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GUIDANCE DOCUMENT ON THE DETERMINATION OF IN VITRO INTRINSIC CLEARANCE USING CRYOPRESERVED HEPATOCYTES (RT-HEP) OR LIVER S9 SUB-CELLULAR FRACTIONS (RT-S9) FROM RAINBOW TROUT AND EXTRAPOLATION TO IN VIVO INTRINSIC CLEARANCE

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INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

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Environment Directorate
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Paris 2018

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Foreword

This document contains the Guidance Document (GD) on aspects of the two OECD Test Guidelines (TG) TG 319A and TG 319B.

The project to develop this Guidance Document was co-led by the European Commission (EC-JRC) and the United States.

This GD was developed to provide additional information on how to best perform the two TGs in terms of selection of in vitro system (i.e. RT-HEP or RT-S9), considerations for the testing of specific chemicals, potential applications of the in vitro intrinsic clearance determined with the two TGs (e.g. in vitro-in vivo (IVIVE) model to predict BCFs in fish) as well as uncertainties and potential limitations of predicted BCFs.

The Guidance Document was approved by the Working Group of the National Co-ordinators of the Test Guidelines Programme (WNT) at its 30th meeting in April 2018. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 30 June 2018.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

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1. General Introduction and Scope

1. This Guidance Document (GD) accompanies OECD Test Guideline (TG) 319A on Determination of *in vitro* intrinsic clearance using cryopreserved rainbow trout hepatocytes (RT-HEP) (OECD, 2018a) and OECD TG 319B on Determination of *in vitro* intrinsic clearance using rainbow trout liver S9 sub-cellular fractions (RT-S9) (OECD, 2018b).

2. The two TGs describe the use of cryopreserved hepatocytes (RT-HEP (OECD, 2018a) or liver S9 sub-cellular fractions (RT-S9 (OECD, 2018b) of rainbow trout (*Oncorhynchus mykiss*) to determine the *in vitro* intrinsic clearance ($CL_{IN\ VITRO,\ INT}$) of a test chemical using a substrate depletion approach. The $CL_{IN\ VITRO,\ INT}$ can be incorporated into *in silico* models to predict bioconcentration factors (BCFs) in fish which substantially improves their performance as demonstrated over the last decade by several authors (Cowan-Ellsberry et al., 2009; Fay et al., 2014b; Gomez et al., 2010; Han et al., 2007; Han et al., 2009; Laue et al., 2014).

3. The GD provides information on how to best perform these methods and the following points are addressed:

- selection of the *in vitro* system, i.e., RT-HEP (OECD, 2018a) or RT-S9 (OECD, 2018b), including biological and technical considerations, and an overview of published efforts to compare both methods (chapter 2).
- specific considerations for the testing of chemicals regarding the analytical method, chemical stock solutions, extraction solvents, test chemical concentrations, poor water solubility, volatility, adsorption, abiotic degradation and quantification of chiral chemicals or structural isomers. Furthermore, the testing of ionizable chemicals, mixtures, multi-constituent substances and UVCBs is briefly addressed (chapter 3).
- use of negative control incubations (enzymatically inactive RT-HEP and RT-S9) and positive control incubations using reference chemicals (chapter 4).

4. The GD further describes in chapter 5 how the *in vitro* intrinsic clearance ($CL_{IN\ VITRO,\ INT}$) determined with OECD TG RT-HEP (OECD, 2018a) or RT-S9 (OECD, 2018b) can be incorporated into *in silico* models to predict BCFs in fish. It includes an example *in vitro-in vivo* (IVIVE) model developed by Nichols et al. (Nichols et al., 2013b) Furthermore, guidance is provided on how to apply these predicted BCFs and uncertainties and potential limitations are addressed.

5. Chapter 6 discusses other potential applications of RT-S9 and RT-HEP, e.g. adaptation to other fish species and metabolites identification.

2. Selection of the *in vitro* system

6. This chapter aims to provide some guidance for the user on which *in vitro* system, i.e., OECD TG RT-HEP or TG RT-S9 (OECD, 2018a, b), should be chosen. There are only limited numbers of studies published which directly compare *in vitro* intrinsic clearance rates of the same set of chemicals in liver S9 sub-cellular fractions and hepatocytes from the same fish species.

2.1. Biological and technical considerations

7. There are significant differences between the two *in vitro* systems which should be considered before choosing one. Hepatocytes contain the whole set of metabolic enzymes and cofactors at physiological levels (Li, 2007). Thus, hepatocyte-based assays do not require addition of cofactors for biotransformation studies. However, rate-limiting factors specifically associated with hepatocytes may include cofactor depletion and / or restricted chemical diffusion across the cell membrane as discussed for human hepatocytes (Godoy et al., 2013).

8. In contrast to liver S9 sub-cellular fractions, hepatocytes contain intact cell membranes. Thus, uptake of the test chemical by passive diffusion or active transport is required for biotransformation in hepatocytes (Li, 2007). If uptake is rate-limiting on biotransformation, hepatocytes may be closer to the *in vivo* situation and a more appropriate choice for the *in vitro* system.

9. Liver S9 sub-cellular fractions are cell-free systems containing cytosolic and microsomal enzymes, but require the addition of cofactors. Cofactors for Phase I (nicotinamide adenine dinucleotide phosphate; NADPH) and the Phase II enzymes UDP-glucuronosyltransferases (uridine 5'-diphospho-glucuronic acid; UDPGA), sulfotransferases (3'-phosphoadenosine-5'-phosphosulfate; PAPS) and glutathione transferases (glutathione; GSH) have to be added to the S9 incubations (Johanning et al., 2012). The addition of alamethicin is needed to reduce latency associated with glucuronidation activity (Ladd et al., 2016). Other Phase II enzymes may be involved in the biotransformation of certain chemicals like the conjugation of carboxylic groups with amino acids, e.g., taurine (James, 1987; Plakas and James, 1990). However, cofactors for these minor Phase II reactions are currently not part of the standard S9 protocol. If it is known that such reactions play a role in metabolism of a specific substrate, hepatocytes may be preferred over S9 fractions.

10. Both *in vitro* systems (OECD TG RT-HEP (OECD, 2018a) or TG RT-S9 (OECD, 2018b)) are considered to have a limited working lifetime due to a progressive loss of enzymatic activity. Hepatocytes are thought to maintain their biotransformational integrity longer, so they may be preferred for assessing slowly metabolized chemicals (Fay et al., 2015). Accumulating experience indicates that substrate depletion experiments using RT-HEP incubations may be carried out up to 4 h (Fay et al., 2015). The total incubation time using RT-S9 should not generally exceed 2 h (Johanning et al., 2012); however, incubation times up to 4 h may be possible for very slowly biotransformed test chemicals. To date, the working lifetime of the two *in vitro* systems has not been

rigorously established (Nichols et al., 2017). Because proteases may contribute to loss of activity of RT-S9, the addition of a protease inhibitor may improve assay performance. However, this possibility has not yet been evaluated. Therefore, the difference regarding incubation time between the two assays seems to be minor. The lowest rate of *in vitro* activity which can be reliably quantified is approximately 0.05 h^{-1} to 0.14 h^{-1} (Chen et al., 2016; Nichols et al., 2013b).

11. The major advantage using liver S9 sub-cellular fractions compared to hepatocytes is the ease of preparation and simpler shipment and storage conditions (i.e., shipment on dry ice and storage at -80°C for S9 sub-cellular fractions vs. shipment in liquid nitrogen and storage in liquid nitrogen or at -135°C for hepatocytes).

12. Liver S9 sub-cellular fractions are technically easier to use for substrate depletion experiments compared to cryopreserved hepatocytes, which require more complex procedures for proper thawing and handling of the cells (e.g., cell counting). As such, it may be possible to achieve higher levels of chemical throughput using S9 fractions. In the ring trial supporting the development of RT-S9 and RT-HEP TGs (OECD, 2018c), intra- and inter-laboratory variability in assay performance tended to be somewhat lower for RT-S9 than for RT-HEP. This finding was attributed to variability among individual users with respect to thawing, cell counting technique and viability determination. Generally, however, intra- and inter-laboratory variability associated with both assays was quite low, indicating that both methods are highly reliable (OECD, 2018c).

2.2. Comparison of *in vitro* intrinsic clearance

13. There have been few direct comparisons of the hepatocyte and liver S9 sub-cellular fraction assays. In a study by Han et al., *in vitro* activities determined using liver S9 sub-cellular fractions, liver microsomes and freshly isolated hepatocytes from rainbow trout were compared for five chemicals. Intrinsic clearance values (expressed as mL/h/10^6 cells) determined using microsomes and S9 sub-cellular fractions were 4.5 to 16.6-fold lower than those measured using hepatocytes (Han et al., 2009). *In vitro* depletion assays for 6 polycyclic aromatic hydrocarbons (PAHs) were performed recently using cryopreserved rainbow trout hepatocytes (Fay et al., 2016), and predicted *in vivo* intrinsic clearance values ($CL_{IN\ VIVO,\ INT}$; L/d/kg fish) were compared to values predicted using existing data from trout liver S9 sub-cellular fractions (Nichols et al., 2013a). Importantly, the hepatocytes and S9 sub-cellular fractions employed for this comparison were obtained from the trout of the same size and strain, raised under similar conditions. Moreover, measured levels of chemical binding in both *in vitro* systems were very similar indicating that for each test chemical the free (unbound) concentration in both test systems was approximately the same. Predicted $CL_{IN\ VIVO,INT}$ values for 5 out of 6 PAHs, determined in cryopreserved hepatocytes, were in close agreement with those determined using S9 sub-cellular fractions (<2.5-fold difference). For one chemical (benzo[a]pyrene), the $CL_{IN\ VIVO,INT}$ determined using S9 fractions was ca. 10-fold higher than that obtained using cryopreserved hepatocytes. Based on these findings, Fay et al. concluded that both *in vitro* systems are well-suited for measuring intrinsic clearance in rainbow trout (Fay et al., 2016).

14. In the ring trial (OECD, 2018c) performed to support development of OECD TG RT-HEP and TG RT-S9 (OECD, 2018a, b), $CL_{IN\ VITRO,\ INT}$ for six chemicals were determined using both methods. When these *in vitro* rates were extrapolated to $CL_{IN\ VIVO,}$

$_{INT}$, the clearance rates calculated for each test chemical differed among the two test systems by no more than a factor of ~2, and were generally much closer. Moreover, there was no general trend indicating that one test system or the other consistently yields higher predicted levels of $CL_{IN\ VIVO,\ INT}$. Differences in *in vivo* hepatic clearance (CL_H) predicted using the two test systems were even smaller (< 2.6-fold), because in several cases predicted CL_H was approaching the limit imposed by the liver blood flow (OECD, 2018c). Generally, these findings confirm earlier work by Fay et al., and suggest that current data do not support a preference for one *in vitro* system or the other (Fay et al., 2016). However, additional studies may be needed to determine whether one *in vitro* system is more suitable for certain chemicals, e.g., for larger molecules.

Table 1. Comparison of in vitro hepatocyte and liver S9 sub-cellular fraction test systems to determine intrinsic clearance of chemicals.

	Cryopreserved hepatocytes	S9 sub-cellular fractions
Cofactor addition	Not required	Cofactor addition required
Membrane transporters	Yes, although transporter activity may be impacted by cell isolation procedures	No
Enzymes	Phase I and Phase II metabolic enzymes	Phase I and Phase II metabolic enzymes
Max. recommended incubation time	4 h	2-4 h
Preparation	More sophisticated, has probably to be adapted for each species	Easy, can be adapted, in principle, to any fish species
Storage	Liquid nitrogen or -150°C	-80°C freezer
Shipment	Liquid nitrogen	Dry ice
Standardization of enzyme content	Thawing and counting step may lead to more variability regarding final cell concentration	Protein concentration determined once allows precise adjustment of final protein concentration
Species used so far	Rainbow trout, carp[1]	Rainbow trout, carp, channel catfish, fathead minnow, various other species [2]

[1] (Bischof et al., 2016; Cowan-Ellsberry et al., 2008; Fay et al., 2015; Mingoia et al., 2010)
[2] (Bearr et al., 2012; Cowan-Ellsberry et al., 2008; Dyer et al., 2008; Fay et al., 2015; Gomez et al., 2010; Han et al., 2009; Johanning et al., 2012; Mingoia et al., 2010; Strobel et al., 2015)

3. Considerations specific for the test chemicals

15. This section addresses considerations for the development of a robust test chemical analytical method, including the selection of appropriate solvents for the preparation of test chemical stock solutions and extraction solvents. Additional guidance is provided on the selection of test chemical concentration and how to address potential issues such as poor water solubility, volatility, adsorption, instability, and ionization. The potential for applying these methods to chemical mixtures is also discussed. In general, preliminary incubations with both active and enzymatically inactive biological material should be performed in order to identify potential issues and optimize the test conditions.

16. The OECD guidance document 23 "Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures" (OECD, 2000) provides further support for the testing of substances and mixtures classified as "difficult to test".

3.1. Analytical method

17. A valid (robust and sensitive) analytical method (HPLC, LC-or GC-MS) is needed to determine chemical depletion using TG RT-HEP (OECD, 2018a) or TG RT-S9 (OECD, 2018b). The sensitivity of the analytical method determines the lower limit of concentration of the test chemical which can be used in the test system. As a general rule, the initial test chemical concentration should be around 10-fold higher than the limit of quantification (LOQ).

18. An internal standard with similar properties as the test chemical may be used to correct for potential losses of the test chemical during sample preparation (e.g., due to evaporation of the stopping solvent during the extraction procedure). The internal standard should be added to the stopping solvent.

19. For the analytical measurement, calibration standards should be prepared in the presence of biological matrix (i.e., incubation buffer containing cofactors, alamethicin, and enzymatically inactive S9 sub-cellular fractions for RT-S9 incubations, and L-15 containing enzymatically inactive hepatocytes for RT-HEP incubations) and extracted using the same protocol as for the incubation samples. Alternatively, the calibration standards can be prepared in an organic solvent. In this case, matrix spikes are needed to allow correction for extraction efficiency in order to calculate the concentration of test chemical in the incubation samples.

3.2. Preparation of test chemical stock solutions

20. Stock solution(s) of the test chemical should be prepared in the reaction buffer (i.e., L-15 medium for RT-HEP and potassium phosphate buffer for RT-S9, respectively) if possible. However, since chemicals of interest for bioaccumulation assessment tend to be hydrophobic, water-miscible solvents are commonly used to facilitate introduction of these chemicals to the test system. A concentrated stock solution of the test chemical is

prepared in a solvent. Defined quantities of the stock solution (or intermediate spiking solution) are then added to the incubation mixture to start the reaction, termed “spiking.”

21. The choice of a spiking solvent depends in part on the properties of the test chemical. Solubility of the test chemical in this solvent should be determined. Water-miscible solvents which are commonly used include acetonitrile, acetone, and methanol (Johanning et al., 2012). DMSO is not recommended due to inhibition of certain CYP isoforms as described for human liver microsomes (Chauret et al., 1998). The organic stock solution can be directly added to the incubation mixture or, preferably, diluted into an intermediary spiking solution with lower level of solvent which is finally added to the incubations. This may be in particular necessary for the multiple vial approach (OECD, 2018b). In either case, the solvent concentration in the incubation should not exceed 1% of the total volume (Johanning et al., 2012). In general, final concentrations of organic solvents in the incubation medium should be minimized as much as possible, since they can potently inhibit enzyme activities (Easterbrook et al., 2001; Nichols et al., 2017; Sakalli et al., 2015). If the *in vitro* intrinsic clearance is lower than expected and the final solvent concentration close to 1%, use of a lower solvent concentration or a different solvent may be considered.

22. If stock solutions of test chemicals are stored prior to the incubation experiments, stability tests must be performed under the corresponding storage conditions. Stock solutions should not be stored for longer than 2 weeks at 4°C, and spiking solutions should be freshly prepared at each day of the incubation. Stock solutions and spiking solutions should be stored in the dark or in Amber glass vials for photolabile chemicals.

3.3. Extraction solvents for stopping of the incubation and extraction of the test chemical

23. The organic solvent used to stop the biotransformation activity and extract the chemical in RT-S9 and RT-HEP incubations depends on the properties of the test chemical and on the analytical method used. Commonly used stopping and extractions solvents are e.g., acetonitrile, methanol, dichloromethane (methylene chloride), and methyl *tert*-butyl ether (MTBE)(OECD, 2018a, b). Due to difference of the biological matrix, different extraction solvents may be needed for RT-HEP and RT-S9 incubations.

24. Preliminary experiments should be performed to determine whether the test chemical is extractable from the incubation samples (active and enzymatically inactive RT-S9 or active and enzymatically inactive RT-HEP, respectively). Different organic solvents may need to be compared to ensure sufficient extraction efficacy. In instances where the extraction solvent may interact with plastic, glass tubes (e.g., Hirschmann glass inserts) should be used for extraction.

25. If extracted incubation samples are kept frozen prior to analysis, the chemical stability of these frozen, extracted samples must be determined. It is not recommended to freeze directly incubation samples which have not been extracted due to potential losses e.g., by adsorption. Extracted samples should be stored in tightly closed glass vials, such as HPLC- or GC-vials.

3.4. Selection of test chemical concentrations

26. From theoretical considerations, the starting test chemical concentration should be substantially lower than the Michaelis-Menten affinity constant (K_M[1]) for the reaction in order to result in first-order depletion kinetics (Nichols et al., 2006). Previously, Lo et al. demonstrated a procedure for estimating K_M from substrate depletion data collected across a range starting concentrations (Lo et al., 2015). Using this method, they showed that *in vitro* intrinsic clearance rates may depend strongly on the initial test chemical concentration. A user of TG RT-HEP or TG RT-S9 (OECD, 2018a, b) may wish to evaluate the concentration-dependence of a particular reaction to insure that the starting concentration is $\ll K_M$. Without such an effort, the test chemical concentration may be guided by analytical sensitivity as described above. Selection of the starting test chemical concentration is detailed in TG RT-HEP, Annex 6 and TG RT-S9, Annex 5 (OECD, 2018a, b). This includes preliminary experiments comparing different test chemical concentrations to establish the final reaction conditions needed to reliably measure *in vitro* intrinsic clearance.

27. In certain cases (e.g., if the analytical method is not sensitive enough which may be common for substances with multiple isomers), the use of a test chemical concentration ≥ 1 µM may be valid from the perspective of providing a conservative bioaccumulation assessment. If, however, the starting test chemical concentration is $>K_M$, an *in vitro* test system may underestimate the true rate of *in vivo* activity leading to an over-prediction of the true BCF.

3.5. Poorly water soluble test chemicals

28. For very hydrophobic chemicals (log $K_{ow} > 6$), use of a solvent spiking approach could result in a dynamic system with incomplete dissolution in the aqueous test medium. In particular, the test chemical concentration could locally exceed its aqueous solubility causing the formation of microcrystals (Kwon et al., 2009). This could, in turn, reduce the substrate concentration available to metabolizing enzymes causing underestimation of *in vitro* biotransformation rates (Lee et al., 2014). To minimize this problem, all samples should be mixed immediately after substrate addition.

29. A sorbent-phase dosing approach may be more useful for measuring *in vitro* intrinsic clearance rates for chemicals with very low water solubility (Kwon et al., 2009; Lee et al., 2011). An ethylene vinyl acetate (EVA) thin-film sorbent-phase dosing approach has been developed and applied to measure the *in vitro* intrinsic clearance of PAHs by RT-S9 (Lee et al., 2014). The *in vitro* intrinsic clearance rate of pyrene (log K_{ow} 5.18) was similar in solvent-delivery dosing experiments and the sorbent-phase dosing experiments. In contrast, the *in vitro* intrinsic clearance rate determined for chrysene (log K_{ow} 5.60) using sorbent-phase dosing was 20-fold higher than that achieved using solvent spiking (Lee et al., 2014).

[1] K_M is the substrate concentration at which the reaction rate is ½ Vmax (maximum rate achieved by the system at substrate maximum saturation concentration).

3.6. Volatile test chemicals

30. Volatility of the test chemical must be taken into account as one potential cause for abiotic loss which should be minimized. Generally, if the Henry's law constant (*H)* is >100 Pa m^3/mol, more than 50% of the substance could be lost from the water phase within 3-4 hours (OECD, 2000). The thin-film sorbent-phase dosing approach should be avoided for volatile chemicals as it may produce higher error in the determination of mass-transfer rate constants due to loss of the substance from the sorbent phase during preparation and handling of the thin films.

31. A substantial decrease of the test chemical concentration (i.e., >20%) in the control incubations using enzymatically inactive RT-S9 or RT-HEP may indicate abiotic losses due to volatility. The significance of volatility can be assessed by comparing control incubations with enzymatically inactive RT-S9 or RT-HEP in open and closed vials in preliminary experiments using other types of vials (e.g., GC or HPLC vials rendering smaller headspace).

32. The multiple vial approach as described in Annex 7 and Annex 6 of the two TGs respectively (OECD, 2018a, b) using tightly closed vials (e.g. GC-vials and lids with gas tight septa) is preferred for testing of volatile chemicals. In contrast to the single vial approach which requires withdrawal of aliquots at different time points, incubation vials using the multiple vial approach are only opened once at the sampling point and the headspace volume for each time point is comparable.

3.7. Adsorption of test chemicals

33. Abiotic loss of the test chemical may also be caused by adsorption onto surfaces and onto organic material like protein or lipids.

34. Glass vials have to be used for the RT-HEP and RT-S9 incubations as described in TG RT-HEP and RT-S9 (OECD, 2018a, b). The use of plastic vessels is not recommended for the incubation tests. Additionally, highly adsorptive materials like rubber should be avoided as part of the exposure system. When closed vials are used for incubations, lids should consist of non-adsorptive materials like polytetrafluoroethylene (PTFE) (OECD, 2000).

35. When working with highly hydrophobic chemicals, it is important to minimize the sampling transfers during analysis (Johanning et al., 2012). Thus, the multiple vial approach as described in Annex 7 and Annex 6 of the two TGs (see TG RT-HEP and TG RT-S9 (OECD, 2018a, b)) is recommended in which incubation, stopping of the reaction and extraction are done in the same vial.

3.8. Abiotic degradation of test chemicals

36. Abiotic degradation processes, including hydrolysis and photolysis, may also cause a loss of test chemicals from active and enzymatically inactive RT-S9 or RT-HEP. Amber glass incubation vials (e.g., amber GC vials) may be used to prevent photolysis.

37. If chemical stock solutions are stored, the stability of these solutions must be evaluated prior to testing. Furthermore, spiking solutions of the test chemical should be prepared fresh the day of an experiment (see section 3.2).

3.9. Ionizable test chemicals

38. Relatively small changes in pH can significantly alter the balance between the dissociated and non-dissociated forms of some organic acids and bases (OECD, 2000). Altered dissociation equilibrium may significantly affect the water solubility. Therefore, the relevant dissociation constants (pK_a values) should be known prior to testing ionizable chemicals (IOCs).

39. There are limited data available on the biotransformation of IOCs in fish. Substrate depletion of 12 pharmaceuticals was studied in rainbow trout liver S9 fractions (Connors et al., 2013b). Recently, *in vitro* intrinsic clearances were determined for 50 IOCs representing the most common types of monoprotic acids and bases using RT-S9 (Chen et al., 2016). Similar RT-S9 incubation conditions were used as described in the TG RT-S9 (OECD, 2018b), while test chemical stock solutions were prepared in acetone/incubation buffer (20:80,v:v) instead of pure solvent.

40. Due to the chemical properties that distinguish IOCs from neutral chemicals, specific considerations may have to be applied for IVIVE extrapolation to predict BCFs (Armitage et al., 2017) (see Chapter 5.4, §83).

3.10. Testing of chiral chemicals and structural isomers

41. The substrate depletion approach using RT-S9 or RT-HEP may be employed to compare *in vitro* intrinsic clearance rates for different stereoisomers or structural isomers. For example, trout liver S9 fractions were applied to investigate enantiomer-specific differences in biotransformation of three pharmaceuticals (Connors et al., 2013a).

42. Some chemicals exist as isomeric mixtures containing two or more isomers. *In vitro* intrinsic clearance rates can be determined for the individual isomers in these mixtures if the analytical method is sensitive enough to quantify the isomers separately (Laue et al., 2014). Current BCF predictions based on log K_{ow} and QSAR-estimated biotransformation rates (i.e., the Arnot-Gobas model, (Arnot and Gobas, 2003)) do not distinguish between different stereoisomers except if there are differences in log K_{ow} values.

3.11. Mixtures, MCS and UVCBs as test chemicals

43. In principle, TG RT-HEP (OECD, 2018a) and TG RT-S9 (OECD, 2018b) can be used to determine *in vitro* intrinsic clearance rates of mixtures. With few exceptions, these *in vitro* methods have only been applied to single chemical substances. Using a conventional solvent dosing approach to determine *in vitro* biotranformation with rainbow trout liver S9 fractions, Lee et al. (Lee et al., 2014) found that *in vitro* biotransformation rate constants for three PAHs tested individually were significantly greater than those obtained when the three PAHs were tested as a mixture. Based on this finding, it was suggested that *in vitro* biotransformation of one PAH may be competitively inhibited by the presence of other PAHs.

44. The mixture effect noted by Lee et al. when using a conventional solvent dosing was substantially reduced when the three PAHs were tested using a sorbent-phase dosing approach. The lower initial substrate concentrations in the incubation medium in the thin-film dosing experiments may reduce competitive inhibition. Thus, a sorbent-delivery system may have greater potential for measuring biotransformation rates of multiple chemicals (Lee et al., 2014).

45. In addition to possible inhibitory effects associated with mixtures, the analytical determination of the concentrations of individual components of the mixture may be challenging. The extraction method, analytical method, and test concentration must be suitable for all components of the mixture. Thus, it is recommended to test the biotransformation of the individual components of mixtures if they are available.

46. The same holds true for multi-constituent substances, major constituents may need to be tested individually provided that a sensitive analytical method is available. Testing of UVCBs may be even more challenging and can only be performed on components for which an analytical method is available.

4. Positive and negative control incubations

4.1. Negative control incubations

47. The use of a negative control is necessary to ensure that an observed decrease in test chemical concentration is not due to abiotic processes. This section describes procedures used to prepare negative controls as well as guidance on how the negative control results should be interpreted.

4.1.1. Enzymatically inactive RT-HEP and RT-S9

48. TG RT-HEP and TG RT-S9 (OECD, 2018a, b) state that in addition to incubations with active RT-HEP or RT-S9, negative control incubations with enzymatically inactive RT-HEP or RT-S9 should be performed in parallel to distinguish between enzymatic metabolism and abiotic decrease (e.g., abiotic degradation, volatilization, adsorption to the reaction vessel). Enzymatically inactive RT-HEP or RT-S9 are used for negative control incubations and their routine preparation by heat inactivation is described in TG RT-HEP and TG RT-S9 (OECD, 2018a, b). Heating in a microwave is not recommended (Fay et al., 2015).

49. A decrease of the test chemical by >20% with enzymatically inactive biological material indicates potential issues which are addressed in section 3.6-3.8. In such cases, it is recommended that the incubation conditions are optimized to reduce these abiotic loss processes. Recommendations on how to proceed in case of substantial decreases of the test chemical in enzymatically inactive control incubations are described in Annex 2.

50. For some chemicals, there may be issues due to the inhomogeneous nature (i.e., precipitated protein) of the heat-inactivated matrix, especially for heat-inactivated RT-S9. In such cases, enzymatically inactive RT-S9 can be prepared by incubating active RT-S9 at room temperature for 24 h followed by storage at -20°C for at least 24 h prior to use. Negative control incubations with room-temperature inactivated RT-S9 are carried out in presence of alamethicin, but without addition of any cofactors. Preparation of room-temperature inactivated RT-S9 is described in detail in Annex 3.

51. If, during preliminary incubations, there is negligible loss of substrate in the presence of enzymatically inactive RT-HEP or RT-S9 (see Annex 6, TG RT-HEP (OECD, 2018a) and Annex 5, TG RT-S9 (OECD, 2018b), respectively), a reduced number of time points may be applied for the negative controls in the main incubations, e.g., starting, middle, and ending time points (Johanning et al., 2012).

52. If there is an abiotic loss of test chemical from enzymatically inactive RT-HEP or RT-S9 which cannot be avoided by optimization of test conditions (i.e., abiotic decrease >20%), the rate of this loss process may be subtracted from the measured rate of depletion in active samples to obtain a corrected *in vitro* intrinsic clearance rate (Nichols et al., 2013a). In this case, however, it must be verified that the abiotic loss process follows first-order kinetics. Furthermore, if the difference between abiotic decrease in the negative control and enzymatic decrease in the RT-S9 or RT-HEP incubations is rather small, a correction of the *in vitro* clearance may also be performed.

53. Furthermore, negative controls should demonstrate no apparent increase (i.e., >20%) of the parent chemical over the incubation time. Apparent increase in the negative controls may be due to an increase in solubility or better extractability in presence of (inactive) protein over the incubation time. In this case, the use of other negative controls like the use of room temperature inactivated RT-S9 may be considered.

4.1.2. Additional negative control incubations – RT-S9

54. Control incubations containing active RT-S9, but no added cofactors, can be used to detect cofactor-independent enzymatic reactions such as hydrolysis by carboxylesterases. It should be noted, however, that background concentrations of each cofactor may be present in liver S9 sub-cellular fractions (Johanning et al., 2012).

4.2. Positive control (reference chemical) incubations

55. Before using a new lot of RT-HEP or RT-S9, the enzymatic activity of this material should be characterized by running Phase I and Phase II biotransformation reactions using standard substrates. Assays that have been used to characterize RT-HEP and RT-S9 are described in Annex 3 of TG RT-HEP (OECD, 2018a) and TG RT-S9 (OECD, 2018b). In addition, activities of the RT-HEP and RT-S9 used for the ring trial are provided in Table 2 of the ring trial report (OECD, 2018c).

56. In addition to the initial characterization of a new lot of RT-HEP or RT-S9, it is recommended that users incorporate an appropriate reference chemical into test systems involving a new test chemical in order to verify enzymatic activity of the biological material. If a specific pathway for biotransformation of the new test chemical is known or suspected (e.g., Phase I or Phase II), it may be useful to choose a reference chemical which is transformed by the same metabolic pathway. Incorporation of a reference chemical increases confidence in the experimental outcome and may provide a means of accounting for lot-to-lot differences in activity of biological material.

57. Ideally, the depletion rate for a reference chemical in the chosen test system would be well studied in order to provide a range of expected performance. Preliminary studies and/or depletion rates reported in the literature may be helpful when selecting a suitable reference chemical. However, potential differences in depletion rates due to differences in biological material, initial test chemical concentrations, and experimental conditions must be taken into consideration (Fay et al., 2015). Additional considerations for selecting a reference compound include: commercial availability, volatility, relative hydrophobicity, stability, and availability of analytical methods. Examples for possible reference chemicals are listed in Annex 4, Table 1.

58. *In vitro* intrinsic clearance rates are usually similar when a reference chemical is tested using different aliquots of the same lot of biological material (RT-HEP or RT-S9). If enzymatic clearance of the reference chemical is verified at regular intervals, it may not be necessary to run the reference chemical in parallel with all test chemical incubations.

5. Use of *in vitro* intrinsic clearance to predict BCFs

59. Biotransformation can reduce the extent to which chemicals accumulate in fish. Recent research has led to development of a screening-level QSAR model for estimating biotransformation rates (k_{MET}) based on chemical structure (Arnot et al., 2009). This QSAR had been implemented in the Arnot-Gobas bioaccumulation models (Arnot and Gobas, 2003) within the U.S. EPA's Estimation Program Interface (EPI) Suite (U.S. Environmental Protection Agency, 2013). Despite this progress, biotransformation remains one of the greatest uncertainties in the prediction of bioaccumulation of chemicals in fish. *In vitro* metabolizing systems which directly measure biotransformation rates using fish hepatocytes or liver S9 sub-cellular fractions can be used method to refine *in silico* BCF prediction models (Cowan-Ellsberry et al., 2008; Han et al., 2007; Nichols et al., 2006).

60. In the following paragraphs, examples of an *in vitro-in vivo* extrapolation (IVIVE) model for rainbow trout and a mass balance model for BCF prediction as published by Nichols et al. are explained (Nichols et al., 2013b). However, $CL_{IN\ VITRO,\ INT}$ may be used as an input to physiologically based toxicokinetic (PBTK) models for fish for bioaccumulation assessment (Brinkmann et al., 2016; Stadnicka-Michalak et al., 2014).

5.1. *In vitro-in vivo* extrapolation and mass balance model to predict BCFs: theoretical background (model examples)

61. *In vitro* intrinsic clearance rates determined with OECD RT-HEP or RT-S9 are extrapolated to an estimate of hepatic clearance (CL_H), which is used to estimate a whole-body biotransformation rate constant (k_{MET}). This whole-body rate constant is then used as an input to established mass-balance models for rainbow trout to predict well-known metrics of accumulation such as the BCF. Details on the general approach have been given by various authors (Cowan-Ellsberry et al., 2009; Han et al., 2007; Han et al., 2009; Nichols et al., 2006). In a recent report, Nichols et al., (Nichols et al., 2013b) described two models that employ measured *in vitro* intrinsic clearance rates to predict BCFs in rainbow trout. One model (HEP-BCF) was provided for data derived from RT-HEP, while a second (S9-BCF) was developed for data derived from RT-S9. Both models were configured as Microsoft Excel spreadsheets (for examples, see Annex 5) and are available via the OECD website. Importantly, these models predict the BCF for a "standardized" fish (10 g rainbow trout containing 5% whole-body lipid), which is typical of fish commonly tested *in vivo* under OECD TG305 (OECD, 2012).

62. As described in TG RT-HEP and TG RT-S9 (OECD, 2018a, b), a substrate depletion approach is used to determine an *in vitro* a first-order elimination rate constant (k_e, h^{-1}). Implied by this approach is an assumption that the starting substrate concentration is below K_M, the Michaelis-Menten affinity constant for the reaction (i.e., under first-order conditions). The rate constant k_e is then normalized to cell number or S9 protein content (C_{HEP} and C_{S9}, respectively) to derive the *in vitro* intrinsic clearance ($CL_{IN\ VITRO,INT}$; mL/h/10^6 cells or mL/h/mg protein; see equation 1).

$$CL_{IN\ VITRO,INT} = k_e / C_{HEP} \quad \text{or} \quad CL_{IN\ VITRO,INT} = k_e / C_{S9} \quad [1]$$

63. In the S9-BCF model, the $CL_{\text{IN VITRO,INT}}$ is multiplied by the S9 protein content of liver tissue (L_{S9}; mg/g liver), the liver weight as a fraction of body weight (L_{FBW}; g liver/g fish) and by 24 to yield the *in vivo* intrinsic clearance ($CL_{\text{IN VIVO,INT}}$; L/d/kg fish; equation 2) (Nichols et al., 2013b). The S9 content of liver tissue refers to the total amount of S9 protein in the tissue and not the protein content of the S9 fraction itself. This scaling factor accounts for incomplete recovery of protein during the preparation of S9 sub-cellular fraction (e.g., due to incomplete cell lysis) and was determined using two markers of microsomal protein (total cytochrome P450 content and glucose-6-phosphatase activity). An average value of 163 mg S9 protein /g liver is applied in the S9-BCF model (Nichols et al., 2013b). This correction had not been implemented in previous models (Cowan-Ellsberry et al., 2008; Han et al., 2009). The fractional liver weight (L_{FBW}) used by Nichols et al. (Nichols et al., 2013b) was based on a value determined previously for small trout (Schultz and Hayton, 1999).

$$CL_{\text{IN VIVO,INT}} = CL_{\text{IN VITRO,INT}} \times L_{S9} \times L_{FBW} \times 24 \qquad [2]$$

64. In the HEP-BCF model, the $CL_{\text{IN VITRO,INT}}$ is multiplied by hepatocellularity (L_{HEP}; 10^6 cells/g liver), fractional liver weight (L_{FBW}) and by 24 to yield the $CL_{\text{IN VIVO,INT}}$ (L/d/kg fish) (equation 3). An average hepatocellularity value of 510 x 10^6 cells/g liver is used in the HEP-BCF model (Fay et al., 2014a). This value is appropriate for sexually immature trout and is based on studies performed by the authors as well as studies described previously (Hampton et al., 1989; Han et al., 2008).

$$CL_{\text{IN VIVO,INT}} = CL_{\text{IN VITRO,INT}} \times L_{HEP} \times L_{FBW} \times 24 \qquad [3]$$

65. The $CL_{\text{IN VIVO,INT}}$ is converted to an estimate of *in vivo* hepatic clearance (CL_H; L/d kg) (equation 4) using a well-stirred liver model (Nichols et al., 2013b). Calculation of the CL_H accounts for possible rate limitations imposed by the liver blood flow rate and by possible chemical binding effects.

$$CL_H = Q_H \times f_U \times CL_{\text{IN VIVO, INT}} / (Q_H + f_U \times CL_{\text{IN VIVO, INT}}) \quad [4]$$

where Q_H (L/d/kg fish) is the liver blood flow rate and f_U (unitless) is a binding term (ranging from 0.0 to 1.0) that corrects for the difference in free chemical concentration between blood and the *in vitro* system used to measure activity (Nichols et al., 2013b; Nichols et al., 2006). In the spreadsheets, f_U is calculated as the ratio of free chemical fractions in blood plasma ($f_{U,P}$; unitless) and the *in vitro* system ($f_{U,S9}$ or $f_{U,HEP}$; unitless), each of which is estimated using log K_{ow}–based algorithms. Consistent with the "free chemical hypothesis," these models assume that only the free chemical fraction is available for metabolic transformation *in vitro* and *in vivo*.

66. Presently, the effect of chemical binding on predicted hepatic clearance is one of the major uncertainties in modelled BCF predictions. It was observed in several studies that setting f_U =1.0 (i.e., assuming the same availability of the chemical to metabolic enzymes *in vitro* and *in vivo*) resulted in much better agreement between predicted and measured BCF values (Cowan-Ellsberry et al., 2008; Escher et al., 2011; Laue et al., 2014; OECD, 2018c). The spreadsheet can be adapted by manually changing the term "fu" to "fuone", thereby setting f_U =1.0. This results in the following equation:

$$CL_H = Q_H \times CL_{\text{IN VIVO, INT}} / (Q_H + CL_{\text{IN VIVO, INT}}) \qquad [5]$$

For chemicals with relatively high log K_{ow} values and low intrinsic clearance rates, the two different binding assumptions result in substantial differences in predicted hepatic

clearance rates and measured BCFs (Nichols et al., 2013b). The impact on these binding assumptions is discussed in more detail below (§84-86).

67. A whole-body biotransformation rate constant (k_{MET}; 1/d) is calculated by dividing CL_H by the chemical's apparent volume of distribution, referenced to the chemical concentration in blood ($V_{D,BL}$; L/kg; equation 6) (Nichols et al., 2006). The $V_{D,BL}$ is estimated as the ratio of fish/water and blood/water partition coefficients, each of which is calculated using log K_{ow}-based algorithms.

$$k_{MET} = CL_H / V_{D,BL} \quad [6]$$

68. There are still uncertainties in the extrapolation models with respect to physiological parameters. The second key parameter besides the binding term f_U is the apparent volume of distribution ($V_{D,BL}$). As described above (§67), $V_{D,BL}$ is estimated as the ratio of fish/water and blood/water partition coefficients. This calculation combines errors associated with prediction of these two terms individually. For some chemicals, in particular when high affinity binding to specific proteins or tissues is observed, it may be necessary to measure $V_{D,BL}$ directly (Nichols et al., 2013b).

69. The total chemical concentration in fish at steady state ($C_{FISH,SS}$; mg/kg) is predicted using the 1-compartment model given by Arnot and Gobas (Arnot and Gobas, 2003) (equation 7). The calculation includes rate constants that describe chemical uptake (k_1) and loss across the gills (k_2), and fecal egestion (k_E) (Arnot and Gobas, 2003). Additionally, a rate constant (k_G) can be included accounting for growth. In the current version of the spreadsheets (see Annex 5 for examples) k_G is set to 0, i.e., growth dilution of the chemical is not considered.

$$C_{FISH,SS} = (k_1 \times C_{W,FD}) / (k_2 + k_{MET} + k_G + k_E) \quad [7]$$

k_1 = gill uptake rate constant (L/d/kg); $C_{W,FD}$ = chemical concentration dissolved in water (mg/L);
k_2 = gill elimination rate constant (d^{-1}); k_{MET} = whole-body biotransformation rate constant (d^{-1}); k_G= growth rate constant (d^{-1}); k_E = fecal egestion rate constant (d^{-1})

70. Finally, ($C_{FISH,SS}$; mg/kg) is divided by the total chemical concentration in water ($C_{W,TOT}$) resulting in a predicted BCF expressed on a total chemical basis (BCF_{TOT}; L/kg fish; equation 8). BCF_{TOT} as quotient of the chemical concentration in fish and the concentration in water is in line (i.e. same units) with the BCF measured using OECD TG 305 (OECD, 2012).

$$BCF_{TOT} = C_{FISH,SS} / C_{W,TOT} \quad [8]$$

71. A second BCF is predicted which is normalized for fish lipid content ($BCF_{FD,L}$; L/kg lipid) and expressed on a freely dissolved chemical basis. $BCF_{FD,L}$ is calculated by dividing $C_{FISH,SS}$ by the product of the chemical concentration dissolved in water ($C_{W,FD}$; mg/L) and the fish's whole-body lipid content (v_{LWB}; unitless); equation 9); v_{LWB}; is assumed to be 0.05 (Nichols et al., 2013b).

$$BCF_{FD,L} = C_{FISH,SS} / (C_{W,FD} \times v_{LWB}) \quad [9]$$

5.2. Use of the spreadsheets for BCF prediction: practical application

72. BCFs are predicted for the test chemical based on *in vitro* biotransformation rates determined in the RT-HEP or RT-S9 test system using the corresponding spreadsheets (for examples, see Annex 5). The two different binding assumptions (i.e., f_U modelled

and f_U=1.0) should be considered for BCF prediction to estimate upper and lower limits of hepatic clearance (Nichols et al., 2013b).

73. Parameters that need to be entered by the user into the spreadsheets are the log K_{ow} of the test chemical, the body weight of fish used for RT-HEP or RT-S9 preparation (does not impact the BCF calculations in the spreadsheets as included in Annex 5), the RT-S9 protein concentration or hepatocyte concentration used in the *in vitro* test system, the reaction rate determined, incubation temperature, and the nominal test chemical concentration (Tables 2 and 3; and examples in Annex 5). Measured log K_{ow} values should be used, when available; in the absence of measured values, modelled estimates can be used (e.g., those provided by EpiSuite (U.S. Environmental Protection Agency, 2013)).

74. Additional independent variables such as liver S9 protein content, liver weight as a fraction of body weight, and liver blood flow as a fraction of cardiac output are specified in the spreadsheets. See Annex 5 for details.

75. The spreadsheets calculate a BCF_{TOT} (L/kg fish) for a standardized fish based on the *in vitro* reaction rate determined in the hepatocyte or S9 test system. A second BCF is calculated normalized for fish lipid ($BCF_{FD,L}$; L/kg lipid) (Nichols et al., 2013b). These models can in principle be adjusted for different sizes of fish and for temperature.

Table 2. Independent variable inputs to be set when using the S9-bioconcentration factor (S9-BCF) model. Additional independent variables which are already included in the spreadsheet are not listed (Nichols et al., 2013b).

Parameter	Value	Units
Log K_{ow} of test chemical	Measured or estimated	Unitless
Body weight of fish used as source of S9	Measured	g
S9 protein concentration (C_{S9}) in the test system	Set by user; typically 0.25 to 2.0	mg/mL
Reaction rate (*Rate*)	Measured; from substrate depletion assay	h^{-1}
Modelled temperature (T)	Set by user, should correspond to temperature used for *in vitro* incubation	Celsius
Total aqueous chemical concentration ($C_{w, TOT}$)	Set by user	mg/L

Table 3. Independent variable inputs to be set when using the hepatocyte-bioconcentration factor (HEP-BCF) model. Additional independent variables which are already included in the spreadsheet are not listed (Nichols et al., 2013b).

Parameter	Value	Units
Log K_{ow} of test chemical	Measured or estimated	Unitless
Body weight of fish used as source of hepatocytes	Measured	g
Hepatocyte cell number (C_{HEP})	Set by user; typically 1 to 2 × 10^6 (final cell number is verified by recounting)	cells/mL

First-order elimination rate constant (k_e)	Measured; from substrate depletion assay	h^{-1}
Modelled temperature (T)	Set by user, should correspond to temperature used for *in vitro* incubation	Celsius
Total aqueous chemical concentration ($C_{w, TOT}$)	Set by user; does not impact BCF calculations	mg/L

5.3. Applications and interpretation of BCF predictions

76. BCFs predicted by incorporating measured *in vitro* biotransformation rates into *in silico* BCF models may be used to screen chemicals for bioaccumulative properties and to decide whether a test chemical is B (bioaccumulative) or not B on the screening level according to the corresponding regulatory framework. Incorporation of biotransformation rates enhances the reliability of the *in silico* models for BCF prediction (Treu et al., 2015), since log K_{ow}-based QSARs or other models often neglect the contribution of fish metabolism which can reduce bioaccumulation.

77. The predicted BCFs may be applied to assess the bioaccumulation potential as part of a weight of evidence approach or for read across (i.e., comparison of *in vitro* intrinsic clearance of the test chemical with another chemical for which empirical BCF data are available) as discussed in some publications and regulatory frameworks (ECHA, 2017a, b, c).

78. Furthermore, predicted BCFs based on *in vitro* data may be useful as an additional tool for screening for bioaccumulation properties in order to decide whether a full *in vivo* fish-BCF study is warranted.

79. BCFs predicted in this way should be considered as more uncertain than the BCFs derived from, for example, the dietary exposure or the minimised test design as part of the OECD TG 305 (OECD, 2012) regarding the uncertainties discussed in Chapter 5.4. Therefore, they may not replace *in vivo* fish bioaccumulation tests. Nevertheless, predicted BCFs based on *in vitro* data may be an alternative if *in vivo* testing is technically not feasible or if the corresponding regulatory framework does not allow vertebrate testing.

80. Although the spreadsheets given by Nichols et al. (Nichols et al., 2013b) were developed to predict BCF values for a standardized fish (10 g trout containing 5% lipid), they may be used to predict BCFs for any fish species (lifestage, etc.) of interest by appropriate specification of *in vitro-in vivo* scaling factors. Additional research is needed to develop these scaling factors for other species.

5.4. Uncertainties and limitations of BCF predictions

81. A valid (sensitive and robust) analytical method is mandatory to quantify the test chemical (see §17).

82. In case, the $CL_{IN\ VITRO,\ INT}$ derived is used to inform *in silico* bioaccumulation models on biotransformation, the test chemical should be within the applicability domain of the corresponding model. The models were developed for well-metabolized neutral

organic chemicals with log K_{ow} between 3-8. More studies on additional chemicals are needed to expand the domain of applicability of this method.

83. Due to the different chemical properties of IOCs, special BCF models may be needed for IVIVE extrapolation especially regarding their uptake and elimination rates (Chen et al., 2016). A recent paper by Armitage et al. (Armitage et al., 2017) discusses some of the specific considerations to assess bioaccumulation of IOCs, and proposes a tiered strategy. A mechanistic bioconcentration model had been developed for IOCs in fish. The model is based on an existing approach for neutral organic chemicials (Arnot and Gobas, 2004) and was modified to account for dissociation of IOCs (Armitage et al., 2013). However, empirical data on chemical absorption efficiency and gill uptake rate constants are lacking in particular for cationic IOCs (Armitage et al., 2013) and further research is needed. There is currently no validated IVIVE model for IOCs available due to the small current data set. Further research is needed on *in vitro* biotransformation and *in vivo* bioconcentration on IOCs.

84. Uncertainties in the IVIVE model, e.g., the binding term f_U, volume of distribution (V_{DB}), and the BCF model, e.g., rate constants, will propagate uncertainty in the calculated BCFs. The models and underlying assumptions that present these uncertainties continue to be evaluated in related on-going research and thus the model formulations are expected to continue to evolve.

85. For some hydrophobic chemicals, there was a poor correlation between empirical BCFs and BCFs predicted using the full modelled binding assumption (i.e., $f_U = f_{U,P}/f_{U,HEP}$ or $f_{U,S9}$). Instead, there was better agreement using the binding assumption f_U=1.0, especially for slowly metabolized chemicals (Cowan-Ellsberry et al., 2008; Escher et al., 2011; Laue et al., 2014; OECD, 2018c). These observations suggest that hepatic clearance values predicted using the full binding assumption under-predict true levels of *in vivo* clearance resulting in overestimation of measured BCFs. A systematic bias toward under-prediction of high *in vivo* hepatic clearance rates by *in vitro* systems (hepatocytes, microsomes) derived from mammalian liver tissue has been noted by several authors (Hallifax et al., 2010; Hallifax and Houston, 2012; Wood et al., 2017).

86. Previously, it was suggested that the two different binding assumptions may be used to estimate upper and lower limits on hepatic clearance (Nichols et al., 2013b). More studies are needed to evaluate which binding assumption results in more accurate BCF predictions for hydrophobic chemicals in fish. These studies should ideally be performed at substrate concentrations shown to be well below the K_M for the reaction if the analytical method is sensitive enough. Recent work indicates that the use of substrate concentrations greater than K_M may, by itself, result in lower levels of measured *in vitro* clearance, and by extension lower levels of predicted *in vivo* hepatic clearance and higher predicted BCF values (Lo et al., 2015).

87. Additional factors that may explain differences between measured and predicted BCF values include extrahepatic metabolism and induction of metabolizing enzymes in prolonged contaminant exposures (e.g., a laboratory BCF testing effort). In either case, these factors would tend to result in measured BCFs that are lower than those predicted considering only hepatic metabolism, as well as those predicted using liver S9-subcellular fractions and hepatocytes from trout that have not been induced.

88. The RT-HEP and RT-S9 test systems have practical limitations which limit their use for chemicals metabolized at very low rates. The ability to detect a low rate chemical depletion (i.e., one statistically different from negative controls) depends on the

behaviour of these controls, the quality of the dataset (e.g., the precision of replicated measurements at each time point), and the length of time over which the test is run. As noted above (§10), these tests have a finite working lifetime. Previously, Nichols et al. (Nichols et al., 2013b) estimated that the lowest rate of *in vitro* activity which can be reliably quantified using this approach is about 0.05 h^{-1}, based on historical data for several compounds. A somewhat higher limit value (0.14 h^{-1}) was estimated by Chen et al. (Chen et al., 2016), based on modelled simulations of hypothetical substrate depletion data.

89. When used to evaluate the validity of *in vitro-in vivo* metabolism extrapolation efforts, it should also be kept in mind that even high quality experimental BCF data differ by >0.5 log units for at least 35% of chemicals tested and >1 log unit for at least 10% of chemicals (Nendza et al., 2010) which may result in BCFs values which are below and above a certain B threshold, e.g., as described for lindane (log BCF ranging from 2.16-3.32) (Arnot and Gobas, 2006).

90. Additionally, the impact of the log K_{ow} value used to predict BCFs based on *in vitro* intrinsic clearance has to be considered for the interpretation of the predicted BCFs. Both measured and predicted (e.g., QSAR) log K_{ow} values may be prone to error in certain instances. The accepted variation of log K_{ow} values determined using OECD TG 117 (OECD, 2004) is +/- 0.5 which may result in substantially different predicted BCFs especially for higher log K_{ow} chemicals which are slowly biotransformed.

6. Other potential use of the *in vitro* RT-HEP and RT-S9 test systems

6.1. Adaptation to other fish species and tissues

91. In principle, the RT-HEP and RT-S9 test systems can be adapted to any fish species, thereby allowing a comparison of *in vitro* biotransformation in different fish species. Primary hepatocytes have been successfully isolated from numerous fish species. However, to-date, consistent substrate depletion studies have only been performed in rainbow trout (Bischof et al., 2016; Fay et al., 2014a; Han et al., 2008; Mingoia et al., 2010) and common carp (*Cyprinus carpio*) (Bischof et al., 2016; Cowan-Ellsberry et al., 2008; Dyer et al., 2008, 2009).

92. Because they are easier to prepare, most studies on multiple species have been performed using liver S9 sub-cellular fractions. For example, Phase I and II metabolism of model substrates have been compared in eight finfish species (González et al., 2009) and the metabolism of benzo[*a*]pyrene was compared in liver S9 sub-cellular fractions from red and white blooded antarctic fish (Strobel et al., 2015).

93. In addition to liver, S9 sub-cellular fractions can be prepared from other organs, such as the gills and gut. This facilitates comparisons of biotransformation in different organs and may provide a means for studying the impact of extrahepatic clearance on predicted BCFs. Gomez et al compared biotransformation of pharmaceuticals in S9 sub-cellular fractions prepared from liver and gill of rainbow trout and channel catfish (*Ictalurus punctatus*) (Gomez et al., 2011; Gomez et al., 2010).

6.2. Identification of metabolites

94. The RT-HEP and RT-S9 test systems could be applied to identify metabolites *in vitro*; i.e. the substrate depletion tests may allow a qualitative identification of metabolites and potential metabolic pathways. Furthermore, the tests may be used to quantify the appearance of metabolites and not the disappearance of parent chemical (Bischof et al., 2016; Chen et al., 2016). Identification of metabolites may be a requirement in various regulatory frameworks.

95. For example, numerous published protocols exist for separating the liver S9 fraction into subcellular fractions that reflect compartmentalization of cellular enzyme activity (e.g., lysosomal, mitochondrial, microsomal, and cytosolic fractions). By using these protocols, it may be possible to explore the metabolism of a particular chemical including the site of metabolism and, by manipulating various cofactors, the identities of responsible enzymes.

7. References

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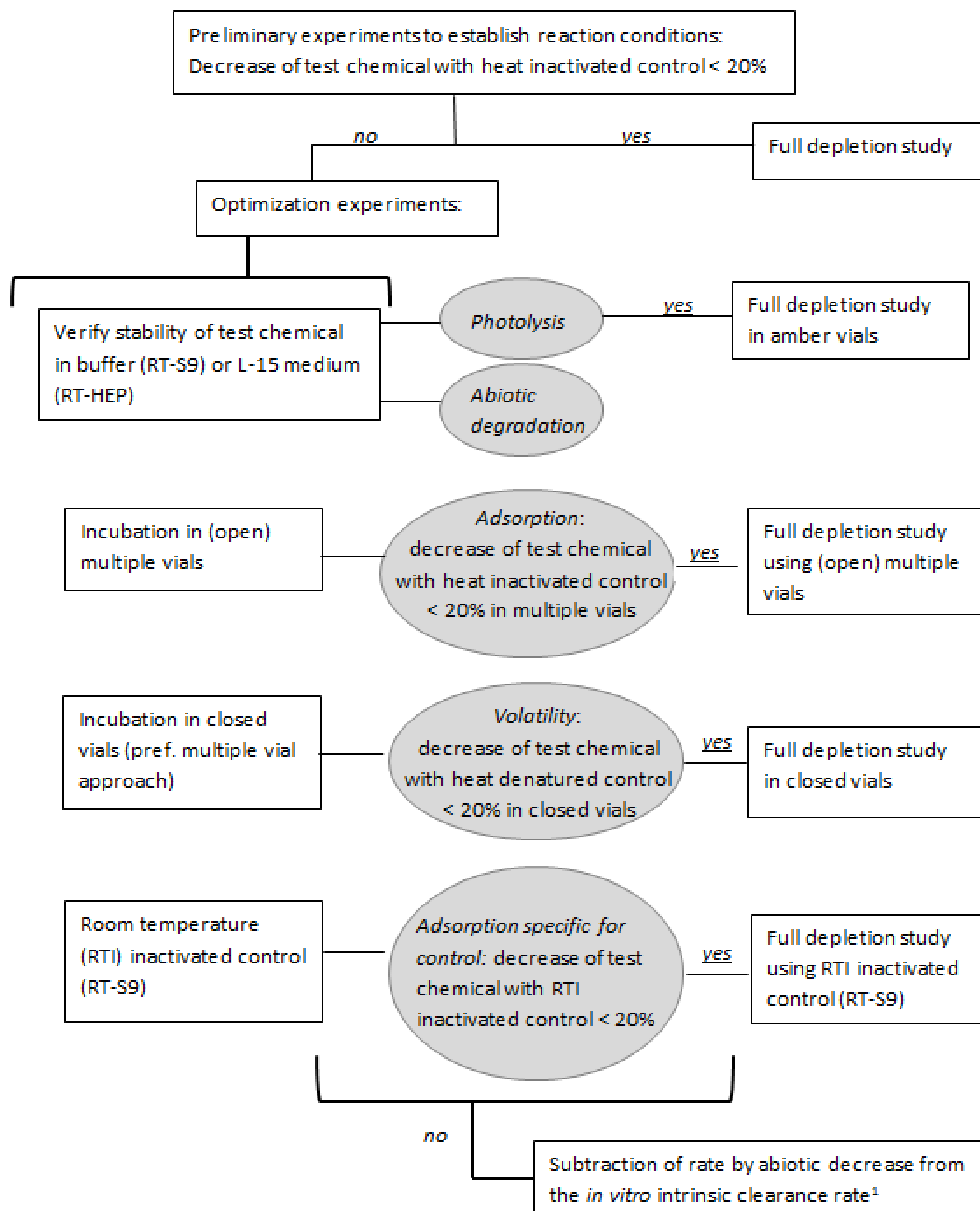
ANNEX 1 - Abbreviations

BCF	Bioconcentration factor
BCF_{TOT}	Bioconcentration factor expressed on a total chemical basis (L/kg)
$BCF_{FD,L}$	Bioconcentration factor normalized for fish lipid content (L/kg lipid)
$C_{FISH,SS}$	chemical concentration in fish at steady state (mg/kg)
CL_{H}	*in vivo* hepatic clearance (L/d kg fish)
C_{HEP}	measured viable RT-HEP cell density (cells/mL)
C_{S9}	S9 protein concentration (mg/mL)
$CL_{IN\ VITRO,\ INT}$	*in vitro* intrinsic clearance (mL/h/10^6 cells or mL/h/mg protein)
$CL_{IN\ VIVO,\ INT}$	*in vivo* intrinsic clearance (L/d/kg fish)
CYP	Cytochrome P450
$C_{W,FD}$	chemical concentration dissolved in water (mg/L)
$C_{W,TOT}$	chemical concentration in water (mg/L)
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EROD	Ethoxyresorufin-O-deethylase
f_{U}	binding term used to correct for binding effects *in vitro* and in plasma
$f_{U,HEP}$	free chemical fractions in the *in vitro* system (RT-HEP; unitless)
$f_{U,\ P}$	free chemical fractions in blood plasma (unitless)
$f_{U,S9}$	free chemical fractions in the *in vitro* system (RT-S9; unitless)
GC	Gas Chromatography
GSH	L-Glutathione
GST	Glutathione transferase
HPLC	High Performance Liquid Chromatography
IOCs	Ionizable organic chemicals
IVIVE model	*In vitro-in vivo* extrapolation model
k_e	Elimination rate constant determined from the slope of the log transformed substrate depletion data (h^{-1})
k_E	Fecal egestion rate constant (d^{-1})
k_G	Growth rate constant (d^{-1})

K_M	Michaelis-Menten constant
k_{MET}	Whole-body biotransformation rate constant (d^{-1})
k_1	Gill uptake rate constant (L/kg/d)
k_2	Gill elimination rate constant (d^{-1})
log K_{ow}	n-Octanol-water partition coefficient
L-15	Leibovitz-15
LC	Liquid Chromatography
LOQ	Limit of quantification
L_{FBW}	Fractional liver weight (g liver/g fish)
L_{S9}	S9 protein content of liver tissue (mg/g liver)
L_{HEP}	Liver hepatocyte content (10^6 cells/g liver)
MCSs	Multi-constituent substances
MS	Mass spectrometry
MTBE	methyl *tert*-butyl ether
NADPH	Nicotinamide adenine dinucleotide 2'-phosphate
PAHs	Polycyclic aromatic hydrocarbons
PAPS	Adenosine 3'-phosphate 5'-phosphosulfate
pK_a	Acid dissociation constant
Q_H	Liver blood flow rate (mL/h/g liver)
RT-HEP	Rainbow trout hepatocytes
RT-S9	Rainbow trout liver S9 sub-cellular fraction
SULT	Sulfotransferase
TG	Test Guideline
UDPGA	Uridine 5'-diphosphoglucuronic acid
UGT	Uridine 5'-diphospho-glucuronosyltransferase
UVCBs	Substances of unknown or variable composition, complex reaction products or biological materials
$V_{D, BL}$	chemical's apparent volume of distribution (L/kg)
V_{max}	maximum enzymatic rate at saturating test chemical concentration

ANNEX 2 - Recommendations on how to proceed in case of substantial decreases of the test chemical in enzymatically inactive (heat-inactivated) control incubations.



ANNEX 3 - Preparation of room temperature inactivated RT-S9

1. For certain chemicals the use of enzymatically inactive RT-S9 generated by heat-inactivation as negative control may lead to substantial abiotic decrease in the control as discussed in section 4.1.1. If the decrease of the test chemical is >20% in presence of heat-inactivated RT-S9 which cannot be diminished as described in sections 3.6-3.8, room-temperature inactivated (RTI) RT-S9 fractions may be used as an alternative negative control.

2. This enzymatically inactive RT-S9 can be prepared by incubating active RT-S9 at room temperature for 24 h as described below. Negative control incubations with RTI RT-S9 are carried out in presence of alamethicin, but without addition of any cofactors.

3. To prepare RTI RT-S9, an appropriate volume of active RT-S9 is thawed and diluted to 10.0 mg/mL protein by adding 100 mM potassium phosphate buffer, pH 7.8.

4. The vial(s) with the diluted RT-S9 are incubated for 24 h at room temperature for inactivation and stored for at least 24 h at -20°C prior to use.

5. Negative control incubations with RTI RT-S9 should be carried out in presence of alamethicin, but without addition of any cofactors. The final composition of the reaction mixture for the RTI RT-S9 control in one 7 mL scintillation vial prior to dosing is as follows:

   a. 800 µL 100 mM K-PO_4 buffer
   b. 100 µL pre-diluted RTI S9 (10 mg/mL protein)
   c. 100 µL 250 µg/mL alamethicin

6. Excess RTI RT-S9 material may be refrozen at -20°C for further use.

ANNEX 4 - Potential reference chemicals for incubations using RT-HEP or RT-S9

Table 1. Chemicals which may be applied for positive control incubations using RT-HEP or RT-S9.

Assay / Activity	Phase	Enzyme	Test chemical	Chemical class	Reference
Ester cleavage	I	Esterase	fluroxypyr methylheptyl ester, haloxyfop methyl ester	pesticide	Cowan-Ellsberry et al., 2008
Aromatic ring hydroxylation	I	CYP3A	testosterone	hormone	Han et al., 2009; Nabb et al., 2006
lauric acid hydroxylation	I		lauric acid	fatty acid	Nabb et al., 2006
Aryl hydrocarbon hydroxylation	I	CYP	pyrene	PAH	Fay et al., 2017; Nichols et al., 2013; OECD, 2018
Aryl hydrocarbon hydroxylation	I	CYP	benzo[a]pyrene	PAH	Fay et al., 2014; Lo et al., 2015; Nichols et al., 2013
Hydroxylation, gluruconidation, sufation	I & II	CYP, UGT, ST	4-n-nonylphenol	alkylphenol	Coldham et al., 1998; Fay et al., 2014; Mingoia et al., 2010; OECD, 2018
O-demethylation, hydroxylation, glucuronidation	I & II	CYP, UGT	methoxychlor	pesticide	Bischof et al., 2016;; Fay et al., 2014
			fenthion	pesticide	Fay et al., 2014
Hydroxylation, glucuronidation	I & II	CYP, UGT	cyclohexyl salicylate	fragrance chemical	Laue et al., 2014; OECD, 2018
Glucuronidation, sulfation	II	UGT, SULT	7-hydroxy-coumarin		Laue et al., 2014
Hydroxylation		CYP	propranolol diclofenac	pharmaceutical	Chen et al., 2016; Connors et al., 2013a; Connors et al., 2013b

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ANNEX 5 - Spreadsheets for calculation of BCFs for methoxychlor as example using the hepatocyte-bioconcentration factor model (HEP-BCF) and the S9-bioconcentration factor (S9-BCF) model

Note: The HEP-BCF and the S9-BCF models from Nichols et al. is given as example. Additional details and full references for the model equations are available in Nichols et al. (Nichols et al., 2013).

Table 1. Independent variable inputs to be set to the hepatocyte-bioconcentration factor (HEP-BCF) model for the BCF prediction of methoxychlor as example (Nichols et al., 2013).

Parameter	Value	Units
Log K_{ow} of test chemical[1]	5.1	Unitless
Body weight of fish used as source of hepatocytes[2]	364	g
Hepatocyte cell number (C_{HEP})[3]	2.2×10^6	cells/mL
Reaction rate (*Rate*)	0.128	h^{-1}
Modelled temperature (T)	12	Celsius
Total aqueous chemical concentration ($C_{w,\ TOT}$)[2]	1.0	mg/L

[1]log K_{ow} of test chemical can be measured or estimated
[2]does not impact BCF calculations
[3]final cell number was verified by recounting

Table 2. Independent variable inputs to be set to the S9 bioconcentration factor (S9-BCF) model for the BCF prediction of methoxychlor as example (Nichols et al., 2013).

Parameter	Value	Units
Log K_{ow} of test chemical[1]	5.1	Unitless
Body weight of fish used as source of S9[2]	310	g
S9 protein concentration (C_{S9}) in the assay	1.0	mg/mL
Reaction rate (*Rate*)	0.359	h^{-1}
Modelled temperature (T)	12	Celsius
Total aqueous chemical concentration ($C_{w,\ TOT}$)[2]	1.0	mg/L

[1]log K_{ow} of test chemical can be measured or estimated
[2]does not impact BCF calculations

Table 3. Spreadsheet of the HEP-BCF model using the full modeled binding assumption applied to predict the BCF of methoxychlor as example.[1]

Hepatocyte substrate depletion (linear) data; Standard fish		The BCF portion of this model incorporates the Arnot and Gobas (2003)	
Hepatocyte spreadsheet_Public_062617		model equations	
Input Parameters for the source of in vitro data			
Parameter	Value	Units	
Reaction rate (Rate)	0.1275597	1/h	Determined from the slope of the log-transformed substrate depletion data
Fish body weight in grams (Bwg_{HEP})	364	g	Hepatocyte donor fish
Hepatocyte concentration (C_{HEP})	2.2	10^6 cells/ml	Set by researcher
Liver hepatocyte content (L_{HEP})	510	10^6 cells/g liver	Avg. of values for juvenile males and females (Nichols et al., 2013)
Inputs for the modeled fish (10 g fish, 5% lipid, @ 15 C)			Modeled after fish commonly used for BCF testing. These are also the parameters assumed by Arnot et al. (2008) for his evaluation of measured BCFs (from which Jon estimated apparent whole-body K_{MET} values)
Parameter	Value	Units	
Modeled body weight in grams (Bwg_M)	10	g	Standard value (assumed)
Modeled body weight in kilograms ($Bwkg_M$)	0.01	kg	Calculated from previous
Modeled temperature (T)	12	C	Assumed
Fractional liver weight (L_{FBW})	0.015	g liver/g fish	From Schultz et al. (1999)
Liver blood flow as fraction of cardiac output (Q_{HFRAC})	0.259	Unitless	From Nichols et al. (1990)
Fractional whole-body lipid content (v_{LWB})	0.05	Unitless	Assumed
Fractional blood water content (v_{WBL})	0.84	Unitless	From Bertelsen et al. (1998)
Additional Input Parameters			
Parameter	Value	Units	
Log K_{OW}	5.1	Unitless	
Total aqueous chemical conc. ($C_{W,TOT}$)	1	mg/l	
Dissolved organic carbon (C_{DOC})	0.0000046	kg/L	From US EPA (2003) Table 6-10, mean of all types
Particulate orgaic carbon (C_{POC})	0.000001	kg/L	From US EPA (2003) Table 6-10, mean of all types
POC binding constant (α_{POC})	0.35	Unitless	From Seth et al. (1999), cited by Arnot and Gobas (2004)
DOC binding constant (α_{DOC})	0.08	Unitless	From Burkhard et al. (2000)
Calculated Parameters			**Equations**
Parameter	Value	Units	
K_{OW}	125892.54118	Unitless	K_{OW} = 10^Log K_{OW}
Blood:water partition coefficient (P_{BW})	846.35240	Unitless	P_{BW} = (10^(0.73*Log K_{OW}) * 0.16) + v_{WBL}
Binding correction term (f_U)	0.01632	Unitless	f_U= (v_{WBL}/P_{BW})/((C_{HEP}/2)/(10^(0.676*Log K_{OW} - 2.215) + 1.0))
Binding correction term assuming fu = 1.0 (fu,1)	1.00000	Unitless	fu=1.0
Partitioning-based BCF (BCF_P)	6294.62706		BCF_P = v_{LWB}*K_{OW}
Volume of distribution ref. to blood plasma ($V_{D,BL}$)	7.44	l/kg	$V_{D,BL}$ = BCF_P/P_{BW}
In vitro intrinsic clearance ($CL_{IN\ VITRO,INT}$)	0.06	ml/h/10^6 cells	$CL_{IN\ VITRO,INT}$ = Rate/C_{HEP}
In vivo intrinsic clearance ($CL_{IN\ VIVO,INT}$)	10.6454	l/d/kg fish (or ml/d/g fish)	$CL_{INVITROINT}$ * L_{HEP} * L_{FBW} * 24
Scaled clearance for 10 g fish ($CL_{IN\ VITRO,INT,10}$)	10.6454	l/d/kg fish (or ml/d/g fish)	$CL_{IN\ VIVO,INT,10}$ = $CL_{IN\ VIVO,INT}$ * ((Bwg_M/Bwg_{HEP})^0)
			weight-normalized clearance is constant across body sizes (allometric exponent set to 0)
			Alternative assumptions are implemented by changing the exponent to a user-assigned
			value (in cell 74C)
Temperature adjusted Cardiac output (Q_C)	70.2706	l/d/kg fish	Q_C = (((0.23*T)-0.78)*(BwgM/500)^-0.1)*24
Liver blood flow (Q_H)	18.2001	l/d/kg fish	Q_H = Q_C*Q_{HFRAC}
Hepatic clearance (CL_H)	0.1721	l/d/kg fish	CL_H = ((Q_H*f_U*$CL_{IN\ VIVO,INT,10}$)/(Q_H+(f_U*$CL_{IN\ VIVO,INT,10}$)))
			To adopt the assumption that binding is functionally identical in vitro and in plasma
			the user must manually change the term "fu" to "fuone" in the equation (in cell 82C)
Whole-body metabolism rate (k_{MET})	0.0231	/d	k_{MET} = CL_H/$V_{D,BL}$
Chemical concentration dissolved in water ($C_{W,FD}$)	0.917102345	mg/l	$C_{W,FD}$ = $C_{W,TOT}$ *(1/(1+C_{DOC}*α_{DOC}*K_{OW}+C_{POC}*α_{POC}*K_{OW}))
Gill uptake rate constant (k_1)	630.456555	l/kg/d	k_1 = 1/((0.01 + 1/K_{OW})*BwkgM^0.4)
Gill elimination rate constant (k_2)	0.100157888	/d	k_2 = k_1/(v_{LWB}*K_{OW})
Fecal egestion rate constant (k_E)	0.00510752	/d	k_E = 0.125*(0.02*$Bwkg_M$^-0.15*e(0.06*T))/(0.000000051*K_{OW} + 2)
Growth rate constant (k_G)	0	/d	K_G = 0 (or 0.000502*$Bwkg_M$^-0.2 as in BCFBAF)
Concentration in fish ($C_{FISH,SS}$)	4503.067125	mg/kg	$C_{FISH,SS}$ = (k_1*$C_{W,FD}$)/(k_2+k_{METAB}+k_G+k_E)
BCF, on a total conc basis, w/out lipid norm. (BCF_{TOT})	**4503.067125**	**l/kg or ml/g**	BCF_{TOT} = $C_{FISH,SS}$/$C_{W,TOT}$
BCF, on freely diss. basis, norm. for fish lipid ($BCF_{FD,L}$)	98202.06332	l/kg lipid or ml/g lipid	$BCF_{FD,L}$ = $C_{FISH,SS}$/($C_{W,FD}$*v_{LWB})

[1]Parameters in red have to be set for each experiment (see Tab. 1). The full modelled binding assumption (i.e., $f_U = f_{U,P}/f_{U,HEP}$ is explained in §66 and in Nichols et al. (Nichols et al., 2013).

Table 4. Spreadsheet of the HEP-BCF model using the binding assumption f_U=1.0 applied to predict the BCF of methoxychlor as example.[1]

Hepatocyte substrate depletion (linear) data; Standard fish		The BCF portion of this model incorporates the Arnot and Gobas (2003)	
Hepatocyte spreadsheet_Public_062617		model equations	
Input Parameters for the source of in vitro data			
Parameter	Value	Units	
Reaction rate (Rate)	0.1275597	1/h	Determined from the slope of the log-transformed substrate depletion data
Fish body weight in grams (Bwg_{HEP})	364	g	Hepatocyte donor fish
Hepatocyte concentration (C_{HEP})	2.2	10^6 cells/ml	Set by researcher
Liver hepatocyte content (L_{HEP})	510	10^6 cells/g liver	Avg. of values for juvenile males and females (Nichols et al., 2013)
Inputs for the modeled fish (10 g fish, 5% lipid, @ 15 C)			Modeled after fish commonly used for BCF testing. These are also the parameters assumed by Arnot et al. (2008)
			for his evaluation of measured BCFs (from which Jon estimated apparent whole-body K_{MET} values)
Parameter	Value	Units	
Modeled body weight in grams (Bwg_M)	10	g	Standard value (assumed)
Modeled body weight in kilograms ($Bwkg_M$)	0.01	kg	Calculated from previous
Modeled temperature (T)	12	C	Assumed
Fractional liver weight (L_{FBW})	0.015	g liver/g fish	From Schultz et al. (1999)
Liver blood flow as fraction of cardiac output (Q_{HFRAC})	0.259	Unitless	From Nichols et al. (1990)
Fractional whole-body lipid content (v_{LWB})	0.05	Unitless	Assumed
Fractional blood water content (v_{WBL})	0.84	Unitless	From Bertelsen et al. (1998)
Additional Input Parameters			
Parameter	Value	Units	
Log K_{OW}	5.1	Unitless	
Total aqueous chemical conc. ($C_{W,TOT}$)	1	mg/l	
Dissolved organic carbon (C_{DOC})	0.0000046	kg/L	From US EPA (2003) Table 6-10, mean of all types
Particulate orgaic carbon (C_{POC})	0.000001	kg/L	From US EPA (2003) Table 6-10, mean of all types
POC binding constant (α_{POC})	0.35	Unitless	From Seth et al. (1999), cited by Arnot and Gobas (2004)
DOC binding constant (α_{DOC})	0.08	Unitless	From Burkhard et al. (2000)
Calculated Parameters			**Equations**
Parameter	Value	Units	
K_{OW}	125892.54118	Unitless	K_{OW} = 10^Log K_{OW}
Blood:water partition coefficient (P_{BW})	846.35240	Unitless	P_{BW} = (10^(0.73*Log K_{OW}) * 0.16) + v_{WBL}
Binding correction term (f_U)	0.01632	Unitless	f_U= (v_{WBL}/P_{BW})/((C_{HEP}/2)/(10^(0.676*Log K_{OW} - 2.215) + 1.0))
Binding correction term assuming fu = 1.0 (fu,1)	1.00000	Unitless	fu=1.0
Partitioning-based BCF (BCF_P)	6294.62706		BCF_P = v_{LWB}*K_{OW}
Volume of distribution ref. to blood plasma ($V_{D,BL}$)	7.44	l/kg	$V_{D,BL}$ = BCF_P/P_{BW}
In vitro intrinsic clearance ($CL_{IN\ VITRO,INT}$)	0.06	ml/h/10^6 cells	$CL_{IN\ VITRO,INT}$ = Rate/C_{HEP}
In vivo intrinsic clearance ($CL_{IN\ VIVO,INT}$)	10.6454	l/d/kg fish (or ml/d	$CL_{INVITROINT}$ * L_{HEP} * L_{FBW} * 24
Scaled clearance for 10 g fish ($CL_{IN\ VITRO,INT,10}$)	10.6454	l/d/kg fish (or ml/d	$CL_{IN\ VIVO,INT,10}$ = $CL_{IN\ VIVO,INT}$ * ((Bwg_M/Bwg_{HEP})^0)
			weight-normalized clearance is constant across body sizes (allometric exponent set to 0)
			Alternative assumptions are implemented by changing the exponent to a user-assigned
			value (in cell 74C)
Temperature adjusted Cardiac output (Q_C)	70.2706	l/d/kg fish	Q_C = (((0.23*T)-0.78)*(BwgM/500)^-0.1)*24
Liver blood flow (Q_H)	18.2001	l/d/kg fish	Q_H = Q_C*Q_{HFRAC}
Hepatic clearance (CL_H)	6.7167	l/d/kg fish	CL_H = ((Q_H*f_U*$CL_{IN\ VIVO,INT,10}$)/(Q_H+(f_U*$CL_{IN\ VIVO,INT,10}$)))
			To adopt the assumption that binding is functionally identical in vitro and in plasma
			the user must manually change the term "fu" to "fuone" in the equation (in cell 82C)
Whole-body metabolism rate (k_{MET})	0.9031	/d	k_{MET} = CL_H/$V_{D,BL}$
Chemical concentration dissolved in water ($C_{W,FD}$)	0.917102345	mg/l	$C_{W,FD}$ = $C_{W,TOT}$ *(1/(1+C_{DOC}*α_{DOC}*K_{OW}+C_{POC}*α_{POC}*K_{OW}))
Gill uptake rate constant (k_1)	630.456555	l/kg/d	k_1 = 1/((0.01 + 1/K_{OW})*BwkgM^0.4)
Gill elimination rate constant (k_2)	0.100157888	/d	k_2 = k_1/(v_{LWB}*K_{OW})
Fecal egestion rate constant (k_E)	0.00510752	/d	k_E = 0.125*(0.02*$Bwkg_M$^-0.15*e(0.06*T))/(0.000000051*K_{OW} + 2)
Growth rate constant (k_G)	0	/d	K_G = 0 (or 0.000502*$Bwkg_M$^-0.2 as in BCFBAF)
Concentration in fish ($C_{FISH,SS}$)	573.3919884	mg/kg	$C_{FISH,SS}$ = (k_1*$C_{W,FD}$)/(k_2+k_{METAB}+k_G+k_E)
BCF, on a total conc basis, w/out lipid norm. (BCF_{TOT})	**573.3919884**	**l/kg or ml/g**	BCF_{TOT} = $C_{FISH,SS}$/$C_{W,TOT}$
BCF, on freely diss. basis, norm. for fish lipid ($BCF_{FD,L}$)	12504.42749	l/kg lipid or ml/g li	$BCF_{FD,L}$ = $C_{FISH,SS}$/($C_{W,FD}$*v_{LWB})

[1]Parameters in red have to be set for each experiment (see Tab. 1). To adopt the assumption that binding is functionally identical *in vitro* and in plasma (see §66), the term "fu" was manually changed to "fuone" in the equation for calculation of hepatic clearance (CL_H) (Nichols et al., 2013).

Table 5. Spreadsheet of the S9-BCF model using the full modeled binding assumption applied to predict the BCF of methoxychlor as example.[1]

S9 substrate depletion (linear) data; Standard fish		The BCF portion of this model incorporates the Arnot and Gobas (2003)	
S9 spreadsheet_Public_062713		model equations	
Input Parameters for the source of in vitro data			
Parameter	Value	Units	
Reaction rate (Rate)	0.3593967	1/h	Determined from the slope of the log-transformed substrate depletion data
Fish body weight in grams (Bwg_{S9})	310	g	S9 Donor fish
S9 Protein concentration (C_{S9})	1	mg/ml	Set by researcher
Liver S9 protein content (L_{S9})	163	mg/g liver	Avg. of recovery corrected values obtained using the G6P and CYP content assays (Nichols et al., 2013)
Inputs for the modeled fish (10 g fish, 5% lipid, @ 15 C)			Modeled after fish commonly used for BCF testing. These are also the parameters assumed by Arnot et al. (2008) for his evaluation of measured BCFs (from which Jon estimated apparent whole-body K_{MET} values)
Parameter	Value	Units	
Modeled body weight in grams (Bwg_M)	10	g	Standard value (assumed)
Modeled body weight in kilograms ($Bwkg_M$)	0.01	kg	Calculated from previous
Modeled temperature (T)	12	C	Assumed
Fractional liver weight (L_{FBW})	0.015	g liver/g fish	From Schultz et al. (1999)
Liver blood flow as fraction of cardiac output (Q_{HFRAC})	0.259	Unitless	From Nichols et al. (1990)
Fractional whole-body lipid content (v_{LWB})	0.05	Unitless	Assumed
Fractional blood water content (v_{WBL})	0.84	Unitless	From Bertelsen et al. (1998)
Additional Input Parameters			
Parameter	Value	Units	
Log K_{OW}	5.1	Unitless	
Total aqueous chemical conc. ($C_{W,TOT}$)	1	mg/l	
Dissolved organic carbon (C_{DOC})	0.0000046	kg/L	From US EPA (2003) Table 6-10, mean all types
Particulate orgaic carbon (C_{POC})	0.000001	kg/L	From US EPA (2003) Table 6-10, mean all types
POC binding constant (α_{POC})	0.35	Unitless	From Seth et al. (1999), cited by Arnot and Gobas (2004)
DOC binding constant (α_{DOC})	0.08	Unitless	From Burkhard et al. (2000)
Calculated Parameters			**Equations**
Parameter	Value	Units	
K_{OW}	125892.54118	Unitless	K_{OW} = 10^Log K_{OW}
Blood:water partition coefficient (P_{BW})	846.35240	Unitless	P_{BW} = (10^(0.73*Log K_{OW}) * 0.16) + v_{WBL}
Binding correction term (f_U)	0.02488	Unitless	f_U= (v_{WBL}/P_{BW})/(1/(C_{S9}*10^(0.694*Log K_{OW} - 2.158) + 1.0))
Binding correction term, assuming fu = 1.0 (fu,1)	1.00000	Unitless	f_U=1.0
Partitioning based BCF (BCF_P)	6294.62706	l/kg	BCF_P = v_{LWB}*K_{OW}
Volume of distribution ref. to blood plasma ($V_{D,BL}$)	7.44	l/kg	$V_{D,BL}$ = BCF_P/P_{BW}
In vitro intrinsic clearance ($CL_{IN\ VITRO,INT}$)	0.36	ml/h/mg S9 protein	$CL_{IN\ VITRO,INT}$ = Rate/C_{S9}
In vivo intrinsic clearance ($CL_{IN\ VIVO,INT}$)	21.0894	/kg fish (or ml/d/g fis	$CL_{IN\ VITRO,INT}$ * L_{S9} * L_{FBW} * 24
Scaled clearance for 10 g fish ($CL_{IN\ VIVO,INT,10}$)	21.0894	/kg fish (or ml/d/g fis	$CL_{IN\ VIVO,INT,10}$ = $CL_{IN\ VIVO,INT}$ * ((Bwg_M/Bwg_{S9})^0) weight-normalized clearance is constant across body sizes (allometric exponent set to 0) Alternative assumptions are implemented by changing the exponent to a user-assigned value (in cell 74C)
Temperature adjusted Cardiac output (Q_C)	70.2706	l/d/kg fish	Q_C = (((0.23*T)-0.78)*(Bwg_M/500)^-0.1)*24
Liver blood flow (Q_H)	18.2001	l/d/kg fish	Q_H = Q_C*Q_{HFRAC}
Hepatic clearance (CL_H)	0.5100	l/d/kg fish	CL_H = ((Q_H*f_U*$CL_{IN\ VIVO,INT,10}$)/(Q_H+(f_U*$CL_{IN\ VIVO,INT,10}$))) To adopt the assumption that binding is functionally identical in vitro and in plasma the user must manually change the term "fu" to "fuone" in the equation (in cell 82C)
Whole-body metabolism rate (k_{MET})	0.0686	/d	k_{MET} = CL_H/$V_{D,BL}$
Chemical concentration dissolved in water ($C_{W,FD}$)	0.917102345	mg/l	$C_{W,FD}$ = $C_{W,TOT}$ *(1/(1+C_{DOC}*α_{DOC}*K_{OW}+C_{POC}*α_{POC}*K_{OW}))
Gill uptake rate constant (k_1)	630.456555	l/kg/d	k_1 = 1/((0.01 + 1/K_{OW})*BwkgM^0.4)
Gill elimination rate constant (k_2)	0.100157888	/d	k_2 = k_1/(v_{LWB}*K_{OW})
Fecal egestion rate constant (k_E)	0.00510752	/d	k_E = 0.125*(0.02*$Bwkg_M$^-0.15*e(0.06*T))/(0.000000051*K_{OW} + 2)
Growth rate constant (k_G)	0	/d	k_G = 0 (or 0.000502*BwkgM^-0.2 as in BCFBAF)
Concentration in fish ($C_{FISH,SS}$)	3326.16137	mg/kg	$C_{FISH,SS}$ = (k_1*$C_{W,FD}$)/(k_2+k_{METAB}+k_G+k_E)
BCF, on a total conc basis, w/out lipid norm. (BCF_{TOT})	**3326.16137**	**l/kg or ml/g**	BCF_{TOT} = $C_{FISH,SS}$/$C_{W,TOT}$
BCF, on freely diss. basis, norm. for fish lipid ($BCF_{FD,L}$)	72536.31812	/kg lipid or ml/g lipid	$BCF_{FD,L}$ = $C_{FISH,SS}$/($C_{W,FD}$*v_{LWB})

[1]Parameters in red have to be set for each experiment (see Tab. 1). The full modelled binding assumption (i.e., $f_U = f_{U,P}/ f_{U,S9}$ is explained in §66 and by Nichols et al. (Nichols et al., 2013).

Table 6. Spreadsheet of the S9-BCF model using the binding assumption f_U=1.0 applied to predict the BCF of methoxychlor as example.[1]

S9 substrate depletion (linear) data; Standard fish		The BCF portion of this model incorporates the Arnot and Gobas (2003)	
S9 spreadsheet_Public_062713		model equations	
Input Parameters for the source of in vitro data			
Parameter	Value	Units	
Reaction rate (Rate)	0.3593967	1/h	Determined from the slope of the log-transformed substrate depletion data
Fish body weight in grams (Bwg_{S9})	310	g	S9 Donor fish
S9 Protein concentration (C_{S9})	1	mg/ml	Set by researcher
Liver S9 protein content (L_{S9})	163	mg/g liver	Avg. of recovery corrected values obtained using the G6P
			and CYP content assays (Nichols et al., 2013)
Inputs for the modeled fish (10 g fish, 5% lipid, @ 15 C)			Modeled after fish commonly used for BCF testing. These are also the parameters assumed by Arnot et al. (2008)
			for his evaluation of measured BCFs (from which Jon estimated apparent whole-body K_{MET} values)
Parameter	Value	Units	
Modeled body weight in grams (Bwg_M)	10	g	Standard value (assumed)
Modeled body weight in kilograms ($Bwkg_M$)	0.01	kg	Calculated from previous
Modeled temperature (T)	12	C	Assumed
Fractional liver weight (L_{FBW})	0.015	g liver/g fish	From Schultz et al. (1999)
Liver blood flow as fraction of cardiac output (Q_{HFRAC})	0.259	Unitless	From Nichols et al. (1990)
Fractional whole-body lipid content (v_{LWB})	0.05	Unitless	Assumed
Fractional blood water content (v_{WBL})	0.84	Unitless	From Bertelsen et al. (1998)
Additional Input Parameters			
Parameter	Value	Units	
Log K_{OW}	5.1	Unitless	
Total aqueous chemical conc. ($C_{W,TOT}$)	1	mg/l	
Dissolved organic carbon (C_{DOC})	0.0000046	kg/L	From US EPA (2003) Table 6-10, mean all types
Particulate orgaic carbon (C_{POC})	0.000001	kg/L	From US EPA (2003) Table 6-10, mean all types
POC binding constant (α_{POC})	0.35	Unitless	From Seth et al. (1999), cited by Arnot and Gobas (2004)
DOC binding constant (α_{DOC})	0.08	Unitless	From Burkhard et al. (2000)
Calculated Parameters			**Equations**
Parameter	Value	Units	
K_{OW}	125892.54118	Unitless	K_{OW} = 10^Log K_{OW}
Blood:water partition coefficient (P_{BW})	846.35240	Unitless	P_{BW} = (10^(0.73*Log K_{OW}) * 0.16) + v_{WBL}
Binding correction term (f_U)	0.02488	Unitless	f_U= (v_{WBL}/P_{BW})/(1/(C_{S9}*10^(0.694*Log K_{OW} - 2.158) + 1.0))
Binding correction term, assuming fu = 1.0 (fu,1)	1.00000	Unitless	f_U=1.0
Partitioning based BCF (BCF_P)	6294.62706	l/kg	BCF_P = v_{LWB}*K_{OW}
Volume of distribution ref. to blood plasma ($V_{D,BL}$)	7.44	l/kg	$V_{D,BL}$ = BCF_P/P_{BW}
In vitro intrinsic clearance ($CL_{IN\ VITRO,INT}$)	0.36	ml/h/mg S9 protein	$CL_{IN\ VITRO,INT}$ = Rate/C_{S9}
In vivo intrinsic clearance ($CL_{IN\ VIVO,INT}$)	21.0894	/d/kg fish (or ml/d/g fish	$CL_{IN\ VITRO,INT}$ * L_{S9} * L_{FBW} * 24
Scaled clearance for 10 g fish ($CL_{IN\ VIVO,INT,10}$)	21.0894	/d/kg fish (or ml/d/g fish	$CL_{IN\ VIVO,INT,10}$ = $CL_{IN\ VIVO,INT}$ * ((Bwg_M/Bwg_{S9})^0)
			weight-normalized clearance is constant across body sizes (allometric exponent set to 0)
			Alternative assumptions are implemented by changing the exponent to a user-assigned
			value (in cell 74C)
Temperature adjusted Cardiac output (Q_C)	70.2706	l/d/kg fish	Q_C = (((0.23*T)-0.78)*(Bwg_M/500)^-0.1)*24
Liver blood flow (Q_H)	18.2001	l/d/kg fish	Q_H = Q_C*Q_{HFRAC}
Hepatic clearance (CL_H)	9.7692	l/d/kg fish	CL_H = ((Q_H*f_U*$CL_{IN\ VIVO,INT,10}$)/(Q_H+(f_U*$CL_{IN\ VIVO,INT,10}$)))
			To adopt the assumption that binding is functionally identical in vitro and in plasma
			the user must manually change the term "fu" to "fuone" in the equation (in cell 82C)
Whole-body metabolism rate (k_{MET})	1.3135	/d	k_{MET} = CL_H/$V_{D,BL}$
Chemical concentration dissolved in water ($C_{W,FD}$)	0.917102345	mg/l	$C_{W,FD}$ = $C_{W,TOT}$ *(1/(1+C_{DOC}*α_{DOC}*K_{OW}+C_{POC}*α_{POC}*K_{OW}))
Gill uptake rate constant (k_1)	630.456555	l/kg/d	k_1 = 1/((0.01 + 1/K_{OW})*BwkgM^0.4)
Gill elimination rate constant (k_2)	0.100157888	/d	k_2 = k_1/(v_{LWB}*K_{OW})
Fecal egestion rate constant (k_E)	0.00510752	/d	k_E = 0.125*(0.02*$Bwkg_M$^-0.15*e(0.06*T))/(0.000000051*K_{OW} + 2)
Growth rate constant (k_G)	0	/d	k_G = 0 (or 0.000502*BwkgM^-0.2 as in BCFBAF)
Concentration in fish ($C_{FISH,SS}$)	407.521917	mg/kg	$C_{FISH,SS}$ = (k_1*$C_{W,FD}$)/(k_2+k_{METAB}+k_G+k_E)
BCF, on a total conc basis, w/out lipid norm. (BCF_{TOT})	**407.521917**	**l/kg or ml/g**	BCF_{TOT} = $C_{FISH,SS}$/$C_{W,TOT}$
BCF, on freely diss. basis, norm. for fish lipid ($BCF_{FD,L}$)	8887.163345	l/kg lipid or ml/g lipid	$BCF_{FD,L}$ = $C_{FISH,SS}$/($C_{W,FD}$*v_{LWB})

[1]Parameters in red have to be set for each experiment (see Tab. 1). To adopt the assumption that binding is functionally identical *in vitro* and in plasma (see §66), the term "fu" was manually changed to "fuone" in the equation for calculation of hepatic clearance (CL_H) (Nichols et al., 2013).

References

Nichols, J.W., D.B. Huggett, J.A. Arnot, P.N. Fitzsimmons and C.E. Cowan-Ellsberry (2013). Towards improved models for predicting bioconcentration of well-metabolized compounds by rainbow trout using measured rates of in vitro intrinsic clearance. Environmental Toxicology and Chemistry 32: 1611-1622.