)c1ccccc1c2 |
| GS9 | 85-01-8 | Phenanthrene | 4.46 | 178.24 | c(c(c(c(c1)ccc2)c2)ccc3)(c1)c3 |
| GS1 | 91-20-3 | Naphthalene | 3.3 | 128.18 | c(c(ccc1)ccc2)(c1)c2 |
| GS2 | 91-20-3 | Naphthalene | 3.3 | 128.18 | c(c(ccc1)ccc2)(c1)c2 |
| GS13 | 3674-75-7 | 9-Ethylphenanthrene | 5.38 | 206.29 | c(ccc1c(ccc2)c3c2)cc1cc3CC |
| GS10 | 85-01-8 | Phenanthrene | 4.46 | 178.24 | c(c(c(c(c1)ccc2)c2)ccc3)(c1)c3 |
| GS11 | 883-20-5 | 9-Methylphenanthrene | 4.89 | 192.26 | c(ccc1c(ccc2)c3c2)cc1cc3C |

| | | | | | |
|---|---|---|---|---|---|
| GS12 | 883-20-5 | 9-Methylphenanthrene | 4.89 | 192.26 | c(ccc1c(ccc2)c3c2)cc1cc3C |
| GS14 | 3674-75-7 | 9-Ethylphenanthrene | 5.38 | 206.29 | c(ccc1c(ccc2)c3c2)cc1cc3CC |
| GS16 | 129-00-0 | Pyrene | 4.88 | 202.26 | c(c(c(cc1)ccc2)c2cc3)(c1ccc4)c34 |
| GS15 | 129-00-0 | Pyrene | 4.88 | 202.26 | c(c(c(cc1)ccc2)c2cc3)(c1ccc4)c34 |

[1)] Reference number from the original data set

**Table A – 13: Gold standard data set – bioconcentration data**

| Ref#[1)] | Substance | Common name | Scientific name | Experimental data | | | | | Reference[3)] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | $k_1$ (l $kg^{-1}$ $day^{-1}$) | Fish weight (kg)[2)] | Lipid (kg $kg^{-1}$) | Temperature (°C) | Dissolved oxygen (mg $l^{-1}$) | |
| GS32 | 1,2,4-Trichlorobenzene | Guppy | Poecilia reticulata | 492 | $4.8\times10^{-4}$ | not determined | 21 | not reported in database | van Eck *et al*. (1997) |
| GS45 | Hexachlorobenzene | Mosquito fish | Gambusia affinis | 1,850 | $1.9\times10^{-4}$ | 0.031 | 23.1 | not reported in database | Chaisuksant *et al*. (1997) |
| GS44 | 1,2,4-Tribromobenzene | Mosquito fish | Gambusia affinis | 1,040 | $1.9\times10^{-4}$ | 0.031 | 23.1 | not reported in database | Chaisuksant *et al*. (1997) |
| GS43 | 1,2,3,5-Tetrachlorobenzene | Mosquito fish | Gambusia affinis | 631 | $1.9\times10^{-4}$ | 0.031 | 23.1 | not reported in database | Chaisuksant *et al*. (1997) |
| GS42 | 1,2,3-Trichlorobenzene | Mosquito fish | Gambusia affinis | 470 | $1.9\times10^{-4}$ | 0.031 | 23.1 | not reported in database | Chaisuksant *et al*. (1997) |
| GS41 | 1,4-Dibromobenzene | Mosquito fish | Gambusia affinis | 272 | $1.9\times10^{-4}$ | 0.031 | 23.1 | not reported in database | Chaisuksant *et al*. (1997) |
| GS40 | 1,4-Dichlorobenzene | Mosquito fish | Gambusia affinis | 112 | $1.9\times10^{-4}$ | 0.031 | 23.1 | not reported in database | Chaisuksant *et al*. (1997) |
| GS7 | 2-Isopropylnaphthalene | Sheepshead minnow | Cyprinodon variegatus | 4,188 | $2.47\times10^{-3}$ | 0.097 | 25 | not reported in database | Jonsson *et al*. (2004) |
| GS8 | 2-Isopropylnaphthalene | Sheepshead minnow | Cyprinodon variegatus | 3,746 | $2.47\times10^{-3}$ | 0.097 | 25 | not reported in database | Jonsson *et al*. (2004) |
| GS5 | 1,3-Dimethylnaphthalene | Sheepshead minnow | Cyprinodon variegatus | 2,909 | $2.47\times10^{-3}$ | 0.097 | 25 | not reported in database | Jonsson *et al*. (2004) |
| GS3 | 2-Methylnaphthalene | Sheepshead minnow | Cyprinodon variegatus | 2,659 | $2.47\times10^{-3}$ | 0.097 | 25 | not reported in database | Jonsson *et al*. (2004) |
| GS4 | 2-Methylnaphthalene | Sheepshead minnow | Cyprinodon variegatus | 2,142 | $2.47\times10^{-3}$ | 0.097 | 25 | not reported in database | Jonsson *et al*. (2004) |
| GS6 | 1,3-Dimethylnaphthalene | Sheepshead minnow | Cyprinodon variegatus | 1,854 | $2.47\times10^{-3}$ | 0.097 | 25 | not reported in database | Jonsson *et al*. (2004) |
| GS9 | Phenanthrene | Sheepshead minnow | Cyprinodon variegatus | 1,783 | $2.47\times10^{-3}$ | 0.097 | 25 | not reported in database | Jonsson *et al*. (2004) |
| GS1 | Naphthalene | Sheepshead minnow | Cyprinodon variegatus | 1,450 | $2.47\times10^{-3}$ | 0.097 | 25 | not reported in database | Jonsson *et al*. (2004) |

| Ref#[1)] | Substance | Common name | Scientific name | Experimental data | | | | | Reference[3)] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | $k_1$ (l kg$^{-1}$ day$^{-1}$) | Fish weight (kg)[2)] | Lipid (kg kg$^{-1}$) | Temperature (°C) | Dissolved oxygen (mg l$^{-1}$) | |
| GS2 | Naphthalene | Sheepshead minnow | Cyprinodon variegatus | 1,137 | $2.47\times10^{-3}$ | 0.097 | 25 | not reported in database | Jonsson *et al*. (2004) |
| GS13 | 9-Ethylphenanthrene | Sheepshead minnow | Cyprinodon variegatus | 731 | $2.47\times10^{-3}$ | 0.097 | 25 | not reported in database | Jonsson *et al*. (2004) |
| GS10 | Phenanthrene | Sheepshead minnow | Cyprinodon variegatus | 680 | $2.47\times10^{-3}$ | 0.097 | 25 | not reported in database | Jonsson *et al*. (2004) |
| GS11 | 9-Methylphenanthrene | Sheepshead minnow | Cyprinodon variegatus | 623 | $2.47\times10^{-3}$ | 0.097 | 25 | not reported in database | Jonsson *et al*. (2004) |
| GS12 | 9-Methylphenanthrene | Sheepshead minnow | Cyprinodon variegatus | 290 | $2.47\times10^{-3}$ | 0.097 | 25 | not reported in database | Jonsson *et al*. (2004) |
| GS14 | 9-Ethylphenanthrene | Sheepshead minnow | Cyprinodon variegatus | 263 | $2.47\times10^{-3}$ | 0.097 | 25 | not reported in database | Jonsson *et al*. (2004) |
| GS16 | Pyrene | Sheepshead minnow | Cyprinodon variegatus | 129 | $2.47\times10^{-3}$ | 0.097 | 25 | not reported in database | Jonsson *et al*. (2004) |
| GS15 | Pyrene | Sheepshead minnow | Cyprinodon variegatus | 116 | $2.47\times10^{-3}$ | 0.097 | 25 | not reported in database | Jonsson *et al*. (2004) |

1) Reference number from the original data set.
2) The initial fish weight.
3) References for the bioconcentration data are as follows:

Chaisuksant Y., Yu Q. and Connell D.W. (1997). Bioconcentration of bromo- and chlorobenzenes by fish (*Gambusia affinis*). Wat. Res. 31: 61-68.

Jonsson G., Bechmann R.K., Bamber S.D. and Baussant T. (2004). Bioconcentration, biotransformation, and elimination of polycyclic aromatic hydrocarbons in sheepshead minnows (*Cyprinodon variegatus*) exposed to contaminated seawater. Environ. Toxicol. Chem. 23: 1538-1548.

van Eck J.M.C., Koelmans A.A. and Deneer J.W. (1997). Uptake and elimination of 1,2,4-trichlorobenzene in the guppy (*Poecilia reticulata*) at sublethal and lethal aqueous concentrations. Chemosphere. 34: 2259-2270.