Protocols for Measuring In Vitro and In Vivo Biotransformation Rates of Hydrophobic Substances in Fish

by

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Abstract

The effective management of commercial chemicals is important for achieving sustainable development goals while reducing risks to human and ecological health. National and international practices for the management of chemicals involve identifying substances with bioaccumulative properties which can lead to elevated chemical concentrations in organisms and associated toxic effects. Current metrics for identifying bioaccumulative properties of chemicals are the fish-water bioconcentration factor (BCF) and the octanol-water partition coefficient (K_{OW}). The currently recommended method for measuring the BCF is expensive, difficult, time consuming, and requires the use of many animals. The objective of this research is to develop and test alternative methods for assessing the bioaccumulation of substances. Methods include a dietary in-vivo bioaccumulation test, in-vitro biotransformation tests and in-vitro to in-vivo extrapolation.

The research involved the development and testing of a dietary bioaccumulation test for determining the BCF as well as biotransformation rates in the intestines and the body of the fish that involved the use of non-metabolizable reference chemicals. The results show that gastro-intestinal biotransformation plays a dominant role in the bioaccumulation of a large number of the tested hydrophobic organic chemicals when fish are exposed via the diet; while somatic biotransformation (including hepatic biotransformation) plays a dominant role in the bioaccumulation of tested chemicals in fish exposed via the water. The results demonstrate that the BCF can be measured in a dietary bioaccumulation tests and that biotransformation pathways and rates differ between aqueous and dietary tests.

The research also involved the development and testing of an in-vitro fish liver S9 biotransformation testing method. The results show that biotransformation rates using fish liver fractions are highly dependent on the concentration of the test chemical in the test. As a result, the recommended 1 µM initial substrate concentration may underestimate the in vitro biotransformation rate constant and, therefore, an overestimation of the whole fish BCF. To avoid challenges presented by concentration dependence, multiple solvent delivery based depletion experiments at a range of initial concentrations are recommended for determining the maximum depletion rate constant. Meanwhile, a single sorbent phase dosing experiment may also provide reasonable approximations of maximum depletion rates of very hydrophobic substances.

Lastly, the research involved extrapolating in vitro maximum depletion rate constants to somatic biotransformation rate constants and comparing the results with those measured from in vivo dietary tests. The results show a good agreement with empirical measurements from various in vivo experiments for the majority of test chemicals. However, a significant underestimation of the in vitro-extrapolated somatic biotransformation rate constant for 9-methylanthracene may suggest that the fish liver S9 in vitro system may not contain all of the enzymes and/or co-factors to biotransform the chemical compared to the whole fish. Overall, the results demonstrate potential for fish liver S9 extracts to assess in vivo biotransformation potential in the fish body.

Both in vivo and in vitro research indicate that extrahepatic may not be considered using standardized in vivo (BCF) and liver S9 in vitro testing. For gastro-intestinal biotransformation to be considered, streamlined in vivo dietary bioaccumulation tests are recommended. Meanwhile, in vitro S9 protocols may be best supplemented with in vitro gastro-intestinal biotransformation tests in future research, especially when extrapolating to endpoints such as BMF and BAF where the diet is a significant route of exposure.

Keywords: Bioaccumulation; biotransformation; sorbent-phase dosing; bioaccumulation modeling; OECD 305; extrahepatic biotransformation

Dedication

I dedicate this work to my parents and my sister. Thank you for your support, love and for always being there for me.

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Glossary

POPs	Persistent Organic Pollutants
PBT	Persistent, bioaccumulative, and toxic
UNEP	United Nations Environmental Programme
CEPA	Canadian Environmental Protection Act
TSCA	United States Toxic Substances Control Act
REACH	European Union's Registration, Evaluation, Authorization and Restriction of Chemicals
ECHA	European Chemicals Agency
GC/MS	Gas chromatography/mass spectrometry
BAF	Bioaccumulation factor ($C_{Organism}/C_{Water}$)
BCF	Bioconcentration factor ($C_{Organism}/C_{Water}$)
BMF	Biomagnification factor ($C_{Organism}/C_{Diet}$)
TMF	Trophic magnification factor
K_{OW}	Octanol-water partition coefficient ($C_{Octanol}/C_{Water}$)
k_{BM}	Somatic biotransformation rate constant (d^{-1})
k_{GM}	Gastrointestinal biotransformation rate constant (d^{-1})
k_{dep}	In vitro depletion rate constant (min^{-1})
V_{MAX}	Maximum formation rate of the metabolite ($\mu M \cdot min^{-1}$)
K_M	Michaelis constant (µM)
S9	Postmitochondrial supernatant fraction extract
f_U	Fraction unbound
EVA	Ethylene vinyl acetate
IVIVE	In vitro to in vivo extrapolation
QSAR	Qualitative structure-activity relationships
SE	Standard error
PAHs	Polycyclic aromatic hydrocarbons
9MA	9-methylanthracene
PYR	Pyrene
CHR	Chrysene
BaP	Benzo[*a*]pyrene
PCBs	Polychlorinated biphenyls
CBZs	Chlorobenzenes

Chapter 1.

Introduction

1.1. Background

Tens of thousands of commercial chemicals are in use, with thousands of new substances produced each year (USEPA 1976, Government of Canada 1999, UNEP 2001). Toxic effects of chemicals in the environment have been studied for many years. However, more attention has been drawn to the effects of anthropogenic chemicals on human and environmental health in the 1950s and 60s (Government of Japan 2002, Carson 1962, Jensen et al. 1969). This environmental movement brought awareness and the need to enforce regulations on chemical substances. The United States Environmental Protection Agency was formed in 1970, and the Department of the Environment was created in Canada in 1971.

Advancements in toxicological science have brought attention to key properties of contaminants that could cause harmful effects to humans and the environment. These key chemical properties include high persistence in the environment (have long environmental half-lives), bioaccumulation (have a tendency to accumulate to higher concentrations in organisms, and being toxic (can cause lethal or adverse effects to organisms at low chemical concentrations). Under the Stockholm Convention on Persistent Organic Pollutants (UNEP 2001) international environmental treaty, 29 chemicals (or chemical congener groups) that exhibited these properties are identified and targeted for elimination, restriction, or reduction of unintentional production in the environment. Specific national regulatory programs (Government of Canada 1999, USEPA 1976, Japanese Ministry of Environment 2001, European Commission 2006) have adopted regulations that evaluate chemicals for their persistence (P), bioaccumulation (B), and toxicity (T).

Bioaccumulation is the process that involves the absorption, distribution, metabolism, and elimination of chemical substances. Bioaccumulation involves a combination of abiotic (e.g. chemical partitioning) and complex biotic mechanisms (e.g. enzyme systems). Bioaccumulation can result in high chemical concentrations in

organisms that may result in toxic effects. As one of the three major properties considered in chemical evaluations, the assessment of the bioaccumulation of substances plays a large role in the management of commercial chemicals in the environment. Halogenated organic substances that include industrial chemicals such as polychlorinated biphenyls (PCBs), pesticides such as dichlorodiphenyltrichloroethane (DDT), and industrial by-products such as polychlorinated dibenzo-p-dioxins/polychlorinated dibenzofurans (PCDDs/PCDFs) have received much of the interest because of their bioaccumulative properties. However, there are many other types of substances that are bioaccumulative such as siloxanes and perfluorooctanesulfonic acid (PFOS).

1.1.1. Approaches to bioaccumulation assessments

To identify bioaccumulative substances, *in vivo*-based bioaccumulation metrics such as the laboratory derived fish bioconcentration factor (BCF; $C_{Organism}/C_{Water}$) are often the preferred metric in regulatory programs. The Canadian Environmental Protection Act also recognizes the field derived fish bioaccumulation factor (BAF; $C_{Organism}/C_{Water}$) over the BCF. Recently, other *in* vivo-based metrics such as the biomagnification factor (BMF; $C_{Organism}/C_{Diet}$) and trophic magnification factor (TMF) are being considered in regulatory bioaccumulation assessments (ECHA 2014a, ECHA 2014b). Nonetheless, regulatory programs still currently rely largely on the BCF and the BAF in Canada. Unfortunately, empirical BCFs and BAFs are not available for the large majority of commercial chemicals that require assessments. For example, among the Canadian Domestic Substances List's 23,000 chemicals, 11,000 are discrete organic chemicals. Among these 11,000 organic chemicals, only about 350 compounds have empirical bioaccumulation or bioconcentration factor data (Arnot and Gobas 2006). In the absence of empirical BCF and BAF data, regulatory programs often rely on the octanol-water partition coefficient (K_{OW}; $C_{Octanol}/C_{Water}$) or BCF determinations using K_{OW}-based bioaccumulation models. The K_{OW} represents the chemical partitioning between the lipids of an organism and water. This oversimplifies and potentially overestimates the bioaccumulation process of many chemicals. For example, certain chemicals may bioaccumulate mechanistically to certain tissues (such as proteins) and would not be predicted well by a lipid-surrogate compared to other neutral hydrophobic organic comopunds. Additionally, chemicals that are superhydrophobic (log K_{OW}> 8) may not

bioaccumulate to the same degree as less hydrophobic (log K_{OW} 6-8) substances. Moreover, chemicals that biomagnify such as polychlorinated biphenyls (PCBs) may have chemical concentrations in the organism that are higher than what can be predicted through the K_{OW}. More importantly, hydrophobic substances that are biotransformed may be identified as bioacccumulative when they are not. For many chemicals that do not biotransform considerably such as PCBs, chlorobenzenes, and other halogenated contaminants, K_{OW}–based bioaccumulation models such as AQUAWEB and RAIDAR (Arnot and Gobas 2004, Arnot and Mackay 2008) can accurately predict rates of uptake and excretion for these chemicals and have proven to be useful alternatives to solely using K_{OW} for bioaccumulation assessments. However, the inability of current bioaccumulation models to estimate biotransformation rates of absorbed chemicals has remained a key challenge in conducting realistic bioaccumulation assessments. If the rate of chemical excretion is very slow (e.g., for high K_{OW} substances), biotransformation plays an important role in the chemical depuration process. In the absence of data and assessment methods for biotransformation rates, the default assumption for the biotransformation rate of chemicals in organisms is zero. This may cause false positives (type I errors), as many hydrophobic chemicals may be improperly evaluated as bioaccumulative when they are not.

The lack of data on biotransformation rate constant presents a major limitation and source of uncertainty in bioaccumulation assessments. Under current regulatory frame works, the current approach to bioaccumulation assessmentment of biotransforming substances is to conduct laboratory-derived fish BCF testing in accordance to the OECD 305 Test Guideline (OECD 2012). Unfortunately, this current approach can be costly, labor intensive, and make extensive use of animals. A typical aqueous-exposure flow-through test uses and costs approximately US $125,000 per chemical (Weisbrod 2007). The chemical exposure and depuration phases last for approximately 7-28 days each resulting in 14-56 days required for each test, with additional time for chemical extraction and analysis (OECD 2012). Each OECD 305 TG flow-through test also requires the use of around 108 fish per test (de Wolf et al. 2007). With an estimated 3,025 commercial chemicals identified by REACH that requires empirical BCF testing, this would result in over 370 million US dollars, several decades

of work effort, and roughly 327,000 fish required for BCF testing under the OECD 305 TG (de Wolf et al. 2007).

The extensive use of animals for bioaccumulation assessments is especially a large concern for regulatory bodies and animal welfare groups. In the European Union, for example, advancements in animal welfare are implemented in legislation under the Directive 2010/63/EU (EU 2010). These advancements include principles of the 3Rs put forward by Russel and Burch (Russell and Burch 1959) to (1) replace the use of vertebrate animals with alternatives (e.g. less advanced animals or computer programs), (2) reduce the use of animals by maximizing information obtained per animal, and (3) refine alternatives in testing procedures to minimize the pain instilled on an animal from life to death. In addition, it is generally accepted that, for regulatory purposes, implementing the principles of the 3Rs to animal research should not sacrifice biological relevance, reliability, and regulatory acceptability of the testing method (OECD 1996).

1.2. Alternative approaches for bioaccumulation assessments

With thousands of commercial chemicals requiring classification and risk assessments, there are several on-going initiatives for developing alternative approaches to bioaccumulation assessments that are more cost and time effective and use less animals in testing compared to the currently established OECD 305 TG method.

1.2.1. In vitro biotransformation testing

One initiative to improve the efficient delivery of bioaccumulation assessments for commercial substances is through in vitro testing. This involves the measurement of chemical depletion rates in in vitro tests using fish liver homogenates or hepatocytes (Trowell et al. 2018, Laue et al. 2014, Han et al. 2007). This is followed by in vitro to in vivo extrapolation (IVIVE) to make estimates of somatic biotransformation rate constants (Nichols et al. 2013), which are used as inputs to then make estimates of BCF values through a bioaccumulation model such as AQUAWEB (Arnot and Gobas 2004). This approach relies on several assumptions, i.e:

1. the liver is considered the main site of biotransformation in the fish body,

2. the in vitro enzyme system (e.g. fish liver homogenate or hepatocyte) is an adequate model to estimate biotransformation rates in the liver of the fish, and

3. the measured in vitro biotransformation rate is the maximum first order depletion rate constant under conditions where the substrate concentrations are well below enzyme saturation (i.e. [substrate] << Michaelis constant, KM).

If one or more of these assumptions are not met, then in vitro tests may significantly underestimate the extrapolated somatic biotransformation rate constant, which in turn can cause the BCF to be overestimated which would result in a false positive in the bioaccumulation assessment. Moreover, there is a need for good quality in vivo biotransformation rate constant data and methods to measure empirical in vivo biotransformation rates in order to validate and implement in vitro biotransformation testing for use in bioaccumulation assessments.

1.2.2. In silico biotransformation estimates

A second initiative involves the back calculation of somatic biotransformation rate constants from empirical BCF data using a bioaccumulation model (e.g. AQUAWEB) for a large set of chemicals, and using the chemical structure of each chemical to parameterize and develop a quantitative structure activity relationship (QSAR) model to predict somatic biotransformation rate constants based on chemical structure (Arnot et al. 2009). These in turn are again used as inputs in bioaccumulation models to calculate subsequent BCF estimates. This method has been integrated into the U.S. Environmental Protection Agency EPI Suite program through the BCFBAF module for estimating BCFs (US EPA 2012).

This method requires a large data set of reliable in vivo somatic biotransformation data to parameterize the QSAR models. Although in silico methods are currently not sufficiently accurate for regulatory purposes, they have the potential to speed up the bioaccumulation assessment process by prioritizing for chemical candidates for testing more effectively. Moreover, these methods can improve as empirical somatic biotransformation rate constant data become available.

1.2.3. Dietary in vivo testing

The current state of bioaccumulation science still requires in vivo testing as the generally accepted method for conducting bioaccumulation tests. The OECD 305 TG remains the only standardized approach to derive BCF values for use in bioaccumulation assessments. However, alternative in vivo testing approaches have the potential to fulfill global objectives to reduce large data gaps in bioaccumulation assessments through time and cost-efficient streamlined methods that also require less animal testing. The OECD 305 TG method was revamped in 2012 to include the diet as an alternative chemical exposure route compared to aqueous exposure tests (OECD 2012). The use of dietary bioaccumulation tests reduces overall costs because measuring very low concentrations of hydrophobic chemicals in water is difficult to perform. Moreover, it may be a challenge to dissolve hydrophobic chemicals which are limited by low aqueous solubility, and maintain constant exposure concentrations in the presence of fish and organic matter introduced by fish waste (Gobas and Zhang 1992, McCarthy and Jimenez 1985). The dietary-exposure approach also allows for the measurement of the biomagnification factor (BMF; $C_{Organism}/C_{Diet}$), a more ecologically relevant bioaccumulation metric than the BCF as it provides direct information on the magnification of a chemical in ecological environments. However, the dietary approach outlined in the OECD 305 TG also relies on the use of theoretical models for the estimation of the BCF. Therefore, the BCF derived from a dietary exposure test may have substantial error compared to aqueous exposure tests through current protocols outlined in the OECD 305 TG (OECD 2012).

As previously discussed, in vivo testing is also required to validate in vitro and in silico approaches for use in bioaccumulation assessment protocols. Alternate approaches for in vivo tests would ideally allow for the direct and empirical measurements for somatic biotransformation rate constants through modified testing and modeling methods. As a result, a direct comparison of in vitro-extrapolated and in vivo measured somatic biotransformation rate constants can be made for testing alternative in vitro approaches. Moreover, direct measurements of in vivo somatic biotransformation rate constants could provide necessary reliable data for parameterizing future QSAR developments for estimating somatic biotransformation rate constants.

1.3. Research Objectives

The main objective of my research is to develop and investigate methods for the measurement and assessment of the biotransformation and bioaccumulation capacity of neutral organic chemicals in fish with the goal to support global initiatives to identify chemicals of concern to environmental and human health. These methods will be based on scientifically-sound principles that are suitable for risk assessment and chemical management purposes, while also requiring less cost, time, and animal use for testing. In order to meet these objectives, two initiatives through in vitro testing and streamlined in vivo testing approaches will be developed, examined, and validated. A third initiative (in silico) for improving bioaccumulation assessments will be supported indirectly through an improvement of the availability of reliable in vivo data for QSAR parameterization and development.

1.3.1. Chapter 2: in vitro biotransformation - concentration dependence

The main objective of Chapter 2 is to investigate the dependence of in vitro biotransformation rates on the concentration of very hydrophobic test chemicals that are known to biotransform in fish. A current convention for initial substrate convention of 1 µM could underestimate the in vitro biotransformation rate constant and may cause biocncentration factors to be over estimated if in vitro biotransformation rates are used to assess bioconcentration factors in fish. To investigate this, depletion rate constants were measured at multiple substrate concentrations through multiple conventional solvent delivery–based tests and fitted to a nonlinear Michaelis-Menten kinetic model to determine kinetic parameters for the hydrophobic test chemicals such the maximum formation rate of the metabolite, V_{MAX} (µM min^{-1}) and the Michaelis constant, K_M (µM) i.e. the substrate concentration at 0.5 V_{MAX}.

The second objective of the study is to explore how a sorbent-phase dosing method may improve methods for assessing the BCF from in vitro bioassays. Using the same liver S9, depletion rate constants will also be measured using a sorbent-phase dosing system and compared with the kinetic parameters estimated from the solvent delivery–based tests.

1.3.2. Chapter 3: in vivo biotransformation - deriving somatic and gastrointestinal biotransformation rates in fish

The main objective of Chapter 3 is developing a method for measuring biotransformation rate constants in fish. A 2-compartment bioaccumulation fish model was developed that considers biotransformation rate constants in the body (soma) of the fish and within the gastrointestinal tract of the fish. This allows for the consideration of biotransformation in the gastrointestinal tract, which previously could not be distinguished in a single compartment bioaccumulation fish body.

A second objective of this chapter is to develop a testing method, similar to the OECD 305 Test Guideline, that would allow for the empirical measurement of biotransformation rate constants in the fish body. This would allow for the direct comparison of somatic biotransformation rate constants extrapolated from in vitro studies to those measured directly in in vivo studies. This testing methodology would help in the generating of high quality in vivo biotransformation rate data that can be used to further develop methods for bioaccumulation assessment in fish.

1.3.3. Chapter 4: in vivo biotransformation - a simplified method to derive somatic biotransformation rates in fish and to measure BCF from dietary studies

The aim of Chapter 4 is to advance dietary-exposure bioaccumulation testing by providing methods to determine an empirical BCF, which is of regulatory relevance. In this chapter, a simplified theoretical framework to describe the bioaccumulation and biotransformation in fish is developed, evaluated, and applied to illustrate its potential use; and finally test the performance of the method.

Currently, the OECD 305 Test Guideline's dietary exposure rely on estimates of chemical uptake through the water for the estimation of a BCF value. As a secondary objective, the simplified modeling framework described in this chapter will allow for the estimation of empirical BCF values from a dietary exposure test, and the results will be compared with empirical BCF values for the same chemicals from other literature sources.

1.3.4. Chapter 5: in vivo biotransformation: application of method to a larger set of OECD 305 fish tests

The objective of Chapter 5 is to develop and apply the previously-developed in vivo method for simultaneously determining in vivo gastrointestinal biotransformation rates, somatic biotransformation rates, BCFs, and BMFs to a larger set of OECD 305 fish tests.

A second goal is to provide measurements of in vivo biotransformation rates for a number of structurally diverse chemicals to allow the testing and further development of quantitative structure activity relationships for predicting biotransformation rates and the testing of extrapolation methods for estimating in vivo rates from in vitro biotransformation rate data. Such a fish biotransformation database is also not available to date.

A third objective is to investigate the relationship between the BCFs and BMFs for substances subject to somatic and gastrointestinal biotransformation. This information is also not available.

1.3.5. Chapter 6: in vitro to in vivo extrapolation

The objective of Chapter 6 will be to develop and test an IVIVE approach for fish to predict in vivo somatic biotransformation rate constants in fish. This study details the results of both in vitro (Chapter 2) and in vivo biotransformation experiments in rainbow trout (Chapters 3-5). First order in vitro biotransformation rate constants of 4 poly aromatic hydrocarbons, measured using both thin-film sorbent dosing experiments and multiple solvent delivery-based experiments, are extrapolated and compared with first order in vivo biotransformation rate constants of the same test chemicals in the same species.

Previous efforts in testing the IVIVE approach involved the comparison of extrapolated and empirical bioconcentration factors. In this study, the IVIVE approach is tested by comparing extrapolated and empirical biotransformation rate constants. This approach avoids the potential influence of confounding factors associated with the uptake of chemicals from water and the depuration of chemicals by depuration routes other than biotransformation. In the present study, in vivo biotransformation rates

derived from in vitro studies using S9 rainbow trout liver extracts, which are then extrapolated to in vivo somatic biotransformation rates, which are then compared to in vivo somatic biotransformation rates in rainbow trout measured in dietary in vivo bioaccumulation experiments. The direct comparison of predicted and observed in vivo biotransformation rate constants in rainbow trout allows for the assessment of the performance of the IVIVE method for bioaccumulation assessments.

The ultimate goal of this research is to improve bioaccumulation assessments of environmental contaminants through the development of testing protocols for the evaluation of bioaccumulation of commercial chemicals that are more precise, time efficient, less costly, while also reducing animal use.

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Chapter 2.

Concentration Dependence of Biotransformation in Fish Liver S9: Optimizing Substrate Concentrations to Estimate Hepatic Clearance for Bioaccumulation Assessment*

Justin C. Lo designed the experiments, prepared the fish liver S9 homogenate with assistance from S. Victoria Otton, conducted the experiments with assistance from Gayatri N. Allard, performed chemical extraction and analysis with assistance from Gayatri N. Allard, performed the data analysis, and wrote the chapter with writing assistance from Frank A.P.C. Gobas and other co-authors.

2.1. Summary

In vitro bioassays to estimate biotransformation rate constants of contaminants in fish are currently being investigated to improve bioaccumulation assessments of hydrophobic contaminants. The present study investigates the relationship between chemical substrate concentration and in vitro biotransformation rate of 4 environmental contaminants (9-methylanthracene, pyrene, chrysene and benzo[*a*]pyrene) in rainbow trout (*Oncorhynchus mykiss*) liver S9 fractions and methods to determine maximum first order biotransformation rate constants. Substrate depletion experiments using a series of initial substrate concentrations showed that in vitro biotransformation rates exhibit strong concentration dependence, consistent with a Michaelis-Menten kinetic model. Results indicate that depletion rate constants measured at initial substrate concentrations of 1 µM (a current convention) could underestimate the in vitro biotransformation potential and may cause bioconcentration factors to be overestimated if in vitro biotransformation rates are used to assess bioconcentration factors in fish. Depletion rate constants measured using thin-film sorbent dosing experiments were not statistically different from the maximum depletion rate constants derived using a series of solvent delivery based depletion experiments for 3 of the 4 test chemicals. Multiple solvent delivery based depletion experiments at a range of initial concentrations are recommended for determining the concentration dependence of in vitro biotransformation rates in fish liver fractions, while a single sorbent phase dosing

experiment may be able to provide reasonable approximations of maximum depletion rates of very hydrophobic substances.

2.2. Introduction

Bioaccumulation of organic contaminants is usually assessed using the octanol-water partition coefficient (K_{OW}) and/or bioconcentration factor (BCF) in fish [**1**]. Because experimentally determined BCFs of most chemicals requiring evaluation are unknown, the bioaccumulation potential of chemicals is often assessed by the K_{OW} or K_{OW}-based bioaccumulation models [**1**]. However, K_{OW} and K_{OW} -based bioaccumulation models cannot provide *a priori* estimates of biotransformation rates. To avoid incorrectly classifying chemicals as bioaccumulative based solely on a high K_{OW} value, there is interest in developing methods for assessing chemical biotransformation rates. One approach is the development of Quantitative Structure-Activity Relationship (QSAR) models to predict biotransformation rates and corresponding BCFs based on chemical structure [**2,3**]. Another approach involves measuring biotransformation rates in vitro using subcellular liver preparations [**4,5**] or hepatocytes [**6**], followed by scaling and extrapolating the results to whole fish [**7**]. Compared to in vivo testing, in vitro testing is time and cost-effective, and reduces animal use [**8**].

In vitro biotransformation assays were first developed to estimate hepatic clearance rates of prospective drugs during the early stages of drug development [**9,10,11**]. In these assays, the initial increase in metabolite concentration C_M (µM) (i.e., $\frac{dC_M}{dt}$ in units of µM min^{-1}) at various substrate concentrations in the incubation medium, C_I (µM), is measured and kinetic parameters are determined by fitting the concentration data to the classical Michaelis-Menten equation:

$$\frac{dC_M}{dt} = \frac{V_{MAX}.C_I}{K_M + C_I} \quad (2.1)$$

where V_{MAX} (µM min^{-1}) is the maximum formation rate of the metabolite and K_M is the Michaelis constant (µM) for the reaction, i.e., the substrate concentration at 0.5 V_{MAX}. This method requires knowledge of the major metabolite formed and the analytical tools (including authentic standards) for their quantification. However, the metabolic pathways of the vast majority of environmental chemicals requiring bioaccumulation assessment

are unknown. Furthermore, the formation of more than one metabolite may need to be characterized to obtain complete information on the chemical's metabolic stability.

An alternative method measures the rate of loss (or depletion) of the chemical in the incubation medium due to biotransformation [**4-8,12**]. Here, the first order depletion rate constant k_{dep} (min^{-1}) is measured as the slope of the relationship between the natural logarithm of the remaining substrate concentration in the incubation medium (C_I) at incubation time (t):

$$k_{\mathrm{dep}} = \frac{\ln\left(\frac{C_{\mathrm{I,t=0}}}{C_{\mathrm{I}}}\right)}{t} \quad (2.2)$$

where $C_{I,t=0}$ is the initial substrate concentration in the incubation medium. The substrate depletion method has been proposed for measuring hepatic clearance rates of environmental contaminants whose metabolic pathways are unknown [**4-8,12**]. These studies were generally conducted using a starting substrate concentration of 1 µM. Johanning et al. [**12**] recommended starting concentrations of test chemical ($C_{I,t=0}$) to be 1 µM or less. Such concentrations are assumed to be sufficiently below the K_M of the major biotransformation reaction(s) to provide a k_{dep} that reflects the initial reaction rate, i.e., the biotransformation rate at substrate concentrations that are well below enzyme saturation. To date, there has been no investigation of the potential dependence of a contaminant's in vitro biotransformation rate (as measured by k_{dep}) using a range of initial chemical concentrations.

Instead of relying on the assumption that $C_{I,t=0} << K_M$, in vitro depletion rates (k_{dep}) at multiple initial chemical concentrations can be determined and fitted to a re-written form of the Michaelis-Menten equation as described by Obach and Reed-Hagen [**13**]:

$$k_{\mathrm{dep}} = k_{\mathrm{dep,C\rightarrow 0}}\left(1 - \frac{C_{\mathrm{I,t=0}}}{C_{\mathrm{I,t=0}} + K_{\mathrm{M}}}\right) \quad (2.3)$$

where $k_{dep,C\rightarrow 0}$ is the theoretical k_{dep} at an infinitesimally low substrate concentration. Equation 2.3 describes a decrease of k_{dep} as $C_{I,t=0}$ increases through the range of the K_M value and approaches zero when the enzymes are presumably saturated. The inflection point in the fitted curve represents K_M. This is the substrate concentration that

correspond with half of the maximum depletion rate constant ($k_{dep,C\to0}$). Equation 2.3 can be solved to yield both K_M and $k_{dep,C\to0}$. If the depletion rate represents a simple one-enzyme, one-product type of metabolic reaction, then the K_M value is theoretically identical to the K_M determined in classical product formation assays [**14**]. This has been observed to be the case for drugs oxidized by predominantly one cytochrome P450 enzyme to form one product [**13,15,16**]. However, for a chemical metabolized by mutliple enzymes present in a liver preparation, the K_M determined from substrate depletion experiments represents a composite of the K_M values of all the major metabolic reactions of the test chemical.

A method developed in our laboratory for measuring in vitro biotransformation rates of highly hydrophobic chemicals employs sorbent phase dosing [**17,18**]. In this method, the test chemical is dissolved in a thin-film of ethylene vinyl acetate (EVA) sorbent phase and exposed to the enzyme preparation. The chemical is delivered solvent-free via passive diffusion into the incubation medium over the course of the incubation. The thin-film configuration intends to provide a high surface to volume ratio to accelerate the release of the substrate in the incubation medium. A high surface area to volume relationship is of particular importance for very hydrophobic substrates because they diffuse very slowly from the sorbent phase into the largely aqueous incubation medium. One relevant feature of this method is that the initial substrate concentration in the incubation medium is essentially 0, while in a conventional solvent delivery based system, the initial concentration is at its maximum value. Due to the low substrate concentrations in the incubation medium, the initial biotransformation rate can be expected to represent the maximum depletion rate constant $k_{dep,C\to0}$. This feature may provide a method for measuring biotransformation rates without the need to determine K_M and the associated concentration dependence of the biotransformation rate. In a sorbent delivery based test, the transfer of the test chemical between the sorbent (EVA in the present study) and the incubation medium can be described by a 2-compartment mass-transfer model:

$$\frac{dC_E}{dt} = k_2 \frac{V_I}{V_E} C_I - k_1 C_E \quad (2.4)$$

$$\frac{dC_I}{dt} = k_1 \frac{V_E}{V_I} C_E - (k_2 + k_{dep,EVA}) C_I \quad (2.5)$$

where C_E and C_I are the chemical substrate concentrations (mol/m^3) in the EVA and the incubation medium, respectively; V_E and V_I are the volumes (m^3) of the EVA and the incubation medium; k_1 and k_2 are the mass transfer rate constants (min^{-1}) between the EVA and incubation medium; and $k_{dep,EVA}$ is the in vitro biotransformation rate constant (min^{-1}) derived in EVA thin film sorbent delivery experiments. The value of $k_{dep,EVA}$ is derived from the mass transfer constants between the two phases as described in Lee et al. 2012 [**17**]. By adding the appropriate amount of test chemical to the sorbent phase, the maximum possible concentration in the incubation medium can be controlled to avoid exceeding a specific target value (e.g. 1 μM). Hence, if K_M is known, experiments can be conducted where C_I does not exceed K_M.

The main objective of the present study is to investigate the dependence on substrate concentration of in vitro biotransformation rates of several very hydrophobic test chemicals. To investigate this, depletion rate constants is measured at multiple substrate concentrations through multiple conventional solvent delivery based tests and fitted to a non-linear model (Equation 2.3) to determine kinetic parameters for the hydrophobic test chemicals. Using the same S9, depletion rate constants are measured using a sorbent phase dosing system [17, 18] and compared with the kinetic parameters estimated from the solvent delivery based tests. The second objective of the present study is to explore how a sorbent phase dosing method may avoid the scientific challenges presented by concentration dependence compared to a conventional dosing system. The ultimate goal of this research is to improve bioaccumulation assessments of environmental contaminants.

2.3. Materials and Methods

2.3.1. Chemicals

The test chemicals 9-methylathracene (log K_{OW} = 5.07) [**19**], pyrene (log K_{OW} = 5.18) [**20**], chrysene (log K_{OW} = 5.81) [**21**], benzo[*a*]pyrene (log K_{OW} = 6.13) [**21**] and the internal standard chrysene-d^{12}, methanol, high-performance liquid chromatography (HPLC)-grade n-hexane, and β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt hydrate (β-NADPH) (≥97% purity) were purchased from Sigma-Aldrich. Ethylene vinyl acetate (Elvax 40W) was obtained from DuPont. Potassium dihydrogen phosphate and HPLC–grade acetonitrile were from Caledon Laboratories. Potassium

phosphate dibasic was obtained from Anachemia Canada. Potassium chloride was purchased from EMD Millipore. Unless specified, all other materials were purchased from Sigma-Aldrich.

2.3.2. Animals

Eight rainbow trout (*Oncorhynchus mykiss*) were obtained from Miracle Springs fish hatchery (Mission, BC). The fish were held in a flow-through tank supplied with dechlorinated water. Water temperatures were kept at 12.5-14.5 °C (mean temperature = 13.5 °C). The holding environment followed a 16:8-h light:dark cycle. Fish were acclimatized for more than 2 wk and fed EWOS Pacific 3.0-mm pellets at a rate of 1% bodyweight d^{-1}. The average body weight was 386 $\pm$ 68 g (SD, n=8) at the time of euthanasia.

2.3.3. Liver S9 preparation

Fish were euthanized by a 5 min exposure to 0.3 g L^{-1} tricaine methanesulfonate (MS 222) buffered with 0.3 g L^{-1} sodium bicarbonate. Exposure to this concentration of MS222 for 5 min has been shown to have no significant effect on microsomal cytochrome P450 activities in rainbow trout [**22**]. The fish livers were excised and immediately rinsed in ice-cold 1.15% (w/v) KCl. The liver weights were 5.3 $\pm$ 1.0 g (SD, n=8). The livers were minced on ice with a razor blade and homogenized using a Potter-Elvehjem glass tissue grinder with a Teflon pestle (Kontes) in 7 volumes (g mL^{-1}) of ice-cold 0.2 M phosphate buffer (pH 7.4) containing 1.15% (w/v) KCl. The liver homogenates were centrifuged at 10,000 *g* for 20 min at 4 °C (Hermle Z360K centrifuge). Following centrifugation, the top lipid layer was carefully removed and discarded, and the post-mitochondrial supernatant fraction (S9) was collected and stored in aliquots at -80 °C until use (held for < 3 months). The protein concentration of the S9 was determined by the method of Bradford [**23**] using bovine serum albumin (Fraction V) as the standard.

2.3.4. Solvent-delivery dosing preparation

Stock solutions of 9-methylanthracene, pyrene, chrysene, and benzo[*a*]pyrene were prepared in acetonitrile and further diluted in acetonitrile resulting in substrate

concentrations in the incubation medium of 0.056, 0.01, 0.18, 0.32, 0.56, 1.0, 1.78, 3.16, 5.62, 10, and 18 µM. The final volume of acetonitrile in the incubation mixture was < 0.5% (v/v).

2.3.5. Sorbent-phase dosing preparation

An EVA solution of 0.06735 $g.L^{-1}$ in dichloromethane was prepared. The test substrates 9-methylanthracene, pyrene, chrysene, or benzo[*a*]pyrene were added to the EVA solution at 10 µM, 5 µM, 10 µM, and 25 µM respectively. Each substrate was incubated individually. A 50 µL volume of the spiked EVA solution was transferred to a 2 mL silanized amber glass vial (Agilent). Uncapped, the vial was rolled to evaporate the solvent. This resulted in 0.0035 µL (3.4 µg) of EVA in the vial and an estimated EVA film thickness of 4 nm (calculated by dividing the volume of EVA film by the interior surface of the test vial). Assuming that the entire chemical mass in the sorbent phase was released into the incubation medium (0.5 mL), this corresponded to maximum possible concentrations in the incubation medium of 1.0 µM for 9-methylanthracene and chrysene, 0.5 µM for pyrene, and 2.5 µM for benzo[*a*]pyrene.

2.3.6. Selection of sampling time points

In preliminary depletion experiments at an initial substrate concentration of 0.5 µM, substrate concentrations declined in a log-linear fashion for a period of 80 min. Therefore, incubations were conducted for 80 min or less if the concentration fell below the detection limit. In preliminary sorbent phase-delivery experiments with inactive S9, k_1 and k_2 rate constants for the 4 test chemicals were measured. These values were then used in simulations to select eleven sampling time points that produced the smallest variance in $k_{dep,EVA}$ estimates for a range of possible $k_{dep,EVA}$ values. The remaining 9 incubation time points were selected at regular intervals at 0, 10, 20, 30, 40, 50, 60, 70, and 80 min for a total of 20 incubation times (n = 20) per sorbent-phase delivery experiment.

2.3.7. Incubations

All in vitro assays in the present study used a single S9 homogenate, pooled from 8 fish. Multiple (n=20) independent incubations were carried out to measure the

decline of the test chemical concentration for each chemical over time. A single S9 homogenate was used to remove confounding variables resulting from differences in enzymatic activities among S9 liver homogenates used for sorbent and solvent delivery based experiments. Each test chemical was incubated individually (not as mixture). Incubations were not subsampled. The incubations were done in a cold room maintained at 12.9-14.2 °C. In solvent delivery experiments, the test substrate dissolved in acetonitrile was transferred to a 2 mL amber glass vial (Agilent) containing 300 µL of incubation buffer (0.2 M, pH 7.4 phosphate buffer containing 1.15% (w/v) KCl and 1.5 mM β-NADPH). The reaction was started by adding 200 µL of pooled S9. In sorbent phase dosing experiments, 300 µL of incubation buffer (containing 1.5 mM β-NADPH) and 200 µL of pooled S9 were added to the EVA-coated vials to start the reaction. The vials were capped with polytetrafluoroethylene-lined screw caps (Agilent) and rolled horizontally at 60 rpm on a rocker/roller (Stuart SRT9D). Incubations where the test chemical in acetonitrile was added to the liver homogenate produced depletion rates that were not significantly different from those where the liver homogenate was added to the test chemical solution (Figure A1). In solvent delivery experiments, the incubation reactions were stopped by adding 200 µL of ice-cold methanol to the reaction vial, followed by shaking on a vortex mixer for 20 seconds. In sorbent phase dosing experiments, the incubation reactions were stopped by transferring 400 µL of the incubation medium to a 2 mL amber glass vial (Agilent) containing 200 µL ice-cold methanol and shaken on a vortex mixer for 20 seconds. The remaining incubation medium (100 µL) was removed from the EVA-coated vial. The EVA film was then rinsed 4 times with 1.0 mL of deionized water. A no-cofactor control experiment using inactive liver S9 (left at room temperature overnight and with no β-NADPH included in the incubation mixture) was conducted in parallel with each test system. Two EVA-coated vials with no added incubation medium were included to determine the presence of initial concentrations of the test substrate in the EVA thin films.

2.3.8. Chemical extraction

Following the termination of the incubation in solvent-delivery experiments, the internal standard (0.5 nmol chrysene-d^{12} dissolved in 10 µL of methanol) was added to the test vial and mixed using a vortex mixer for 30 seconds. Then 1.0 mL of n-hexane was transferred to the vial and shaken on a vortex mixer for 1 minute to extract the test

chemical and internal standard. The same procedure was carried out for the vials containing the incubation media from the sorbent-phase dosing experiments. For the EVA-coated test vials, 1.0 mL of n-hexane containing the internal standard (0.5 nmol chrysene-d^{12}) was transferred to the vial. Following the hexane extraction, the vials were centrifuged at 1,560 *g* for 10 min (IEC Centra-CL2; Thermo Scientific). The upper organic layer was transferred to a 2-mL amber glass vial (Agilent) for GC/MS analysis. In the sorbent dosing experiments, chemical concentrations were measured in both the EVA films and the incubation media.

2.3.9. GC/MS analysis

Test chemicals were analyzed using an Agilent 6890 GC coupled to an Agilent 5973 MS and an Agilent 7683 autosampler. The GC was fitted with a cool-on-column capillary inlet, and the injection volume was 1 µL. Chemicals were separated on an HP-5MS 5% phenyl methylpolysiloxane-coated column (30 m x 0.25 mm inner diameter, 0.25 µm film thickness) connected to a fused-silica deactivated guard column (5 m x 0.53 mm inner diameter). The oven was held at an initial temperature of 60 °C for 0.5 min, then increased at 25 °C min^{-1} to 200 °C (held for 0.5 min), followed by an increase at 20 °C min^{-1} to a final temperature of 300 (held for 4 min for 9-methylathracene, pyrene, and chrysene, 8 min for benzo[*a*]pyrene analysis). Helium was used as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. Conditions for MS measurements were as follows: electron impact ionization at 70 eV; ion source temperature at 230 °C; and selected ions at mass-to-charge ratios of 192 (9-methylanthracene), 202 (pyrene), 228 (chrysene), 240 (chrysene-d^{12}), and 252 (benzo[*a*]pyrene). Agilent MSD ChemStation software (G1701CA) was used for instrument control and data processing. An 8-point calibration curve (concentration range 0.005-5 µM) was constructed and run for each chemical. Strong linearity ($r^2 > 0.99$) was shown in the calibration curves, with constant relative response factor values obtained over the range. The limits of quantification for 9-methylanthracene, pyrene, chrysene and benzo[*a*]pyrene were approximately 0.02, 0.01, 0.01, and 0.03 µM, during the solvent delivery dosing experiments and 0.1, 0.5, 0.03, and 0.05 µM during the EVA sorbent phase dosing experiments, respectively.

2.3.10. Kinetic analyses

First order depletion rate constants (k_{dep}) were determined as the slope of the regression lines relating the natural logarithm of the remaining substrate concentration to incubation time according to Equation 2.2. Maximum depletion rate constant at infinitesimally low substrate concentrations ($k_{dep,C\rightarrow 0}$) and the apparent K_M were determined by fitting Equation 2.3 to k_{dep} estimates measured over a range of substrate concentrations. Because the k_{dep} estimated at low and high initial substrate concentrations do not carry the same precision, each k_{dep} estimate was weighted by the reciprocal of the standard error of the estimate. A weighted nonlinear least squares regression was used to get the best fit of the k_{dep} data to its corresponding starting substrate concentrations. This weighted nonlinear least squares analysis followed the Analytic Gauss-Newton algorithm in JMP 10.

In sorbent phase dosing experiments, the observed substrate concentrations in the EVA (C_E) and in the incubation media (C_I) for the inactive (control) and active S9 (test) incubations were plotted versus time. To estimate the mass-transfer rate constants (k_1, k_2) and the in vitro depletion rate constant ($k_{dep,EVA}$), the data were fitted by the model described in Equations 2.4 and 2.5 through non-linear least squares. Since the control and test for both the EVA and the incubation medium phases share the same parameters, all 4 data sources (i.e., EVA-control, EVA-test, media-control, media-test) were combined to obtain the least squares parameter estimates. This nonlinear least squares analysis was solved using MATLAB R2014a (Mathworks). To avoid sub-optimal parameter estimates caused by a potentially multimodal likelihood, multiple random starting points for the parameters were selected for initializing the least squares algorithm, with the best overall least squares minima returned. Time zero incubation media concentrations were fixed at 0 µM.

2.4. Results and Discussion

2.4.1. S9 liver preparation

The protein concentration of the pooled S9 from 8 fish livers was 8.8 (0.2 SE, n=3 measurements) mg mL^{-1}. Each incubation contained a final protein concentration of 3.5 mg mL^{-1}. Because the same S9 was used in all incubations, reported depletion rates

are expressed in units of min^{-1} and are not normalized for protein. For all test chemicals, no biotransformation was observed in the control incubations (containing inactive S9 and no added β-NADPH). Extraction efficiencies of the 4 test chemicals from the incubation mixture and from the EVA thin films were not significantly different from 100%. Therefore, chemical concentrations were not corrected for extraction efficiency.

2.4.2. Solvent delivery dosing experiments

Figure 2.1 illustrates substrate depletion over time for pyrene. Similar depletion curves were observed for the other test chemicals (Figure A2). The concentration of the test chemicals declined in a log-linear fashion with time for all test chemicals and at all initial concentrations except for incubations with initial pyrene concentrations greater than 2 μM. At concentrations in excess of 2 μM, the enzyme(s) involved in the biotransformation reaction(s) were presumed to be saturated, and biotransformation had a negligible and undetectable effect on substrate concentrations. At initial pyrene concentrations less than 2 μM, the apparent first order substrate depletion rate, k_{dep}, increased with decreasing initial pyrene concentrations. A similar inverse relationship between the apparent first order substrate depletion rate constant and the initial substrate concentration, consistent with Michaelis-Menten reaction kinetics, was observed for the other test chemicals.

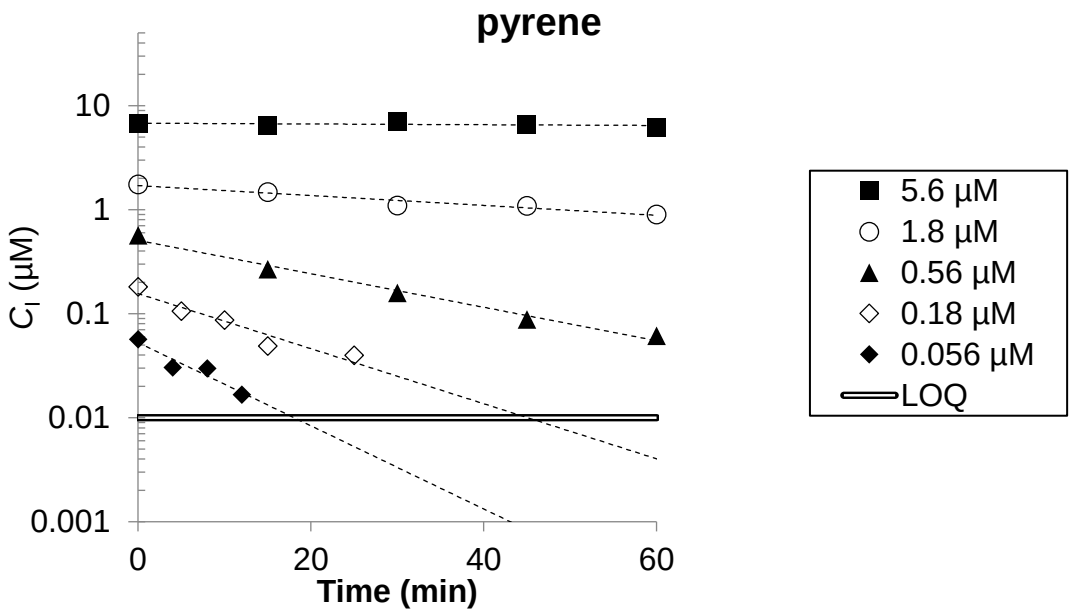
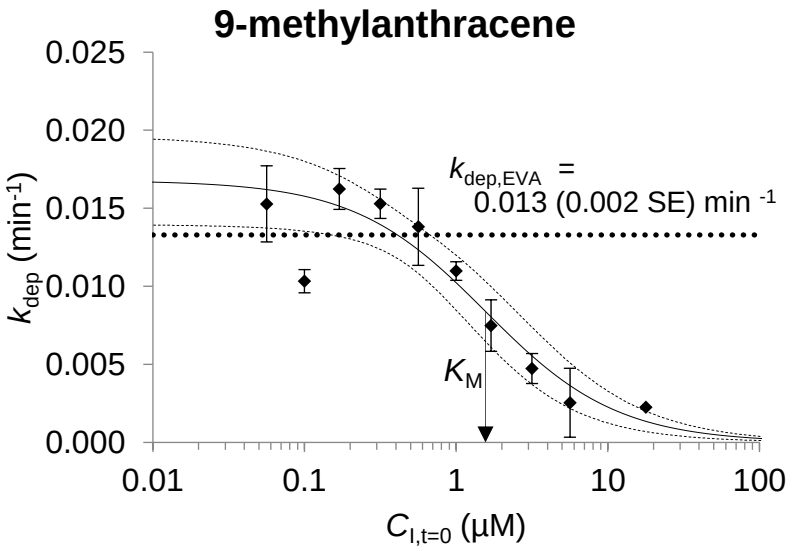
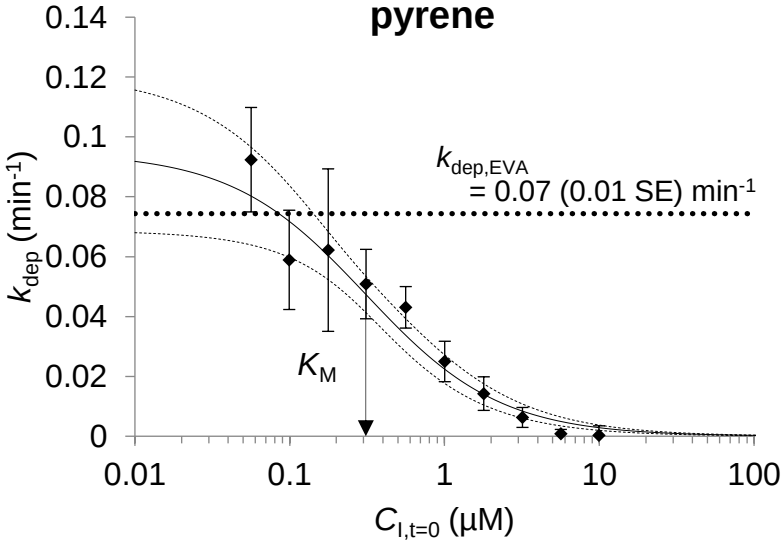


Figure 2.1 **Decline of the pyrene concentration in the incubation medium for different initial substrate concentrations in the incubation medium (C_l) in solvent-delivery dosing experiments. The limit of quantification is illustrated by the double solid line.**


9-methylanthracene
0.025
0.020
0.015
0.010
0.005
0.000
k_{dep} (min^{-1})
$k_{dep,EVA}$ =
0.013 (0.002 SE) min $^{-1}$
K_M
0.01
0.1
1
10
100
$C_{I,t=0}$ (µM)


pyrene
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
k_{dep} (min^{-1})
$k_{dep,EVA}$
= 0.07 (0.01 SE) min^{-1}
K_M
0.01
0.1
1
10
100
$C_{I,t=0}$ (µM)

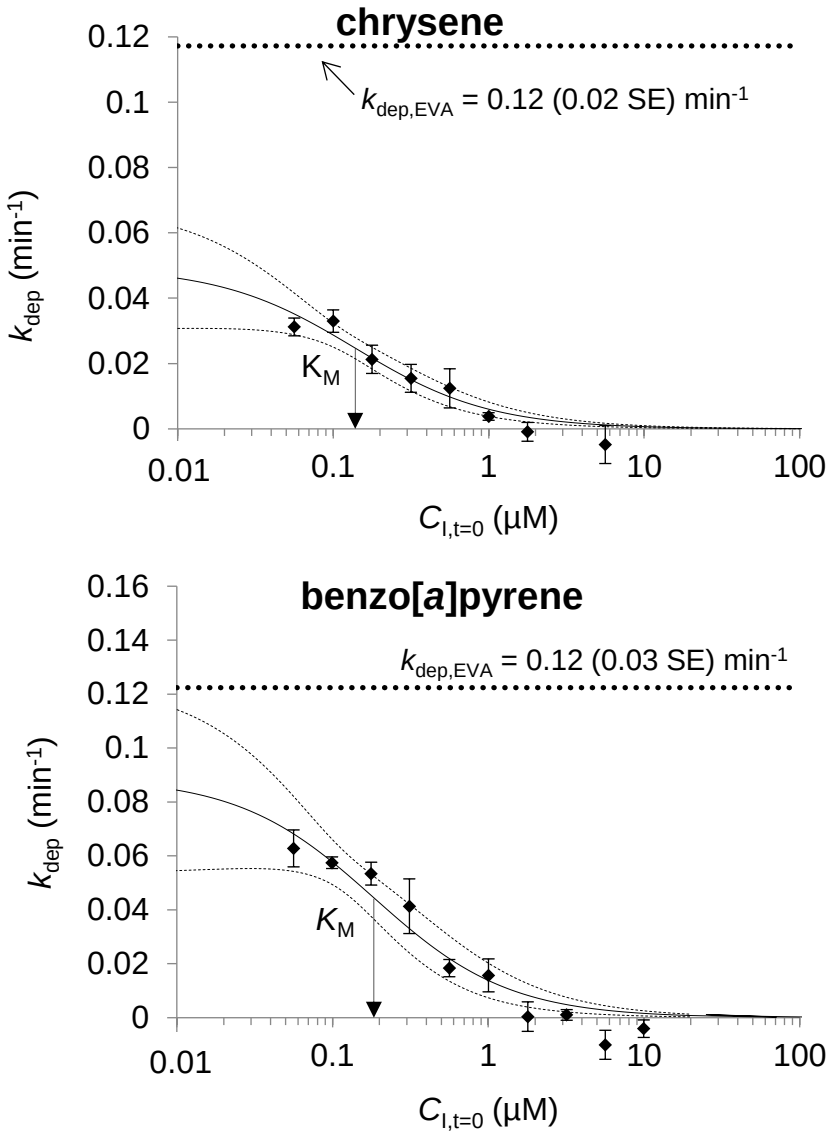


Figure 2.2 **Depletion rate constants (k_{dep}) versus the initial substrate concentrations ($C_{l,t=0}$) in solvent-delivery dosing experiments for 9-methylanthracene, pyrene, chrysene and benzo[*a*]pyrene. Error bars represent the standard errors of the mean. Dashed lines represent the 95% confidence interval of the mean k_{dep} estimate. The arrow presents the apparent Michaelis constant (K_M). The dotted line represents the depletion rate constant estimated from the EVA sorbent-phase delivery dosing method results ($k_{dep,EVA}$).**

Figure 2.2 shows the relationship between k_{dep} and $C_{l,t=0}$, and the good fit of the Michaelis-Menten kinetic model as formulated in Equation 2.3 to the biotransformation rate data. The apparent K_M and the maximum depletion rate constant ($k_{dep,C\rightarrow 0}$) were estimated through non-linear regression of the data to the model. The $k_{dep,C\rightarrow 0}$ values for 9-methylanthracene, pyrene, chrysene, and benzo[*a*]pyrene were estimated at 0.017 (0.001 SE) min^{-1}, 0.09 (0.01 SE) min^{-1}, 0.049 (0.008 SE) min^{-1} and 0.09 (0.02 SE) min^{-1}, respectively. The apparent K_M values were 1.6 (0.4 SE) µM for 9-methylanthracene, 0.31 (0.08 SE) µM for pyrene, 0.14 (0.05 SE) µM for chrysene, 0.18 (0.08 SE) µM for benzo[*a*]pyrene (Table 2.1). Despite a reasonable fit of the depletion rate constants for chrysene to the Michaelis-Menten model (Figure 2.2), $k_{dep,C->0}$ estimates for chrysene should be interpreted with caution as measurements of the depletion rate constants at the lowest concentrations could be not be completed because concentrations were below the detection limit.

Table 2.1 Comparison of the depletion rate constants (min^{-1}) of 4 hydrophobic test chemicals at 1 µM substrate concentration (k_{dep}), the maximum depletion rate constants ($k_{dep,C\rightarrow 0}$), the apparent Michaelis constants (K_M), and the depletion rate constants measured in thin EVA film sorbent phase delivery dosing experiments ($k_{dep,EVA}$). Error is represented by the standard error (SE) of the mean. Water solubility estimates were obtained from EpiSuite v.4.11

Substrate	$k_{dep,1\ uM}$ (min^{-1})	$k_{dep,C\rightarrow 0}$ (min^{-1})	K_M (µM)	$k_{dep,EVA}$ (min^{-1})	Log K_{OW}	Water solubility @ 25 ˚C (mg/L)
9-Methylanthracene	0.011 (SE 0.002)	0.017 (SE 0.001)	1.6 (SE 0.4)	0.013 (SE 0.002)	5.07	0.261
Pyrene	0.021 (SE 0.007)	0.09 (SE 0.01)	0.31 (SE 0.08)	0.07 (SE 0.01)	5.18	0.135
Chrysene	0.004 (SE 0.001)	0.049 (SE 0.008)	0.14 (SE 0.05)	0.12 (SE 0.02)	5.81	0.00345
Benzo[*a*]pyrene	0.016 (SE 0.006)	0.09 (SE 0.02)	0.18 (SE 0.08)	0.12 (SE 0.03)	6.13	0.00162

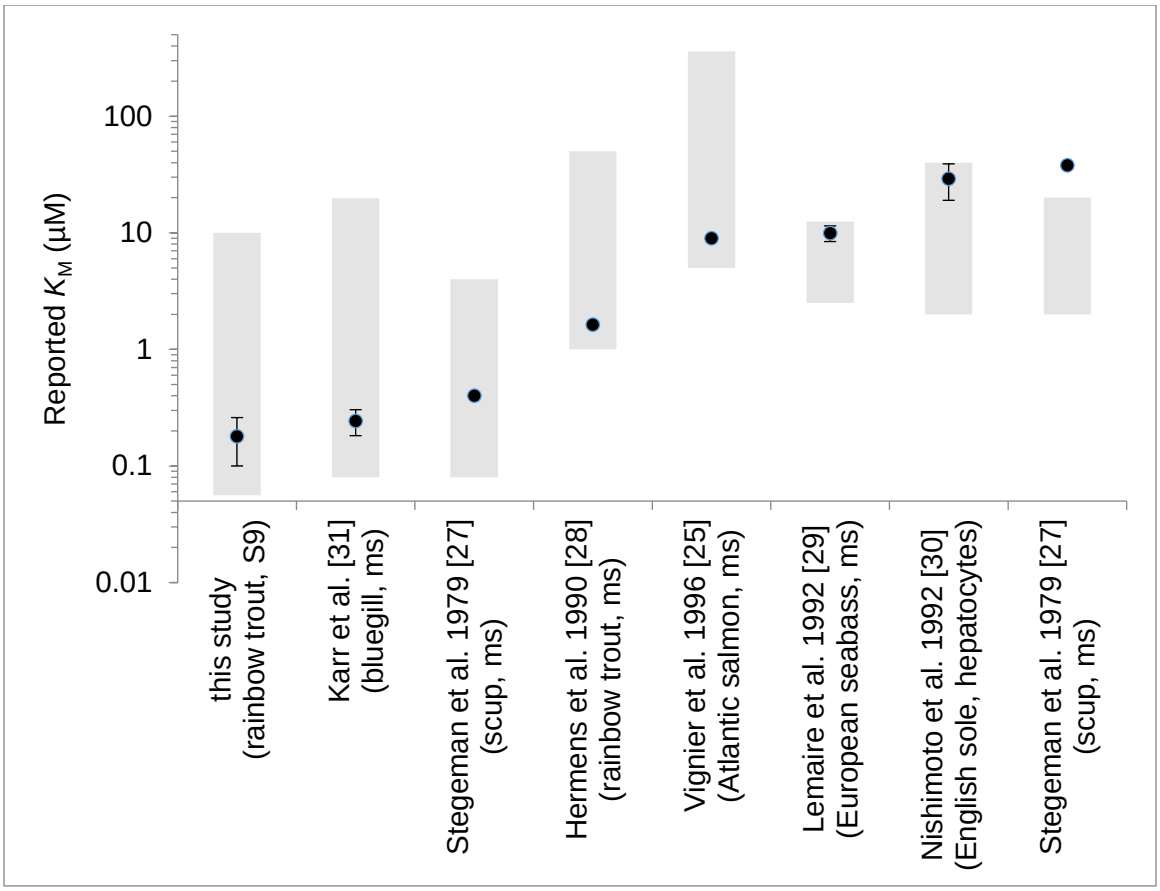


Figure 2.3 A comparison of the concentration range (shaded box) and apparent K_M estimates of benzo[*a*]pyrene (circles) in various fish liver preparations. Error bars are standard errors of the mean. Results from the present study monitored the substrate depletion. All other studies measured the metabolite formation. Details of each study can be found in Table A1.

Figure 2.3 provides a comparison of the Michaelis constant (K_M) of benzo[*a*]pyrene observed here with values reported in other studies using fish liver. It shows that for a single chemical (benzo[*a*]pyrene), literature K_M values can vary by over 3 orders of magnitude. Fitzsimmons et al. [**24**] made a similar observation. The large differences in reported K_M values can be due to differences in the biotransformation study design (i.e., product formation *vs*. substrate depletion), fish species, life stage [**25**], thermal acclimation [**26**], the type of in vitro liver preparation (i.e., S9, microsomes, isolated hepatocytes), and the range of substrate concentrations tested. Interestingly, in the 3 studies (including the present study) that have investigated benzo[*a*]pyrene biotransformation at concentrations less than 1 µM, the apparent K_M measurements vary only 2.2-fold, despite the different fish species and liver preparations used. The other reports examined benzo[*a*]pyrene biotransformation at concentrations from 1 to 360 µM

and would be unable to detect a K_M value of 0.2 µM. The study by Stegeman et al. [**27**] is notable because these authors observed an apparent K_M = 0.4 µM when a range of low benzo[*a*]pyrene concentrations were examined, and a K_M = 38 µM when incubations were done using a higher range of benzo[*a*]pyrene concentrations. Due to the many factors that may affect concentration dependence of the in vitro biotransformation rate, it is important to be cautious when relying on literature K_M measurements as a benchmark for selecting substrate concentrations for biotransformation assays. Conducting multiple depletion experiments using a range of initial substrate concentrations may be the most appropriate approach to obtain meaningful in vitro biotransformation rates for in vitro-to-in vivo extrapolation.

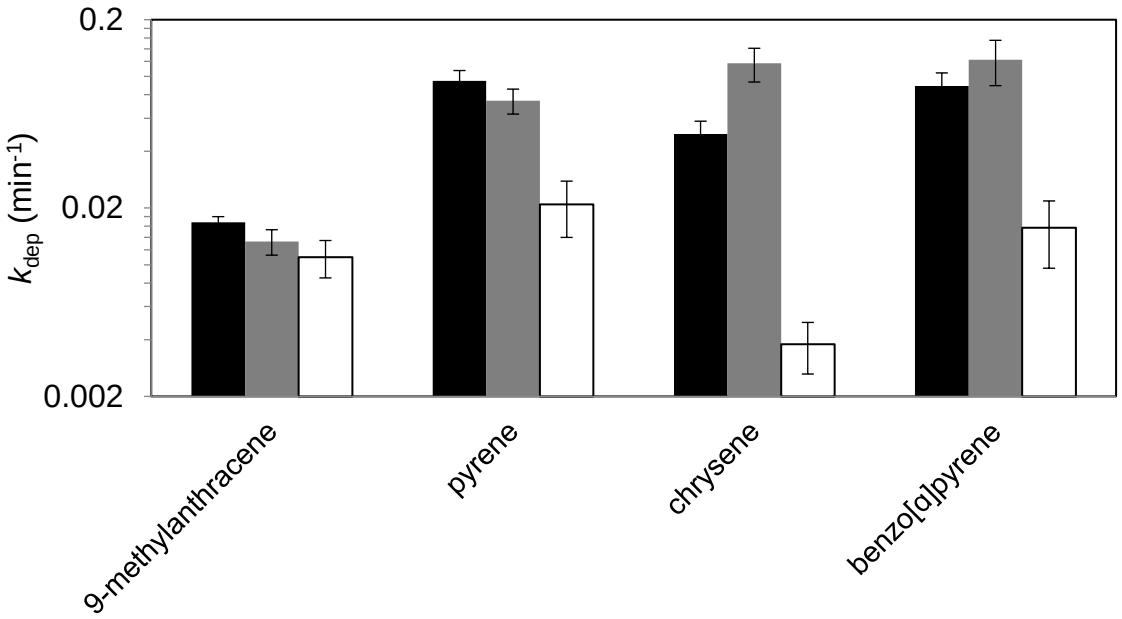
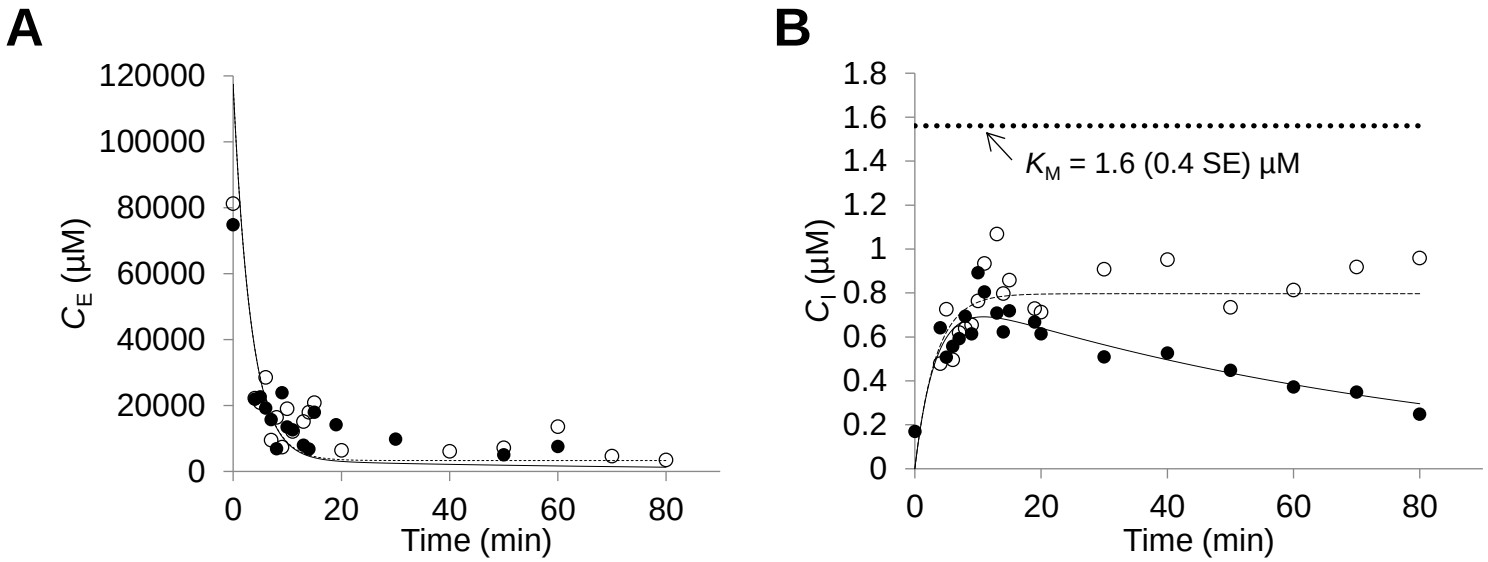
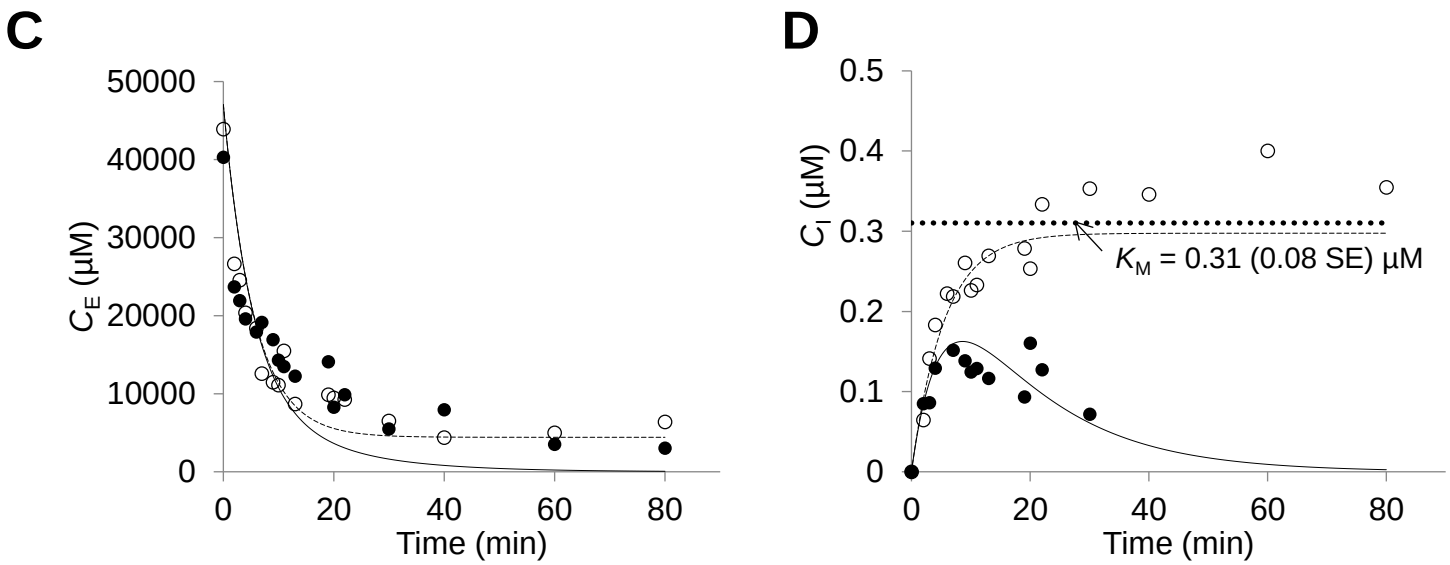


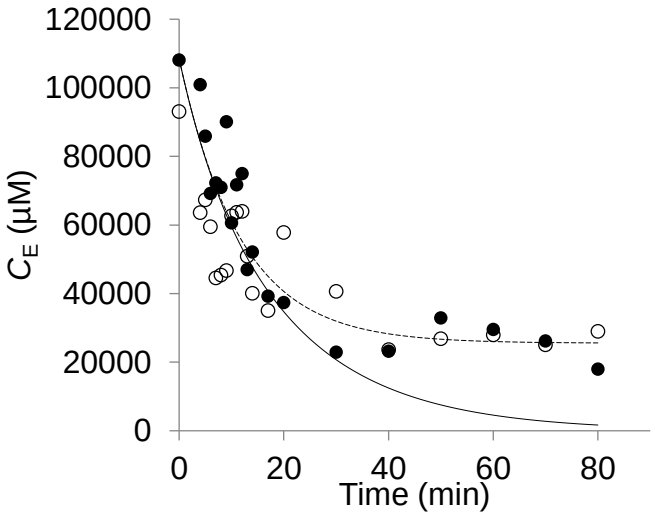
Figure 2.4 Comparison of the maximum depletion rate constant ($k_{dep,C\rightarrow0}$) estimated from the series of solvent delivery dosing experiments (black), the depletion rate constant (kdep,EVA) estimated from the EVA dosing experiments (grey), and the depletion rate constant (kdep) estimated from a solvent delivery experiment at the initial substrate concentration of 1 µM (white). Error bars are standard errors of the mean.

Figure 2.4 shows that maximum, first order depletion rate constants of pyrene, chrysene and benzo[*a*]pyrene ($k_{dep,C\rightarrow0}$) were 4- to 12-times higher than k_{dep} measured at 1 µM initial substrate concentrations (values given in Table 2.1). An initial concentration of 1 µM or less has been suggested as a possible test concentration for measuring first order depletion rate constants [**12**]. Only for 9-methylanthracene, k_{dep} determined at a 1 µM initial concentration (0.017 min^{-1}) was not significantly different from $k_{dep,C\rightarrow0}$ (0.011 min^{-1}). This is due to the relatively high K_M of 9-methylanthracene (1.6 [0.4 SE] µM) which exceeds 1 µM. Figure 2.4 suggests that k_{dep} of these substrates measured at initial concentrations of 1 µM can substantially underestimate the chemical's in vitro biotransformation rate, which in turn can cause hepatic clearance rates to be underestimated and bioconcentration factors to be overestimated in in vitro-to-in vivo extrapolations.

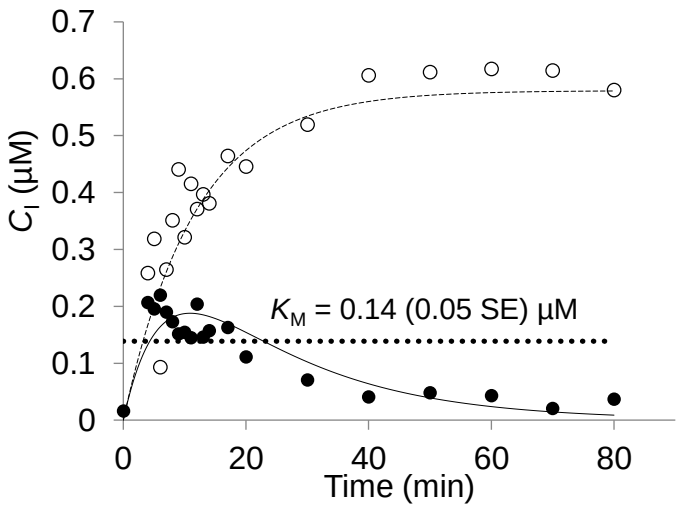

A
120000
100000
80000
60000
40000
20000
0
C_E (μM)
0
20
40
60
80
Time (min)
B
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
C_I (μM)
K_M = 1.6 (0.4 SE) μM
0
20
40
60
80
Time (min)


C
50000
40000
30000
20000
10000
0
C_E (μM)
0
20
40
60
80
Time (min)
D
0.5
0.4
0.3
0.2
0.1
0
C_I (μM)
K_M = 0.31 (0.08 SE) μM
0
20
40
60
80
Time (min)

E


120000
100000
80000
60000
40000
20000
0
C_E (µM)
0
20
40
60
80
Time (min)

F


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
C_I (µM)
K_M = 0.14 (0.05 SE) µM
0
20
40
60
80
Time (min)

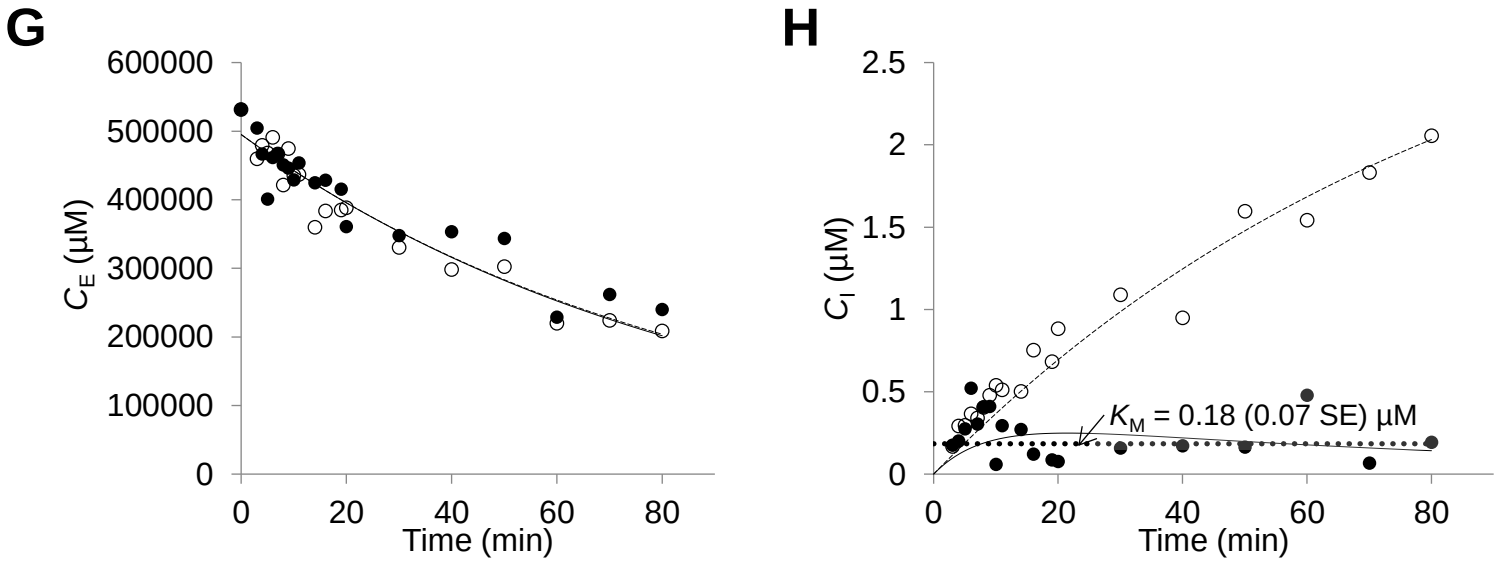


Figure 2.5 **Concentration-time profiles in the sorbent phase delivery dosing experiments for 9-methylanthracene (A,B), pyrene (C,D), chrysene (E,F), and benzo[*a*]pyrene (G,H) in both the EVA sorbent phase (C_E; A, C, E, G) and the incubation medium (C_I; B, D, F, G). Solid circles and solid lines represent the active trout liver S9 data and nonlinear regression estimates. Open circles and dashed lines represent the inactive S9 data and nonlinear regression estimates. The dotted line represents the apparent Michaelis constant (K_M) estimated from the series of solvent delivery dosing experiments.**

Figure 2.5 illustrates the measured concentrations of the test chemicals in EVA thin films and in the incubation medium. Figures 2.5A, 2.5C, 2.5E and 2.5G show that concentrations of the test chemicals in the EVA declined over time as the chemicals were transferred to the incubation medium. There were no statistically significant differences between the EVA depletion curves with active and inactivated liver S9. This behavior has been observed before and is due to biotransformation rates exceeding the sorbent phase delivery rates [**17**]. Figures 2.5B, 2.5D, 2.5F and 2.5H show that concentrations of the chemicals in the incubation medium initially increased with time (as test chemicals were transferred from the EVA into the incubation medium), then reached a maximum (as delivery rates matched biotransformation rates) and finally declined over time in active S9 (but not in inactive S9) as biotransformation rates exceeded thin-film delivery rates. For all test chemicals, there were distinct differences in the concentration-time curves for the active and inactivated S9 incubation media, indicating clear evidence of biotransformation. Figures 2.5B and 2.5D show that pyrene and 9-methylanthracene concentrations in the active S9 liver media were well below the apparent K_M measured from the multiple depletion curve experiments. The sorbent dosing derived depletion rate constants ($k_{dep,EVA}$) of 0.07 (0.01 SE) min^{-1} for pyrene and 0.013 (0.002 SE) min^{-1} for 9-methylanthracene from the EVA-dosing experiments are not statistically different (Student's t-test) from the maximum depletion rate constants ($k_{dep,C->0}$) of 0.09 (0.01 SE) and 0.017 (0.001 SE) min^{-1} discussed earlier (Table 2.1). Figures 2.5F and 2.5H show that concentrations of chrysene and benzo[*a*]pyrene in the active S9 liver media were at or below the apparent K_M value throughout most, but not all, of the incubation period. The benzo[*a*]pyrene depletion rate constant estimated from the EVA-dosing experiment ($k_{dep,EVA}$) of 0.12 (0.03 SE) min^{-1} is also not statistically significantly different (Student's t-test) from the maximum depletion rate ($k_{dep,C->0}$) of 0.09 (0.02 SE) min^{-1}. The depletion rate constant of chrysene of 0.12 (0.07 SE) min^{-1} derived from the EVA-dosing experiment is greater and statistically significantly different from the maximum depletion rate constants ($k_{dep,C->0}$) of 0.049 (0.008 SE) min^{-1} (Figure 2.5). This, we expect, may be due to analytical detection limits for chrysene preventing the determination of the full relationship between k_{dep} and log $C_{l,t=0}$ at low substrate concentrations in the incubation medium, causing $k_{dep,C->0}$ of chrysene to be misidentified. The good agreement between $k_{dep,EVA}$ and $k_{dep,C\rightarrow 0}$ estimates from multiple depletion curves method for 3 of the 4 test chemicals indicates the potential of sorbent phase methods to approximate $k_{dep,C\rightarrow 0}$ without the need to conduct experiments at multiple concentrations and characterize K_M.

However, it should be stressed that sorbent phase dosing methods can produce incubation concentrations above K_M. However, it is advantageous that, in sorbent delivery based experiment, the initial concentration of the test chemical in the incubation medium is at its lowest possible value (i.e., near zero), rather than at its maximum concentration. Furthermore, chemicals that require bioaccumulation assessments (i.e., those having a log K_{OW} > 5) are very hydrophobic and are therefore released slowly into the incubation medium, hence producing low concentrations in the incubation medium. Measured in vitro biotransformation rates from sorbent phase dosing experiments can therefore in many cases be expected to be closer to the theoretical maximum depletion rate constant at an infinitesimally low-substrate concentration ($k_{dep,C\rightarrow 0}$) than comparable measurements in solvent delivery based dosing experiments. This low initial substrate concentration in sorbent delivery based dosing methods may explain that in previous studies in both rat [17] and fish [18] liver S9 preparations depletion rate constants measured in sorbent delivery based dosing methods were higher than those measured in solvent delivery based experiments.

Table 2.2 The thin film to incubation medium transfer rate constant (k_1, min^{-1}), the incubation medium to thin film transfer rate constant (k_2, min^{-1}), the initial chemical concentration in the EVA thin film ($C_{EVA,t=0}$, mM), and the fraction unbound in the incubation medium in the sorbent phase delivery based experiments.

Substrate	k_1 (min^{-1})	k_2 (min^{-1})	$C_{EVA,t=0}$ (mM)	Fraction unbound*
9-methylanthracene	0.29 (SE 0.03)	0.009 (SE 0.004)	117.5 (SE 3.5)	0.038
Pyrene	0.16 (SE 0.01)	0.017 (SE 0.005)	47.0 (SE 1.4)	0.014
Chrysene	0.065 (SE 0.004)	0.020 (SE 0.004)	108.5 (SE 2.9)	0.025
benzo[*a*]pyrene	0.0113 (SE 0.0007)	0.0003 (SE 0.0001)	495.1 (SE 5.5)	0.0009

*The fraction unbound was calculated as K_{EI}/K_{EW}, where K_{EI} is the partition coefficient between the EVA sorbent phase and the incubation medium and K_{EW} is the partition coefficient between EVA sorbent phase and water. K_{EI} was estimated by $k_2.V_I/k1.V_{EVA}$, where V_I = 0.5 mL and V_{EVA} =3.5 . 10^{-6} mL. K_{EW} was calculated from K_{OW} using log K_{EW} = 1.04 (log K_{OW}) $\pm$ 0.22 in George et al. 2011.

The results of the present study indicate that conducting multiple solvent delivery based experiments within an appropriate concentration range provides the most accurate method for determining biotransformation potential as the experiments reveal the full Michaelis-Menten relationship between substrate concentration and biotransformation rate. The sorbent phase delivery based dosing method appears to provide a reasonable alternative to the multiple solvent delivery based experiments as it

provided good estimates of the maximum first order depletion rate constant for the test chemicals in the present study. The main advantage of the EVA-dosing method is that it does not require multiple dosing experiments and hence may be less costly and less time consuming and require fewer animals. In addition, the sorbent phase delivery based dosing method provides an estimate of the fraction unbound (a valuable extrapolation parameter), requires no solvents, and prevents incomplete dissolution of the test chemical in the incubation medium. However, in contrast to the multiple solvent delivery method where $k_{dep,C\rightarrow 0}$ is determined, the sorbent phase delivery based dosing method does not determine K_M and cannot reveal the full relationship between the in vitro biotransformation rate and the substrate concentration. The relationship between chemical concentration and biotransformation rate likely plays an important role in the in vitro-to-in vivo extrapolation of biotransformation rates. Models for extrapolation of in-vitro to in-vivo biotransformation rates may benefit from incorporating the concentration dependence of the biotransformation rate.

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Chapter 3.

Somatic and Gastro-intestinal In vivo Biotransformation Rates of Hydrophobic Chemicals in Fish*

Justin C. Lo designed the experiments, conducted the feeding experiments, performed chemical analysis with assistance from Kexin Catherine Rong (SFU Environmental Science Undergraduate), performed data analysis and wrote the chapter with assistance from Frank A.P.C. Gobas.

3.1. Summary

To improve current bioaccumulation assessment methods, a methodology is developed, applied and investigated for measuring in vivo biotransformation rates of hydrophobic organic substances in the body (soma) and gastro-intestinal tract of the fish. The method resembles the bioaccumulation OECD 305 dietary test but includes reference chemicals to determine both somatic and gastro-intestinal biotransformation rates of test chemicals. Somatic biotransformation rate constants for the test chemicals ranged between 0 and 0.38 (SE 0.03) d^{-1} Gastro-intestinal biotransformation rate constants varied from 0 to 46 (SE 7) d^{-1}. Gastro-intestinal biotransformation contributed more to the overall biotransformation in fish than somatic biotransformation for all test substances but one. Results suggest that biomagnification tests can reveal the full extent of biotransformation in fish. The common presumption that the liver is the main site of biotransformation may not apply to many substances exposed through the diet. The results suggest that the application of quantitative-structure-activity-relationships (QSARs) for somatic biotransformation rates and hepatic in vitro models to assess the effect of biotransformation on bioaccumulation can underestimate biotransformation rates and overestimate the biomagnification potential of chemicals that are biotransformed in the gastro-intestinal tract. With some modifications, the OECD 305 test can generate somatic and gastro-intestinal biotransformation data for the development of biotransformation QSARs and the testing of in vitro-in vivo biotransformation extrapolation methods.

It should be noted that the gastro-intestinal biotransformation rate constant discussed in this research applies to the biotransformation rate constant occurring in the intestinal contents or digesta within the lumen of the gastro-intestinal tract. The fish's body (or soma) discussed in this research, on the other hand, includes all parts of the fish but not the contents of the intestinal tract.

3.2. Introduction

Bioaccumulation is the process that involves the absorption, internal distribution, biotransformation and excretion of chemical substances. Bioaccumulation can lead to high chemical concentrations in organisms that may make organisms more susceptible to toxic effects. Quantitative estimates of the degree of bioaccumulation in biota can be helpful in identifying substances that are bioaccumulative in nature and in estimating internal concentrations in organisms and associated risks. Currently, regulatory programs such as the Canadian Environmental Protection Act (CEPA), the US Toxic Substances Control Act (TSCA), and the European Union (EU) Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) include the assessment of the bioaccumulative behavior of commercial chemicals using laboratory derived bioconcentration factors (BCFs; $C_{Organism}/C_{Water}$), the octanol-water partition coefficients (K_{OW}; $C_{Octanol}/C_{Water}$), and, in Canada, field-derived bioaccumulation factors (BAFs; $C_{Organism}/C_{Water}$) [1]. Recently, the EU included provisions for considering other bioaccumulation metrics such as the biomagnification factor (BMF) and trophic magnification factor (TMF) to increase the weight of evidence in bioaccumulation assessments [2,3]. However, regulatory programs often largely rely on BCF determinations. One of the challenges of this approach is that empirical BCFs and BAFs are typically available for only a small fraction of the many commercial chemicals that require assessment [4]. As a result, the octanol-water partition coefficient is often used to evaluate a chemical's bioaccumulation potential. However, the octanol-water partition coefficient represents a chemical partitioning process between a lipid surrogate (i.e., 1-octanol) and water, which oversimplifies the bioaccumulation process of many chemicals, including those with a very high octanol-water partition coefficient (e.g. $K_{OW} > 10^5$) and those that are biotransformed. Mechanistic bioaccumulation models, which can represent details of uptake and excretion of chemicals, have proven to be useful alternatives to the K_{OW} for the bioaccumulation assessment of many commercial

chemicals in fish and other organisms. However, the inability of bioaccumulation models to a-priori estimate biotransformation rates of absorbed chemicals has remained a key challenge in conducting realistic bioaccumulation assessments. Especially if the rate of chemical excretion is very slow (e.g. for high K_{OW}, potentially bioaccumulative substances), biotransformation can be an important elimination process. Absence of data on biotransformation rates may cause many hydrophobic chemicals to be evaluated as bioaccumulative when they are not, hence resulting in false positives and unnecessary prioritization in chemical management programs.

To develop methods for including biotransformation in bioaccumulation assessment, Quantitative Structure-Activity Relationship (QSAR) models have been developed to predict biotransformation rates and corresponding BCFs of hydrophobic organic chemicals in fish based on chemical structure [5]. These biotransformation rate models can be useful for screening-level assessments and have been incorporated into regulatory software programs such as the U.S. EPA's Estimation Programs Interface Suite [6]. The development of QSARs requires good quality data. However, few data on in vivo biotransformation rates of chemicals in fish and other organisms exist to date and there are no established methods for making direct measurements of in vivo biotransformation rates in fish.

Another initiative aimed at including biotransformation in bioaccumulation assessment involves the development of in vitro biotransformation rate assays using fish hepatocytes and liver S9 and microsomal fractions [7,8]. The success of this initiative depends on the ability to extrapolate in vitro biotransformation rates to in vivo biotransformation rates. The lack of reliable in vivo biotransformation rate data or methods to measure in vivo biotransformation rates provides a barrier for the successful validation and eventual implementation of in vitro bioassays for measuring biotransformation rates.

The lack of reliable methods for measuring in vivo biotransformation rates has precluded the development of a biotransformation rate data base for QSAR model development. Previously, biotransformation rates have been estimated from BCF data and bioaccumulation models [9,10]. In the present study, we propose and apply a new method that uses biotransformation resistant reference chemicals to measure in vivo biotransformation rates of hydrophobic organic chemicals that are useful in

bioaccumulation assessment. This method may be relatively easily included in the current OECD 305 test guideline [11] for measuring bioconcentration and biomagnification factors. The present study illustrates the application and underlying theory of the method. The aim of this method is to provide a methodology for generating high quality in vivo biotransformation rate data that can be used to further develop methods for bioaccumulation assessment in fish.

3.3. Theory

Bioaccumulation of contaminants in fish is often described by a fish-water-diet 3 compartment, 1st order kinetic model [12]:

$$dC_F/dt = k_1 C_W + k_D C_D - (k_2 + k_E + k_G + k_M) C_F \quad (3.1)$$

where C_F is the chemical concentration in the fish (g chemical.kg fish^{-1}); C_W is the chemical concentration in the water (g chemical.L^{-1}); k_1 is the uptake clearance rate for respiratory uptake (L water.kg fish^{-1}.d^{-1}); k_D (kg food.kg fish^{-1}.d^{-1}) is the rate constant for chemical uptake through the diet, C_D (g chemical.kg food^{-1}) is the concentration of the chemical in the diet, k_2 (d^{-1}) is the rate constant for respiratory elimination, k_E (d^{-1}) is the rate constant for elimination via fecal egestion, k_G (d^{-1}) is the rate constant for pseudo elimination via growth dilution, and k_M (d^{-1}) is the rate constant for biotransformation of the chemical in the fish and t is time (d) (Figure 3.1A). This model can represent chemical bioconcentration (i.e. $C_D = 0$) as represented in the OECD 305 test guideline [11], by the steady-state bioconcentration factor (BCF) (g water.g fish^{-1}):

$$BCF = C_F/C_W = k_1/(k_2 + k_E + k_G + k_M) = k_1/k_T \quad (3.2)$$

where k_T (d^{-1}) represents the sum of k_2, k_E, k_M and k_G. The model can also represent dietary bioaccumulation (i.e. C_W=0) in the form of the steady-state biomagnification factor (BMF) (g food.g fish^{-1}), as measured in dietary bioaccumulation tests such as the new OECD 305 test guideline [11].

$$BMF = C_F/C_D = k_D/(k_2 + k_E + k_G + k_M) = k_D/k_T \quad (3.3)$$

It is important to stress that in this modeling approach, the fish is viewed as a single compartment and includes the gastro-intestinal contents (Figure 3.1A). Standard

BCF assays are consistent with this modeling approach as measurements of C_F are usually determined by homogenizing the entire fish including the intestinal contents. The OECD 305 testing protocol [11] also states that the BMF is normally determined using test substance analysis of whole fish, even though the mass of chemical in the intestines can contribute considerably to the total mass of chemical in the fish, especially for substances that biotransformed rapidly in the body of the fish.

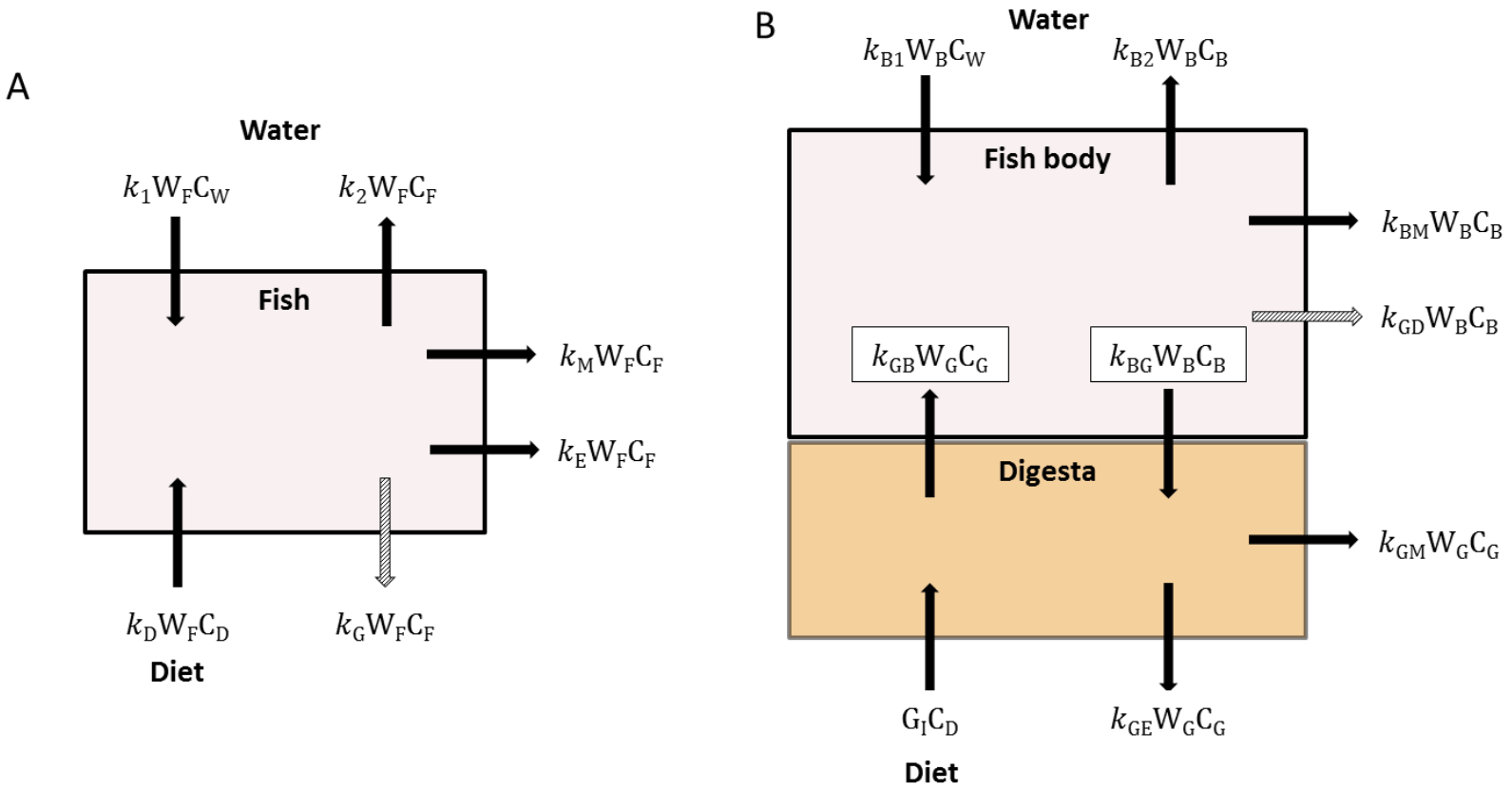

Figure 3.1 **Conceptual diagram of the transport and transformation kinetics of hydrophobic organic chemicals in a single compartment fish (Figure 3.1A) illustrating the role of biotransformation in the whole fish (k_M), and in a 2 compartment model separating the fish body from the contents of the digestive tract (Figure 3.1B) illustrating the role of somatic biotransformation (k_{BM}) and gastro-intestinal biotransformation (k_{GM}).**

To better represent biotransformation and in particular biotransformation in the gastro-intestinal tract, we can refine this model by distinguishing between the intestinal tract and the body (soma) of the fish as is shown in Figure 3.1B. This model can be implemented experimentally by removing the intestinal content from the fish before analysis. The intestinal tract is viewed as consisting of the gut lumen. The lumen contains the intestinal contents or digesta and includes the intestinal flora and gastric enzymes secreted by enterocytes. The fish's body includes all parts of the fish but not the contents of the intestinal tract. Chemical enters the lumen as a result of food ingestion and chemical transfer from the body of the fish into the intestinal content (including bile excretion). Chemical is removed from the lumen through chemical transfer into the body of the fish, fecal egestion and transformation in the intestines. Chemical in the body of the fish is the result of uptake from the intestinal lumen and from water via the gills and the skin. Chemical is removed from the body of the fish via chemical transfer from the fish into the intestinal content (including bile excretion), respiratory elimination via the gills and skin, biotransformation in the body of the fish (somatic biotransformation) and pseudo removed through growth dilution. Enterohepatic re-circulation of contaminants in this model is represented by the chemical exchange between the intestinal content and the fish (i.e., k_{BG} and $k_{GB)}$. The mass balance equations for the body of the fish (B) and the gastro-intestinal contents (G) are:

$$dM_B/dt = k^*_{B1}\ M_W + k_{GB}\, M_G - (k_{B2} + k_{BG} + k_{GD} + k_{BM})\, M_B \qquad (3.4)$$

$$dM_G/dt = G_I\, C_D + k_{BG}\, M_B - (k_{GB} + k_{GE} + k_{GM})\, M_G \qquad (3.5)$$

where M_B and M_G are the chemical masses (g) in the body of the fish and the digesta; G_I is the food ingestion rate (kg food.d^{-1}), C_D is the concentration of the chemical in ingested diet (g chemical.kg food^{-1}); k^*_{B1} $k_{B2,}$ k_{GB}, k_{BG}, k_{GD}, $k_{BM,}$ $k_{GE,}$ and k_{GM} are the rate constants (d^{-1}) for respiratory uptake, respiratory elimination, chemical transfer from the gastro-intestinal content to the fish body; chemical transfer from the fish body to the gastro-intestinal content, growth dilution, biotransformation of the chemical in the body of the fish, fecal egestion of the gastro-intestinal content, and biotransformation of the chemical in the gastro-intestinal content (Figure 3.1B), i.e. somatic biotransformation, respectively. The combined depuration rate constant from the fish's body ($k_{B2} + k_{BG} + k_{GD} + k_{BM}$) is k_{BT}.

Assuming a steady-state in the gastro-intestinal contents, i.e. $dM_G/dt = 0$, Equation 3.5 can be rewritten as

$$M_G = (G_I C_D + k_{BG} M_B) / (k_{GB} + k_{GE} + k_{GM}) \quad (3.6)$$

which after substitution in Equation 3.4 and recognizing that the chemical concentration in the body of the fish C_B (g.kg body weight^{-1}) is the ratio of M_B (g) and the fish's body weight W_B (kg), i.e. $C_B = M_B/W_B$ becomes

$$dC_B/dt = k_{B1} C_W + (k_{GB}/(k_{GB} + k_{GE} + k_{GM})) (G_I / W_B) C_D - (k_{B2} + k_{BG}.((k_{GE} + k_{GM})/(k_{GB} + k_{GE} + k_{GM})) + k_{GD} + k_{BM}) C_B \quad (3.7)$$

where k_{B1} is the uptake clearance rate for respiratory uptake (L water.kg bodyweight^{-1}.d^{-1}) and (G_I / W_B) is the proportional feeding rate of the fish expressed as a percentage of the fish's body weight per day. A comparison of Equations 3.1 and 3.7 shows a similarity in both expressions. However, Equation 3.7 applies to the body of the fish while Equation 3.1 applies to the body of the fish and the intestinal content. Hence, the rate constants k_1 and k_{B1} as well as k_2 and k_{B2} and k_M and k_{BM} are not the same. If the mass of the chemical in the body of the fish is large compared to that in the digesta, then k_{B1} approaches k_1, k_{B2} approaches k_2, k_{BM} approaches k_M and k_{BT} approaches k_T. However, if the mass of chemical in the fish body is comparable to or smaller than that in the digesta, e.g. due to rapid biotransformation of the chemical in the body of the fish, then $k_1 < k_{B1}$, $k_2 < k_{B2}$, $k_M < k_{BM}$ and $k_T < k_{BT}$.

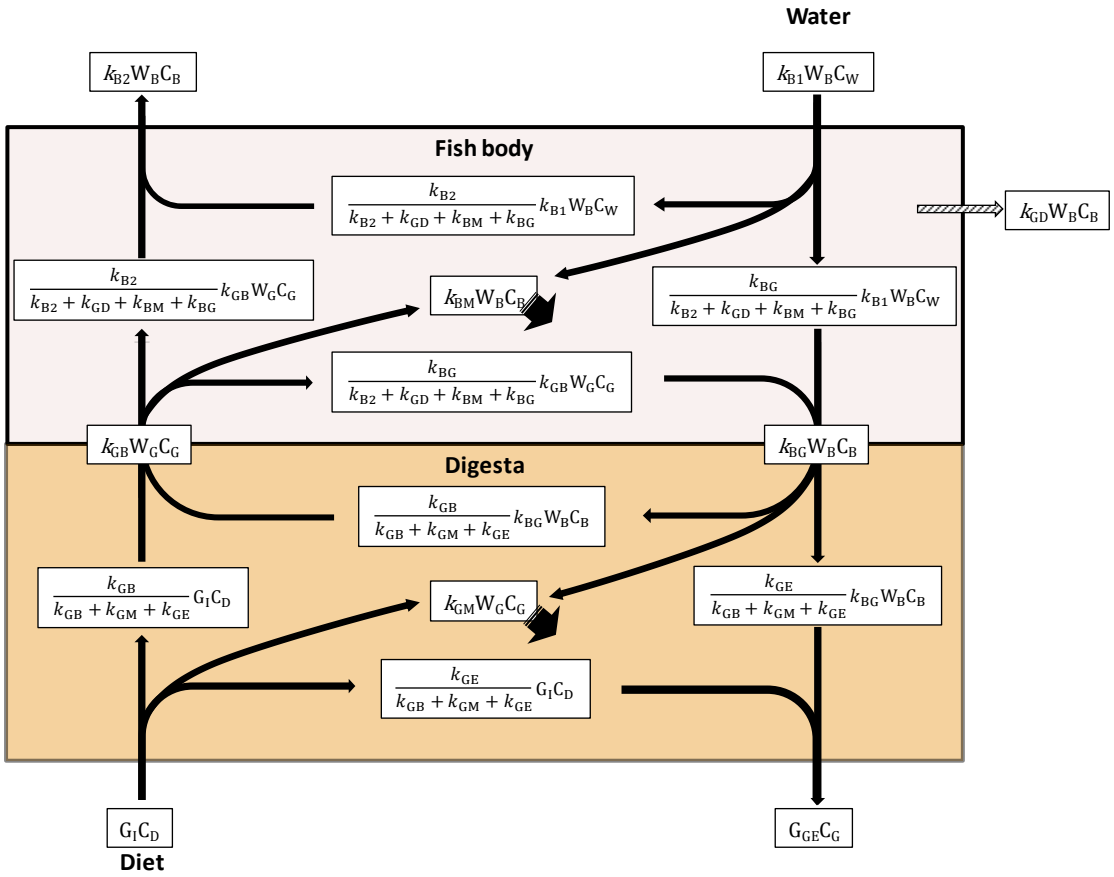

Figure 3.2 A more detailed conceptual diagram of the transport and transformation kinetics of hydrophobic organic chemicals in a 2 compartment model separating the fish body from the contents of the digestive tract illustrating the role biotransformation (represented by the ⬋ arrow) in the body (k_{BM}) and the gastro-intestinal tract (k_{GM}) of the fish.

Figure 3.2 illustrates that in this model the ingested chemical flux (in units of g chemical.d^{-1}), i.e. $G_I.C_D$, is fractionated in the intestinal tract in (i) the flux (g.d^{-1}) that is absorbed by the fish body, i.e. (k_{GB} / (k_{GB} + k_{GE} + k_{GM})).$G_I.C_D$; (ii) the flux (g.d^{-1}) that is egested from the intestinal tract, i.e. (k_{GE} / (k_{GB} + k_{GE} + k_{GM})).$G_I.C_D$; and (iii) the flux (g.d^{-1}) that is transformed in the intestinal tract, i.e. (k_{GM} / (k_{GB} + k_{GE} + k_{GM})).$G_I.C_D$. The chemical flux from the body of the fish to the intestines, i.e. $k_{BG}.W_B.C_B$, is also fractionated in the intestinal tract into (i) the flux (g.d^{-1}) that is recirculated back into the fish body, i.e. ($k_{BG}.k_{GB}$ / (k_{GB} + k_{GE} + k_{GM})).$W_B.C_B$; (ii) the flux (g.d^{-1}) that is egested from the intestinal tract, i.e. ($k_{BG}.k_{GE}$ / (k_{GB} + k_{GE} + k_{GM})).$W_B.C_B$; and (iii) the flux (g.d^{-1}) that is transformed in the intestinal tract, i.e. ($k_{BG}.k_{GM}$ / (k_{GB} + k_{GE} + k_{GM})).$W_B.C_B$. Figure 3.2 illustrates that intestinal biotransformation is made up of 2 contributions, i.e. chemical transformation

upon ingestion and chemical transformation after absorption and subsequent elimination from the body of the fish. Both contributions express the gastro-intestinal biotransformation rate constant k_{GM}. At the start of a dietary bioaccumulation study (t=0), there is only contribution, i.e., $(k_{GM} / (k_{GB} + k_{GE} + k_{GM})).G_I.C_D$ as $C_B=0$, providing an opportunity to determine k_{GM} from information typically collected in a dietary bioaccumulation study.

If the fish is viewed as the body of the fish, it is possible to redefine the dietary uptake rate constant as k_{BD} (kg food.kg bodyweight^{-1}.d^{-1})

$$k_{BD} = (k_{GB} / (k_{GB} + k_{GE} + k_{GM})) (G_I / W_B) \quad (3.8)$$

In Equation 3.8, the dietary uptake efficiency for a substance that is biotransformed in the gastro-intestinal tract ($E_{D,M}$) is

$$E_{D,M} = k_{GB} / (k_{GB} + k_{GE} + k_{GM}) \quad (3.9)$$

which equates k_{BD} to its more recognizable form of $E_{D,M}.G_I/W_B$. In Equation 3.9, k_{GB}, k_{GE} and k_{GM} represent the relative rates of chemical uptake from the intestines into the body of the fish, egestion in fecal matter and biotransformation in the lumen of the digestive tract. The dietary uptake efficiency for a dietary contaminant that is not biotransformed in the gastro-intestinal tract ($E_{D,N}$), i.e. $k_{GM} = 0$, is

$$E_{D,N} = k_{GB} / (k_{GB} + k_{GE}) \quad (3.10)$$

It has been shown that $E_{D,N}$ for non-biotransforming chemicals follows a non-linear relationship with K_{OW}, which can be used to estimate $E_{D,N}$ from K_{OW} for neutral hydrophobic chemicals, i.e.:

$$E_{D,N}^{-1} = \alpha K_{OW} + \beta \quad (3.11)$$

where α and β are coefficients that can be determined via regression of empirical $E_{D,N}$ observations [13]. If the fish is viewed as the body of the fish, it is also possible to redefine the fecal egestion rate constant k_E in Equation 3.1 in terms of the fecal egestion rate constant from the fish body (k_{BE} in d^{-1}) as

$$k_{BE} = k_{BG}\,((k_{GE} + k_{GM}) / (k_{GB} + k_{GE} + k_{GM})) = k_{BG}\,(k_{GE} / (k_{GB} + k_{GE} + k_{GM})) + k_{BG}\,(k_{GM} / (k_{GB} + k_{GE} + k_{GM})) \quad (3.12)$$

where $k_{GE} / (k_{GB} + k_{GE} + k_{GM})$ is the fraction of the ingested chemical that is egested from the intestinal tract in fecal matter and $k_{BG}\,(k_{GE} / (k_{GB} + k_{GE} + k_{GM}))$ is the fraction of the fish absorbed chemical that is eliminated from the fish body untransformed (i.e. as parent chemical) in fecal matter; and $k_{GM} / (k_{GB} + k_{GE} + k_{GM})$ is the fraction of the ingested chemical that is biotransformed in the intestinal tract and $k_{BG}\,(k_{GM} / (k_{GB} + k_{GE} + k_{GM}))$ is the fraction of the chemical mass eliminated by the fish that is biotransformed in the intestinal tract.

The model illustrates that biotransformation rates in the body of the fish (i.e. somatic biotransformation) and in the gastro-intestinal tract (i.e. gastro-intestinal biotransformation) are represented in bioaccumulation metrics in distinctly different fashions. Somatic (including hepatic) biotransformation is represented as k_{BM} in the combined depuration rate constant ($k_{B2} + k_{BG} + k_{GD} + k_{BM}$) or k_{BT}. The k_{BT} can be estimated from the concentrations in the body of the fish (C_B) during the depuration phase of a bioconcentration or biomagnification test in the same fashion as k_T is derived from C_F through log linear regression. Gastro-intestinal biotransformation is reflected in the dietary uptake rate constant (k_{BD}) or the dietary uptake efficiency ($E_{D,M}$). $E_{D,M}$ can be determined from the initial (t=0) increase in chemical concentration in the fish over time when there is no chemical in the fish.

Equation 3.1 shows that k_M can be determined from the total depuration rate constant k_{BT} as long as the elimination rate constants through non-metabolic pathways, i.e. ($k_2 + k_E + k_G$) or k_{SE} is known. One way to determine k_{SE} is to stop biotransformation of the chemical in the fish such that k_T equals $k_{SE.}$ The biotransformation rate constant k_{BM} can then be found in the experiment by subtracting k_{SE} from k_{BT} determined in the experiment where biotransformation is allowed to occur. This approach has been applied by Sijm et al. [14] and Myamoto et al. [15], who used inhibitors of the cytochrome P-450 system to stop or reduce the biotransformation of their test chemicals. The application of this method requires prior knowledge of the metabolic pathway of the test chemical; may not capture all applicable biotransformation pathways and can involve treatment of test animals that may interfere with animal well fare. Another approach, explored in the present study, is to determine k_{SE} by exposing test animals with non-biotransformable

reference chemicals together with a biotransformable test chemical. Given that elimination rates are known to be related to K_{OW}, it would be ideal to use a reference chemical of the same log K_{OW} values as the test chemical. However, such a reference chemical may be difficult to find. Alternatively, a range of non-biotransformable reference chemicals with varying K_{OW} can be used to develop an empirical relationship between k_{SE} and K_{OW} for non-biotransformable chemicals that can be used to derive the k_{SE} for test chemicals of varying K_{OW}. In theory, a range of numerical relationships (e.g. regression, polynomial equations) can be used to fit the relationship between k_{SE} and K_{OW} of the reference chemicals. However, we prefer to use a previously developed mechanistic model [16] to fit the empirical depuration rate constant data for the non-biotransformable reference chemicals to derive the k_{SE} and K_{OW}. This model may provide a better description of the functional relationship between k_{SE} and K_{OW} than regression models which are not based on the same mechanistic considerations. The fitted model allows k_{SE} of the test chemical to be derived from the K_{OW} of the test chemical and k_{BM} then follows from the measurement of the total depuration rate constant k_{BT} of the biotransformable test chemical as

$$k_{BM} = k_{BT} - k_{SE} \tag{3.13}$$

Equations 3.9 and 3.10 illustrate how the biotransformation rate constant in the gastro-intestinal tract (k_{GM}) can be determined from the dietary uptake efficiency of the test and the reference chemicals as:

$$k_{GM} = (E_{D,M}^{-1} - E_{D,N}^{-1})\, k_{GB} \tag{3.14}$$

where k_{GB} can be derived by rearranging Equation 3.14 and considering that k_{GE} is the ratio of the fecal egestion rate G_{GE} (kg digesta.d^{-1}) and the amount of digesta W_G (kg) in the gastro-intestinal tract, i.e. $k_{GE} = G_{GE}/W_{G,}$ as:

$$k_{GB} = (E_{D,N} / (1-E_{D,N}))\, k_{GE} = (E_{D,N} / (1-E_{D,N}))\, (G_{GE} / W_G) \tag{3.15}$$

G_{GE} can be determined experimentally from fecal collection measurements or by adding inabsorbable chromic oxide to the diet and measuring the increase in chromic oxide concentrations in the fecal matter over that in administered food that occurs as a result of food absorption by the fish [17,18]. For example, in previous work in rainbow trout (*O. mykiss*) in our laboratory [18], the ratio of chromic oxide concentrations in the

digesta (g.kg digesta^{-1}) and in the diet (g.kg food^{-1}) was measured as 2.1 (SE 0.4) (kg food dry weight.kg digesta dry weight^{-1}), indicating a G_{GE}/G_I ratio or γ_{GI} of 0.48 kg digesta dry weight.kg food dry weight^{-1} and a corresponding dietary assimilation efficiency ε_f (unitless) of approximately 52% on a dry weight basis. Also, G_{GE} can be estimated from the dietary ingestion rate, the composition of the diet and the assimilation efficiencies of the diet constituents [16] using values for the assimilation efficiencies of the various food constituents. Hence G_{GE} can be determined as

$$G_{GE} = \{(1-\varepsilon_L)\,\phi_{DL} + (1-\varepsilon_P)\,\phi_{DP} + (1-\varepsilon_N)\,\phi_{DN} + (1-\varepsilon_W)\,\phi_{DW}\}\,G_I = \gamma_{GI}\,G_I \qquad (3.16)$$

where ϕ_{DL}, $\phi_{DP,}$, ϕ_{DN} and ϕ_{DW} are the fractions of lipid (kg lipid.kg diet^{-1}), protein (kg protein.kg diet^{-1}), non-digestible organic matter (kg non-digestible organic matter.kg diet^{-1}) and water (kg water.kg diet^{-1}) of the fish's diet and ε_L, $\varepsilon_{P,}$ ε_N and ε_W are the dietary assimilation efficiencies of lipids, protein, non-digestible organic matters and water, and where γ_{GI} is the ratio of the fecal egestion and dietary ingestion rates. The amount of digesta W_G (kg digesta dry weight) in the intestinal tract can be estimated from the feeding rate and the instantaneous evacuation rate of digesta (i.e. through food absorption and fecal egestion) from the gastro-intestinal tract of the fish:

$$dW_G/dt = G_I - \delta\,W_G \qquad (3.17)$$

where δ is the digesta evacuation rate constant expressed as a fraction of digesta W_G that is evacuated (d^{-1}). Equation 3.17 is consistent with observations indicating that the decrease in gastro-intestinal content follows an exponential relationship with time, suggesting that the rate of emptying of the gastro-intestinal tract in units of g.d^{-1} is proportional to the amount of food in the intestinal tract [19]. A mean steady-state amount of digesta can then be estimated as a result of a constant feeding rate (G_I) and a constant digesta evacuation rate (δ) since if dW_G/dt is 0 (i.e. at steady-state), then Equation 3.17 shows that $W_G = G_I/\delta$, where G_I is known from experimental conditions and δ can be estimated from digestive tract emptying times. For example, 100% emptying times ($t_{E,100}$) have been compiled by Fänge and Grove [19] and may reasonably approximate 95% emptying times ($t_{E,95}$), which are related to δ as $3/t_{E,95}$. The rate constant for chemical excretion from the gastro-intestinal tract can then be derived as

$$k_{GE} = G_{GE} / W_G \quad (3.18)$$

Equations 3.14-3.18 provide a method to derive the gastro-intestinal biotransformation rate constant k_{GM} for a test chemical from the dietary uptake efficiencies of the test chemical and a non-biotransfomable reference chemical. For example, a 100 g fish, which is fed 1% of its body weight per day (i.e. 1 g food.d^{-1}) produces a fecal egestion rate of approximately 0.5 g digesta.d^{-1}. If the fish's 95% gastro-intestinal evacuation time ($t_{E,95}$) for a meal is 1.5-d, then δ is 3/1.5 or 2 d^{-1} and the steady-state amount of digesta W_G in the gastro-intestinal tract is 1 g food.d^{-1} /2 d^{-1} or 0.5 g. This means that k_{GE} or G_{GE}/W_G of 0.5 g digesta.d^{-1} / 0.5 g digesta = 1 d^{-1}. If, in our example, the $E_{D,N}$ for the non-biotransformable reference chemical is 0.50, then following Equation 3.15, $k_{GB} = k_{GE} = G_{GE}/W_G = 1$ d^{-1}. If $E_{D,M}$ for the test biotransformable chemical is for example 0.25, then k_{GM} can be determined as ($0.25^{-1} - 0.50^{-1}$).1 d^{-1} = 2 d^{-1}. It should be emphasized that k_{GM} applies to the mass of chemical in the gastro-intestinal tract (M_G), while k_{BM} applies to the chemical mass in the fish's body (M_B). To compare the relative importance of gastro-intestinal and somatic biotransformation, the rate constants need to be multiplied by the corresponding masses of the parent substance in the intestinal tract (M_G) and the fish's body (M_B). The mass of parent test chemical in the fish body can be determined at steady-state ($dM_B/dt = 0$) as:

$$M_B = C_B W_B = C_D W_B k_{BD} / k_{BT} \quad (3.19)$$

where C_D, W_B, k_{BD} and k_{BT} are all parameters determined in a dietary bioaccumulation study.

The mass of parent test chemical in the fish's intestinal tract can be determined from Equation 3.5 at steady-state ($dM_G/dt = 0$) as:

$$M_G = C_G W_G = (C_D G_I + k_{BG} M_B) / (k_{GB} + k_{GE} \ k_{GM}) \quad (3.20)$$

where the chemical concentration in the diet C_D and the feeding rate G_I are known from the experimental conditions; k_{GM}, k_{GE} and k_{GB} can be determined from Equations 3.14-3.18 and k_{BG}, i.e. the rate constant for chemical from the fish into the gastro-intestinal tract, can be estimated as

$$k_{BG} = K_{GB} k_{GB} W_G / W_B \quad (3.21)$$

where K_{GB} is the chemical partition coefficient between the gastro-intestinal content and the fish, which can be estimated from the composition of the digesta and the body of the fish as [16]:

$$K_{GB} = C_G / C_B = (\phi_{GL} K_{OW} + \phi_{GP} \chi K_{OW} + \phi_{GN} \theta K_{OW} + \phi_{GW}) d_G / (\phi_{BL} K_{OW} + \phi_{BP} \chi K_{OW} + \phi_{BN} \theta K_{OW} + \phi_{BW}) d_B \quad (3.22)$$

where ϕ_{GL}, ϕ_{GP}, ϕ_{GN} and ϕ_{GW} are the fractions of lipid (kg lipid.kg digesta^{-1}), protein (kg protein.kg digesta^{-1}), non-digestible organic matter (kg non-digestible organic matter.kg digesta^{-1}) and water (kg water.kg digesta^{-1}) in the gastro-intestinal contents of the fish; ϕ_{BL}, ϕ_{BP}, ϕ_{BN} and ϕ_{BW} are the fractions of lipid (kg lipid.kg fish body^{-1}), protein (kg protein.kg fish body^{-1}), non-digestible organic matter (kg non-digestible organic matter.kg fish body^{-1}) and water (kg water.kg fish body^{-1}) of the body of the fish; d_G and d_B are the densities of the digesta and body of the fish (kg.L^{-1}); χ and θ are proportionality constants comparing the absorptive capacity of proteins and non-digestible organic matter respectively to that of lipids (assumed to be equal to that of octanol) and can be assumed to be 0.05 following deBruyn and Gobas [20]. Whereas the lipid and protein contents of the fish body and fish food are often known or easily measurable, the composition of the digesta is usually not known and difficult to measure, but can be approximated from the dietary composition using estimates of the dietary assimilation efficiencies of lipids (ε_L), protein (ε_P), non-digestible organic matter (ε_N) and water (ε_W) following Arnot and Gobas [16]:

$$\phi_{GL} = (1-\varepsilon_L) \phi_{DL} / \{(1-\varepsilon_L) \phi_{DL} + (1-\varepsilon_P) \phi_{DP} + (1-\varepsilon_N) \phi_{DN} + (1-\varepsilon_W) \phi_{DW}\} \quad (3.23)$$

$$\phi_{GP} = (1-\varepsilon_P) \phi_{DP} / \{(1-\varepsilon_L) \phi_{DL} + (1-\varepsilon_P) \phi_{DP} + (1-\varepsilon_N) \phi_{DN} + (1-\varepsilon_W) \phi_{DW}\} \quad (3.24)$$

$$\phi_{GN} = (1-\varepsilon_N) \phi_{DN} / \{(1-\varepsilon_L) \phi_{DL} + (1-\varepsilon_P) \phi_{DP} + (1-\varepsilon_N) \phi_{DN} + (1-\varepsilon_W) \phi_{DW}\} \quad (3.25)$$

$$\phi_{GW} = (1-\varepsilon_W) \phi_{DW} / \{(1-\varepsilon_L) \phi_{DL} + (1-\varepsilon_P) \phi_{DP} + (1-\varepsilon_N) \phi_{DN} + (1-\varepsilon_W) \phi_{DW}\} \quad (3.26)$$

where ϕ_{DL}, $\phi_{DP,}$, ϕ_{DN} and ϕ_{DW} are the fractions of lipid (kg lipid.kg food^{-1}), protein (kg protein.kg food^{-1}), non-digestible organic matter (kg non-digestible organic matter.kg food^{-1}) and water (kg water.kg food^{-1}) of the fish's diet. The dietary lipid assimilation efficiency is approximately 92% in rainbow trout [18] and protein and water assimilation

efficiencies in fish are approximately 75% and 50%. The dietary assimilation efficiency of non-digestible organic matter can be assumed to be 0%.

There are several areas of uncertainty in the derivation of gastro-intestinal biotransformation rate constants from dietary absorption efficiencies. One of these originates from the practice of using dried fish foods in dietary bioaccumulation experiments and the lack of measuring the amount of water absorbed with the food. Fortunately, as demonstrated in detail in Appendix B, the absorption of water with the food by fish does not have a significant effect on the determination of intestinal biotransformation rates for very hydrophobic neutral organic substances with a very high K_{OW} ($\log K_{OW} > 5$). As a result, calculations based on a dry weight basis will produce estimates of biotransformation rates that are not significantly different from those conducted on a wet weight basis. The main reason for the insignificant role of water on the dietary uptake dynamics of very hydrophobic substances in fish is that water has a negligible capacity to solubilize very hydrophobic chemicals compared to lipids, proteins and other organic materials. As a result, the mass balance equations for the uptake of very hydrophobic chemicals in fish can be described on a wet or dry weight basis without introducing a significant error due to ignoring the chemical in aqueous parts of the diet and digesta in the dry weight based calculations. This is advantageous in dietary bioaccumulation experiments because often, as is the case in the present study, the chemical is administered in the form of dry food that is applied to water. Because the water content of the actual diet and digesta of the fish are in most cases not characterized in dietary bioaccumulation experiments, wet weight based calculations involving the feeding and fecal egestion rates are difficult to perform. Hence, we recommend that calculation of gastro-intestinal biotransformation rates for very hydrophobic organic chemicals are conducted on a dry weight basis.

Another area of uncertainty originates from the inherent assumption of the bioaccumulation model that food consumption is a continuous process. Observations by Fänge and Grove [19] suggest that this assumption may be reasonable for fish in controlled feeding experiments as the dynamics of intestinal evacuation in fish is consistent with the generation of a relatively constant amount of digesta. However, the assumption of continuity and the recognized effect of temperature, meal size, food type, fish size, method of feeding and feeding history on gastric evacuation times [19], contribute uncertainty in the characterization of the amount of digesta W_G in the

intestinal tract of the fish, which contributes uncertainty to the determination of k_{GE}, k_{GB} and k_{GM}. This uncertainty may further increase when extending the applicability of domain of the presented approach (i.e. dietary bioaccumulation experiments) to field applications where fish may not feed for extended periods of time. Fortunately, the uncertainty in W_G is to a large extent removed from the determination of the gastro-intestinal biotransformation rate (expressed in g chemical.d^{-1}), i.e. $k_{GM}.M_G$ or $k_{GM}.W_G.C_G$, because k_{GM} follows an inverse relationship with W_G, while the chemical mass in the digesta is proportional to W_G. Hence, errors in the determination of W_G have a tendency to cancel out when determining gastro-intestinal biotransformation rates.

The model description presented here illustrates that the contribution of gastro-intestinal and somatic in vivo biotransformation can be derived from measurements typically made in a dietary bioaccumulation study if non-biotransformable reference chemicals are added to the experimental protocol of the test and the chemical mass in the intestinal content of the fish is not included in the measurement of the chemical concentration in the fish. The contribution of somatic biotransformation as a proportion of the total mass of chemical biotransformed (Φ_M) in the fish can be estimated as:

$$\Phi_{BM} = k_{BM}\, M_B/(k_{BM}\, M_B + k_{GM}\, M_G) \qquad (3.27)$$

One of consequences of gastro-intestinal biotransformation is that it counteracts the gastro-intestinal magnification effect in the gastro-intestinal tract. Gastro-intestinal magnification is the increase of the chemical fugacity (or thermodynamic) activity in the intestinal content over that in the diet, occurring as a result of food-absorption and food digestion [18]. Gastro-intestinal magnification (which is defined as an increase in the chemical fugacity in the digesta over that in the diet [21]) is generally viewed as the underlying mechanism why hydrophobic organic chemicals biomagnify (defined as an increase in the chemical fugacity in the digesta over that in the diet) in fish [18]. As illustrated in the supporting information, it is possible to determine the magnitude of k^*_{GM} (d^{-1}) that prevents gastro-intestinal magnification and hence biomagnification in fish as:

$$k^*_{GM} = (G_I / W_G)\,(K_{DG} - \gamma_{GI}) \qquad (3.28)$$

where K_{DG} (kg digesta dry weight.kg food dry $weight^{-1}$) is the diet-digesta partition coefficient, which can be estimated from the composition of the diet as:

$$K_{DG} = C_D / C_G = (\phi_{DL}.K_{OW} + \phi_{DP}.\chi.K_{OW} + \phi_{DN}.\theta.K_{OW} + \phi_{DW}) / (\phi_{GL}.K_{OW} + \phi_{GP}.\chi.K_{OW} + \phi_{GN}.\theta.K_{OW} + \phi_{GW}) \quad (3.29)$$

As explained in more detail in the supporting information, substitution of Equation 3.28 into Equation 3.14 provides a threshold dietary uptake efficiency $E^{*}_{D,M}$ which if not exceeded, indicates that the chemical cannot be subject to gastro-intestinal magnification and hence is not expected to biomagnify in fish:

$$\frac{1}{E^{*}_{D,M}} = \frac{K_{DG}}{\gamma_{GI}} \times \frac{1-E_{D,N}}{E_{D,N}} + \frac{1}{E_{D,N}} \quad (3.30)$$

If substances exhibit a dietary uptake efficiency greater than $E^{*}_{D,M}$ then it is still possible that the substance cannot biomagnify as long as the somatic biotransformation rate is sufficiently high.

3.4. Materials and Methods

3.4.1. Fish

Rainbow trout (*Oncorhynchus mykiss*, approximately 30 g body weight.) were purchased from Miracle Springs Inc. Hatchery and Trout Farm and acclimatized for 4 weeks before initiation of the experiments. Fish were held in 4 (i.e. 3 test and 1 control) flow-through glass aquaria supplied with dechlorinated water. The water was oxygenated with air stones, resulting in dissolved oxygen concentrations in water that were 90% of saturation. The aquaria were housed in a cold room at Simon Fraser University with a 14-h light, 10-h dark schedule. Water temperatures were kept at 11.4-13.3 °C (mean temperature = 12.6 °C) throughout the acclimation period and the experiment with a thermostatted water chiller. Fish were fed commercial fish chow at a daily rate of 1.5% of the pre-experiment mean fish bodyweight. Upon administration, fish food (1.5 mm EWOS Pacific Complete Feed for Salmonids) contained 18.6% lipids, 46.6% protein, 32.4% of non-digestible organic materials and 2.4% water.

3.4.2. Chemicals

Fish were administered a control or a contaminated diet containing 15 chemicals at nominal concentrations listed in Table 3.1 along with the K_{OW} of the chemicals. The reference chemicals were selected because of their resistance to biotransformation in fish and microbial degradation and to represent a range in K_{OW}. While the reference chemicals are recognized for their persistence, some biotransformation of PCB 52 has been observed in fish [22]. To prepare the diet, test chemicals were dissolved in 3 mL of corn oil and 15 mL of toluene. This spiking solution was then added to 400 g of fish feed slowly while mechanically stirring the fish feed in an open system overnight. The spiked diet was stored at 2 °C in a sealed container. Fish feed for the control diet was prepared in a similar manner but without the test chemicals.

3.4.3. Dosing design

Fish in the exposure group (n=51) were housed in 3 40 gallon glass flow-through tanks and exposed to the test chemicals in the diet for 14-d, followed by a 114-d depuration period when fish were fed a non-contaminated control diet. Three fish were collected, 1 from each of the 3 exposure tanks, on days 0, 2, 5, 9, 14, 14.17, 14.5, 14.75, 15, 17, 19, 23, 31.4, 48.2, 86, and 128 and analyzed independently. Unexposed control fish (n=12, housed in a single 40 gallon glass flow-through aquarium) were fed a control diet throughout the entire 128-d experiment and shared the same dechlorinated freshwater source as the exposed fish to monitor for any potential chemical uptake from water. Three fish were collected on days 0, 5, 23, and 128 and analyzed independently to test for potential uptake of test and reference chemicals from the water due to chemical leaching from administered fish food and exposed fish to the water. Fish were sacrificed using an overdose of Finquel MS-222 (Argent Laboratories) and split into liver, carcass and intestines. Each compartment was analyzed individually.

Table 3.1 Concentrations of reference and test chemicals in the diet of the fish, the log K_{OW}, the combined depuration rate constant from the fish body k_{BT} (day^{-1}) with its standard error, and the dietary uptake efficiency E_D (unitless) and its standard error for the reference and test chemicals in this study.

Reference Chemicals	Nominal food concentrations ($mg.kg\ wet\ weight^{-1}$)	log K_{OW}	k_{BT} ± SE (day^{-1})	E_D ± SE (%)
1,2,4,5-tetrachlorobenzene	18.1	4.64 [36]	0.045 ± 0.016	63 ± 13
pentachlorobenzene (PCBz)	52.9	5.17 [36]	0.024 ± 0.002	55 ± 8
hexachlorobenzene (HCBz)	8.8	5.73 [37]	0.0088 ± 0.0019	53 ± 10
2,2',5,5'-PCB (PCB 52)	12.5	6.09 [36]	0.0028 ± 0.0020	45 ± 8
2,2',4,4',6,6'-PCB (PCB 155)	12.5	7.55 [36]	0.0012 ± 0.0022	46 ± 6
2,2',4,4',5,5'-PCB (PCB 153)	12.5	7.75 [36]	0.00069 ± 0.0021	46 ± 7
2,2',3,3',4,4',5',5',6,6'-PCB (PCB 209)	12.5	8.27 [37]	0.0011 ± 0.0021	34 ± 3
Test Chemicals	**Nominal food concentrations ($mg.kg\ wet\ weight^{-1}$)**	**log K_{OW}**	**k_{BT} ± SE (day^{-1})**	**E_D ± SE (%)**
1,2,3,4-tetramethyl benzene	120	4.00 [38]	0.36 (± 0.10)	44 ± 12
β-hexachlorocyclohexane (β -HCH)	8.5	4.14 [36]	0.23 (± 0.079)	55 ± 5
trans-decalin	499	4.79 [39]	0.038 (± 0.0025)	19 ± 5
9-methylanthracene	129	5.07 [38]	0.41 (± 0.10)	13 ± 9
chrysene	28.2	5.81 [40]	0.39 (± 0.12)	4.9 ± 1.4
hexylcyclohexane	488	6.05 [6]	0.043 (± 0.020)	14 ± 4
2,6-dimethyldecane	476	6.09 [6]	0.18 (± 0.043)	33 ± 14
benzo[*a*]pyrene	27.8	6.13 [40]	0.094 (± 0.031)	2.3 ± 1.2

3.4.4. Sample extraction

The whole liver was used for sample extraction. For carcass samples, a homogenized fraction of the whole carcass (minus the liver) was used. These samples were homogenized with an Oster 18-speed blender/blade (Sunbeam Products Inc.) used with glass canning jars (Benardin). All experimental equipment were washed with detergent and rinsed with hexane and dichloromethane before use. Liver (ranging in weight from 0.15-1.22 g) and carcass samples (4.17-5.27 g) were weighed and homogenized with 20 g (for liver samples) or 40 g (carcass samples) of sodium sulphate (Caleon laboratory Chemicals). To prevent volatilization of chemicals in the extraction process, 0.2 mL (for liver) and 0.4 mL (for carcass) of corn oil were added. Internal

standards were also added, including d^8-naphthalene (for trans-decalin, 2,6-dimethyl decane, 1,2,3,4-tetramethylbenzene, hexylcyclohexane, 1,2,4,5-tetrachlorobenzene), d^{10} acenaphthene (for pentachlorobenzene), ^{13}C-hexachlorobenzene (for hexachlorobenzene, beta-hexachlorocyclohexane), PCB 115 (for PCB 52, dibutyl phthalate, 9-methylanthracene, PCB 155, PCB 153) d^4-endosulfan (for beta-endosulfan), d^{12}-chrysene (for chrysene, DEHP, benzo[*a*]pyrene), PCB 207 (for PCB 209), and 3(tert-butyl)phenol (for 4(tert-octyl)phenol). Samples were extracted 3 times with 1:1 DCM/Hexane (40 mL, 20 mL, 20 mL for liver samples – 60 mL, 30 mL, 30 mL for Carcass samples) under sonication for 20 min. Under a stream of nitrogen, extracts were concentrated to approximately 5 mL. The concentrated samples were then eluted through a column packed with 10 g of de-activated florisil and eluted with 40 mL hexanes. The samples were then eluted with 90 mL of 1:1 DCM/Hexane, which was concentrated down to 0.3-0.5 mL under a steady nitrogen stream, and diluted with n-hexane to a total volume of 1 mL according to the scale of the GCMS vial. For carcass samples, the samples were diluted 20 fold. No dilution was necessary for the analysis of liver samples.

3.4.5. GSMS analysis

Extracts were analyzed for the test chemicals using an Agilent 6890 gas chromatograph (GC) attached to an Agilent 5973N mass spectrometer (MS), with a programmable cool on-column injection port, a 30m × 250 um × 0.25 um HP -5MS 5% phenyl methyl siloxane-coated column (Agilent), and a 5 m × 530 um × 0.25 um fused-silica deactivated guard column (Agilent). The oven temperature was 45 °C for 1.5 min, increasing to 150 °C at 15 °C.min^{-1}, and finally increasing 10 °C.min^{-1} to 285 °C and held for 5 min. The injection port and ion source temperatures were 45 and 230 °C respectively. The carrier gas was helium at 1 mL.min^{-1} flow rate. The MS data was acquired in the selected ion monitoring mode (m/z 138 for trans-decalin, 85 for 2,6-dimethyldecane, 119 for 1,2,3,4-tetramethylbenzene, 136 for d^8-naphthalene, 82 for hexylcyclohexane, 216 for 1,2,4,5-tetrachlorobenzene, 164 for d^{10}-acenaphthene, 250 for pentachlorobenzene, 290 for ^{13}C-hexachlorobenzene, 284 for hexachlorobenzene, 219 for beta-hexachlorocyclohexane, 292 for PCB 52, 149 for dibutyl phthalate, 192 for 9-methylanthracene, 360 for PCB 155, 343 for d^4-endosulfan, 326 for PCB 115, 195 and 339 for beta-endosulfan, 360 for PCB 153, 240 for d^{12}-chrysene, 228 for chrysene, 149

for DEHP, 464 for PCB 207, 252 for benzo[*a*]pyrene, and 498 for PCB 209). These ions were selected based on the properties of high intensity with low interference. A 1.00 μL sample of the extract was injected into the column by a 5-μL gas-tight glass syringe (Agilent). Peak areas were integrated and used to quantify the test chemicals using Chemstation software (Hewlett Packard). Chemical concentrations were calculated using the relative response factor approach.

3.4.6. Whole body concentrations

Chemical concentrations in the body of the fish (C_B) were determined by adding the chemical masses in the liver and carcass of each fish and dividing by the combined wet weights of the 2 compartments.

3.4.7. Uptake and elimination rate constants

Somatic depuration rate constants (k_{BT}) were derived from the test chemical concentrations in the body of the fish during the depuration phase of the experiment by a weighted linear regression of the natural logarithm of the concentrations in the fish body measured after day 14.5 (i.e. 12-h after the last feeding period) vs. time. The dietary uptake rate constant (k_{BD}) for each chemical was derived using non-linear regression of

$$C_B = (k_{BD} / k_{BT})\, C_D\, (1 - \exp(-k_{BT}\, t)) \qquad (3.31)$$

which is the analytical solution of Equation 3.7 if C_D is constant over time. The dietary uptake efficiency for test and reference chemicals was determined from k_{BD} following Equation 3.4 using a daily feeding rate of 1.2% the mean fish body weight during the uptake period.

3.4.8. k_{SE}

To determine k_{SE}, the fish bioaccumulation model described in Arnot and Gobas [16], was parameterized (Appendix B, Table B1) to represent the experimental fish under the experimental conditions to produce a non-linear relationship between k_{SE} (i.e. k_{BT} with $k_{BM} = 0$) and K_{OW}. This model was fitted to the experimental depuration rate constant data for the reference chemicals using a weighted non-linear least squares Gauss-Newton algorithm under JMP® 9.0.2.The reciprocal of the standard errors of the

depuration rate constants for the reference chemicals were used for weighting. The fitting involved the determination of the fish body lipid content and growth rate constant that best fitted the empirical k_{SE} – K_{OW} relationship.

3.4.9. Somatic biotransformation rate constant (k_{BM})

The rate constant of somatic biotransformation (k_{BM}) of the test chemicals was determined by subtracting k_{SE} from k_{BT} following Equation 3.13.

3.4.10. $E_{D,N}$

To determine the relationship between $E_{D,N}$ and K_{OW} for the reference chemicals, dietary absorption efficiencies for the reference chemicals were fitted to Equation 3.11 using the non-linear weighted least squares Gauss-Newton algorithm under JMP® 9.0.2 with the reciprocal of the standard errors of the $E_{D,N}$ estimates as the weight.

3.4.11. Gastro-Intestinal biotransformation (k_{GM})

The rate constant for gastro-intestinal biotransformation (k_{GM}) of the test chemicals was determined from the dietary uptake efficiency $E_{D,M}$ and $E_{D,N}$ following Equations 3.14-3.18.

3.5. Results and Discussion

3.5.1. Fish

No fish mortalities were observed throughout the experiment in either the exposure or control groups. Behavior and appearance of fish in the exposure and control groups were similar. Concentrations of the test and reference chemicals in the control fish were below their limit of quantitation (0.4 – 6 µmol.g wet weight^{-1}). Growth rate constants (k_{GD}) were calculated using the standard OECD 305 method [11] as the slope of the natural logarithm of 1/weight(g) vs time (day). There was no evidence of a difference in the growth rate constant for the test fish (0.0066 [SE 0.0012] day^{-1}) and control fish (0.0066 [SE 0.0015] day^{-1}) (Appendix B, Figure B1). Fish exhibited an initial body weight of 32 (SD 2, n=3) g, which increased over time to 42 (SD 4, n=3) g at the

end of the 14-d uptake period, and to 81 (SD 39, n=7) g at the end of the 128-d experiment. The average body weight and lipid content of the fish during the uptake period was 37 (SE 4) g and 6.7 (SE 1.0) % respectively and the average feeding rate was 0.012 (SE 0.002) g food wet weight/g fish wet weight or 0.0116 (SE 0.002) g food dry weight/g fish wet weight during the exposure period. Using a dietary assimilation efficiencies for lipids, protein and non-digestible organic matter of 92%, 75% and 0% respectively, the dry weight based dietary assimilation efficiency was calculated to be (0.92 × 0.186 + 0.75 × 0.466 + 0 × 0.324) × (0.012/0.0116) or 53%, similar to the 52 (SD 4.0) % measured previously using chromic oxide concentrations in [18]. Assuming that the 100% digestive emptying time of 35-h or 1.45-d reported for 60-80 g rainbow trout at 13.5 ^{o}C [19] is a reasonable estimate for the 95% digestive emptying time in the fish in this experiment, δ can be estimated as 3/1.45 or 2.1 d^{-1}, and the steady-state amount of digesta in the fish can be estimated as (0.0116 × 37)/2.1 = 0.20 g dry weight (Equation 3.17).

3.5.2. Somatic biotransformation

Appendix B, Figure B2 illustrates that throughout the uptake phase the mean chemical concentration in fish (of 3 replicates) increased over time and reached for certain substances a steady-state concentration, after which the concentration remained constant throughout the remainder of the uptake phase. For other substances the chemical concentration in the fish increased throughout the uptake period and steady-state was never achieved. After fish were fed non-contaminated diet (i.e. depuration phase), mean concentrations declined over time. Whole fish body total depuration rate constants (k_{BT}) for the reference and test chemicals were derived through log linear regression version of concentrations (C_B) versus time (t) and are listed in Table 3.1.

Figure 3.3 shows that the total depuration rate constant (k_{SE}) for the reference chemicals decreased with increasing log K_{OW} to a minimum value of approximately 0.001 d^{-1} for chemicals with a log K_{OW} greater than approximately 7.5. This minimum k_{SE} value may represent growth dilution, as with increasing log K_{OW}, k_{B2} and k_{BE} become increasingly smaller causing k_{SE} to approach k_{GD}. However, it should be emphasized that the error in the depuration rate constants (k_{BT}) of PCBs 52, 155, 153 and 209 is large due to the small decline in concentration achieved over the duration of the depuration phase. This error has several consequences. First, it means that a small rate of

biotransformation of a chemical (i.e. a rate within the margins of error), such as may occur for PCB 52, is not necessarily an impediment to the use of that chemical as a reference chemical. Secondly, because for very hydrophobic chemicals, the error is large enough for the depuration rate constant to be not statistically different from 0, it is sometimes reasonable to assume that k_{SE} is essentially 0 for very hydrophobic reference chemicals. Thirdly, while the relative error in the determination of depuration rate constant of the reference chemical can be large, it may contribute little error in the determination of the somatic biotransformation rate constant (k_{BM}) according to Equation 3.13 as long as the somatic biotransformation rate is sufficiently high. Fourth, the error also means that BMFs of very hydrophobic non-biotransformable substances derived using a kinetic approach (e.g. as the ratio of k_{BD} and k_{BT}) can contain large errors.

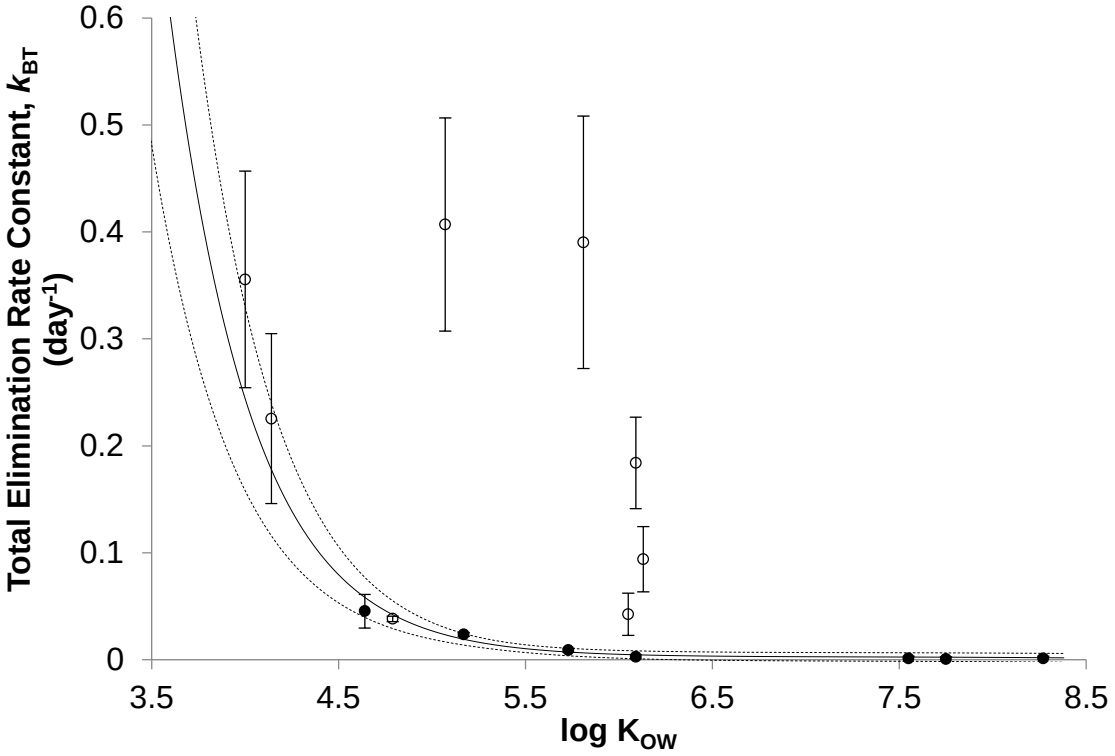

Figure 3.3 Rate constants for total elimination from the fish body k_{BT} (day^{-1}) of reference chemicals (filled round circles) and test chemicals (open round circles) versus log K_{OW} (standard errors reported in error bars). Test chemicals from left to right: 1,2,3,4-tetramethylbenzene, β-HCH, trans-decalin, 9-methylanthracene, chrysene, hexylcyclohexane, 2,6-dimethyldecane, benzo[*a*]pyrene. The solid line represents the model used to fit the depuration rate constant data for the non-biotransformable reference chemicals. The dotted lines represent the 95% confidence intervals for the predicted model values.

Figure 3.3 illustrates that the bioaccumulation model provides a good fit of the relationship between the depuration rate constants (k_{BT}) and log K_{OW} for the reference chemicals, hence providing a method for determining k_{SE} of non-ionized hydrophobic test chemicals. Figure 3.2 shows that the depuration rate constants (k_{BT}) of the test chemicals 1,2,3,4-tetramethylbenzene and β-HCH were close to their corresponding k_{SE} values, indicating a small somatic biotransformation rate constant (k_{BM}), not different from 0. These findings do not necessarily indicate that these substances do not biotransform in the body of the fish, but that the biotransformation rate constant k_{BM} is small compared to k_{SE} for these substance. Both 1,2,3,4-tetramethylbenzene and β-HCH have a relatively low log K_{OW} and eliminate from fish to the water (via the gills) relatively quickly. For substances with a relatively low log K_{OW} (e.g. less than 3.5), which are

eliminated quickly in fish by non-metabolic pathways, the reference chemical method applied here may not be a suitable method for measuring somatic biotransformation rates.

Figure 3.3 illustrates that the depuration rate constants (k_{BT}) of 2,6-dimethyldecane, 9-methylanthracene, chrysene and benzo[*a*]pyrene are greater than their corresponding k_{SE} values. For these substances, values for the somatic biotransformation rate constant (k_{BM}) can be determined with confidence (Table 3.2) because the difference between k_{BT} and k_{SE} is large compared to the error in the measurements of k_{BT} and k_{SE}. The biotransformation of some of these test chemicals in this experiment as well as related substances has been reported before [23, 24]. QSAR predictions of biotransformation rate constants obtained from EpiSuite 4.11 [6] for a 62 g fish (i.e. mean weight of the experimental fish during the depuration phase) at 12.6^{o}C (Table 3.2) also indicate that these chemicals can be expected to be biotransformed by fish. The k_{BM} values derived for the test chemicals in the present study can provide a preliminary test of the QSAR based biotransformation rate predictions by EpiSuite 4.11. Appendix B, Figure B5 illustrates that the QSAR predicted k_M values of 2,6-dimethyldecane, 9-methylanthracene, chrysene, transdecalin, β-HCH and hexylcyclohexane are in reasonable agreement with the observed k_{BM} values. However, the EpiSuite predictions of the biotransformation rate constant of 1,2,3,4-tetramethylbenzene and benzo[*a*]pyrene are approximately 10 times greater than the observed values in the present study. The lack of good agreement between QSAR predicted and observed biotransformation rate constants for 1,2,3,4-tetramethylbenzene may be due to the relatively low K_{OW} of 1,2,3,4-tetramethylbenzene, which produces a relatively high k_{SE} and hence makes it difficult to detect and accurately quantify the contribution of biotransformation to the overall depuration rate constant. This limitation affects both the determination of the biotransformation rate constant in the present study as well as the training set of chemicals used in the development of the biotransformation QSAR. The lack of good agreement between QSAR predicted and observed k_{BM} values for benzo[*a*]pyrene is more difficult to explain. However, it is possible that in a multiple chemical dosing design (as conducted in the present study), involving several aromatic hydrocarbons, competitive inhibition among the various test chemicals may cause biotransformation rates to be lower than in single chemical exposure studies. Competitive inhibition of biotransformation rates of benzo[*a*]pyrene, chrysene and 9-

methylanthracene has been observed in in-vitro biotransformation studies involving S9 rainbow trout liver homogenates [25]. It may also be possible that due to the common practice of analyzing whole fish (fish body and intestines), the biotransformation rates used to develop the QSARs may have been influenced by biotransformation in the gastro-intestinal tract.

Table 3.2 Somatic biotransformation rate constants (k_{BM}), modeled somatic k_{SE} (sum of k_1, k_E and k_G) rate constants, intestinal biotransformation rate constants (k_{GM}), BCFBAF (v.3.00) [6] predicted fish biotransformation rate constants (k_M), proportion of total mass biotransformed in the gut (Φ_{GM}), and proportion of total mass biotransformed in the fish body (Φ_{BM}). BCFBAF predicted k_M values were adjusted to 62 g fish in water at a temperature of 12.6 °C.

chemical	k_{BM} ± SE (1/day)	k_{SE} ± SE (1/day)	k_{GM} ± SE (1/day)	$k_{M,BCFBAF}$ (1/day)	Φ_{GM}	Φ_{BM}
1,2,3,4-tetramethylbenzene	0.11 ± 0.03	0.24 ± 0.04	0.45 ± 0.15	0.66	0.63	0.37
β-HCH	0.047 ± 0.025	0.18 ±0.03	-0.09 ± 0.03	0.025	0	1
trans-decalin	-0.004 ± 0.003	0.042 ±0.007	3.7 ± 1.0	0.06	1.0	0
9-methylanthracene	0.38 ± 0.03	0.023 ±0.003	6.6 ± 2.0	0.22	0.87	0.13
chrysene	0.38 ± 0.04	0.006 ±0.002	20 ± 5	0.14	0.95	0.05
hexylcyclohexane	0.0378 ± 0.005	0.005 ±0.002	5.7 ± 1.4	0.07	0.92	0.08
2,6-dimethyldecane	0.18 ± 0.01	0.005 ±0.002	1.2 ± 0.4	0.09	0.64	0.36
benzo[*a*]pyrene	0.09 ± 0.01	0.004 ±0.002	46 ± 10	0.48	0.98	0.02

3.5.3. Gastro-Intestinal Biotransformation

Non linear regression of the reciprocal of observed dietary uptake efficiencies of non-biotransformable reference chemicals ($E_{D,N}$) as a function K_{OW} using Equation 3.11 produced the following relationship (Figure 3.4):

$$E_{D,N}^{-1} = 5.6.10^{-9} \text{ (SE } 1.8.10^{-9}) K_{OW} + 1.9 \text{ (SE } 0.1) \quad (3.32)$$

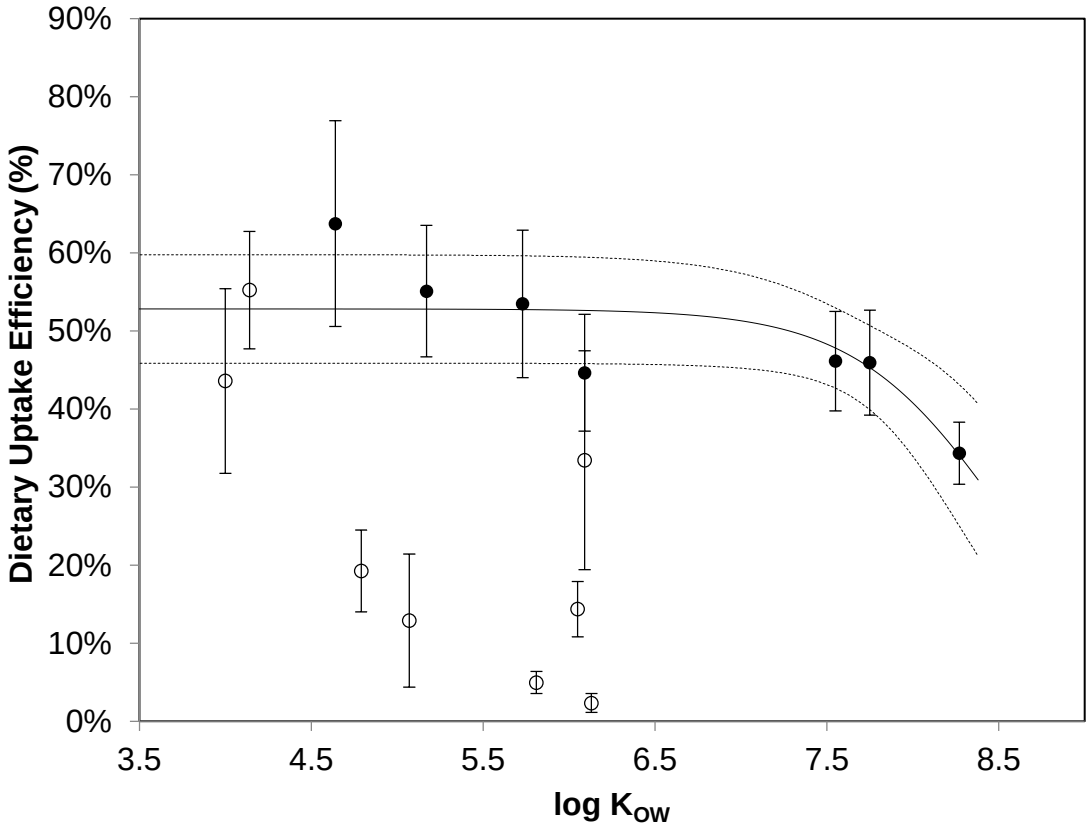

Figure 3.4 **Dietary uptake efficiencies of the reference chemicals ($E_{D,N}$, filled round circles) and test chemicals ($E_{D,M}$, open round circles) versus log K_{OW} (standard errors reported in error bars). Test chemicals from left to right: 1,2,3,4-tetramethylbenzene, β-HCH, trans-decalin, 9-methylanthracene, chrysene, hexylcyclohexane, 2,6-dimethyldecane, benzo[*a*]pyrene. The line represents non-linear regression fit to the dietary uptake efficiency data of the reference chemicals (Equation 3.32). The dotted lines represent the 95% confidence intervals for the predicted mean.**

This relationship is similar to the relationship between the dietary uptake efficiency and K_{OW} observed in previous studies for similar chemical substances [13, 18] administered in the diet over a prolonged period of time. The basic relationship is also apparent in a recent study by Xiao et al. [26], who reported benchmarked dietary uptake efficiencies and applied a different methodology for determining dietary uptake efficiencies than that used in the present study. Figure 3.4 shows that the mean dietary uptake efficiency for non-biotransformable chemicals is approximately constant at 52 (SE 4) % for substances with a log K_{OW} up to approximately 7 and then declines with increasing log K_{OW}. Figure 3.4 shows that with the exception of 1,2,3,4-tetramethyl benzene and β-HCH, all test chemicals exhibit dietary uptake efficiencies ($E_{D,M}$)that are significantly smaller than those derived by Equation 3.26 for the same chemical in absence of gastro-intestinal biotransformation. The highest dietary uptake efficiency (55 [SE 8] %) was observed for β-HCH and was not significantly different ($p>0.05$) from that derived by Equation 3.32 for the predicted reference compound at the same log K_{OW}. The smallest dietary uptake efficiencies were observed for benzo[*a*]pyrene (2.3 [SE 1.2] %) and chrysene (4.9 [SE 1.4] %). These findings are in good agreement with a number of studies observing low dietary uptake efficiencies of benzo[*a*]pyrene [25, 27, 28], 9-methyl anthracene [29] and related substances [29] in fish and trophic dilution in field studies [30, 31, 32]. The low dietary uptake efficiencies of 6 of the 8 test chemicals relative to those of reference chemicals indicate significant gastro-intestinal biotransformation of these test chemicals. Gastro-intestinal biotransformation rate constants (k_{GM}) for these substances can be derived from the dietary uptake efficiencies of the test and reference chemicals according to Equations 3.14 and 3.15 if the fecal egestion rate G_{GE} and W_G are known. Equations 3.10 and 3.11 indicate that throughout the uptake phase of the experiment in which fish exhibit an average weight of 37 g, G_{GE} was (1-0.53) × 0.0116 × 37 = 0.20 g digesta dry weight.d^{-1} and W_G was approximately 0.20 g. Intestinal biotransformation rate constants ranged between 0 (for β-HCH) to 46 d^{-1} (Table 3.2) and were, with the exception of k_{GM} for β-HCH, greater than their corresponding somatic biotransformation rate constants (k_{BM}). A direct comparison between k_{GM} and k_{BM}, however, is not meaningful because the rate constants apply to different compartments, i.e. the digesta in the intestinal tract for k_{GM} and the fish's body weight for k_{BM}. To estimate the relative importance of somatic and gastro-intestinal biotransformation, k_{GM} and k_{BM} need to be multiplied by the mass of test chemical in the gastro-intestinal tract M_G and in the fish body weight M_B respectively, which can be

derived at steady-state from the empirical observations according to Equations 3.16-3.22. Figure 3.5, which shows the relative importance of somatic and intestinal biotransformation at steady-state as Φ_{BM}, suggests that for all test chemicals except β-HCH, gastro-intestinal biotransformation is the main contributor to biotransformation within the fish. β-HCH did not appear to be biotransformed in the gastro-intestinal tract of the fish. β-HCH is a substance that is known to biomagnify in certain terrestrial organisms but not in fish due to a high degree of elimination through respiratory ventilation of water [33]. Gastro-intestinal biotransformation accounted for 63% of the total biotransformation for 1,2,3,4-tetramethylbenzene, 64% for 2,6-dimethyldecane, 87% for 9-methylantharcene, 92% for hexylcyclohexane, 95% for chrysene, and 98% for benzo[*a*]pyrene. Appendix B, Figure B3 shows that for those substances that were biotransformed in both the gastro-intestinal tract and in the body of the fish, there is a weak and statistically insignificant ($p=0.059$, $n=6$) correlation between somatic and gastro-intestinal biotransformation rates (g parent test chemical biotransformed.d^{-1}), but this correlation does not apply to trans-decalin and β-HCH. Figure B3 suggests that somatic biotransformation rates may in some cases be indicative of gastro-intestinal biotransformation rates, but that there can also be distinct differences in the capacity for biotransformation between the fish's body and the gastro-intestinal tract. Appendix B, Figure B4 shows that there was no correlation between k_{BM} and K_{OW} or between k_{GM} and K_{OW}.

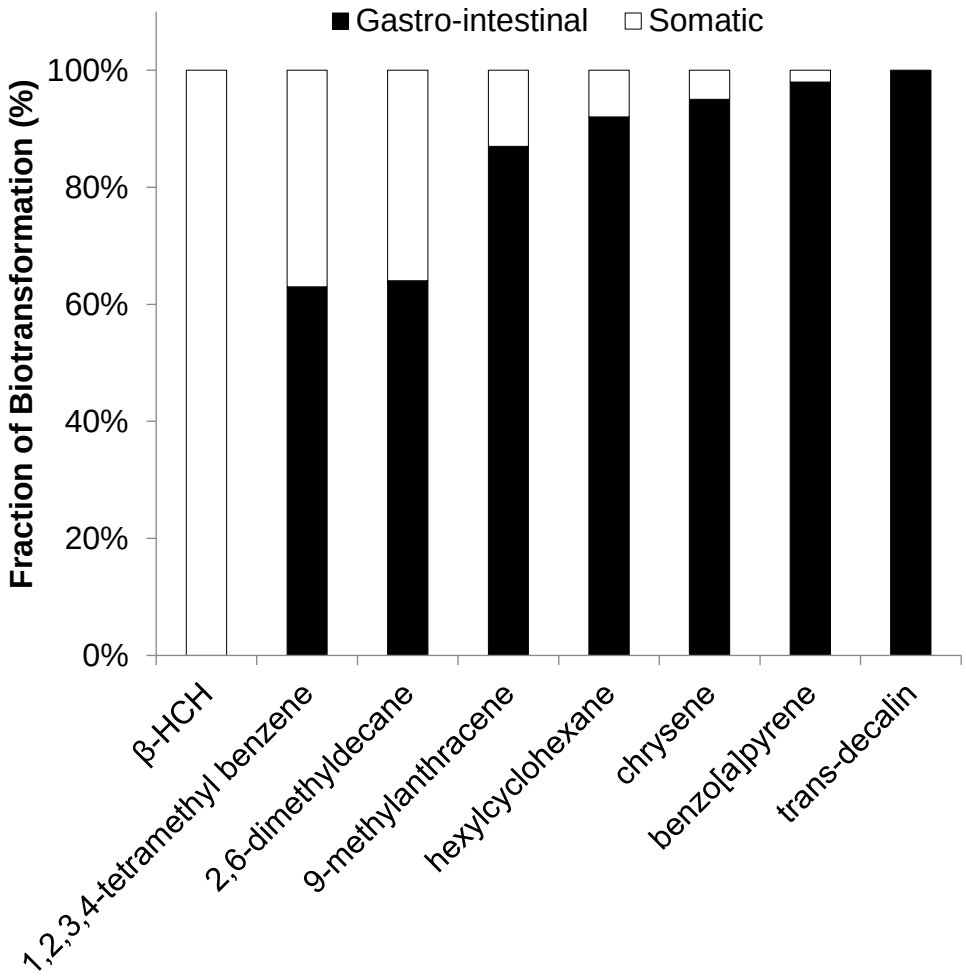

Figure 3.5 Contribution of somatic (black) and gastro-intestinal (grey) biotransformation to the overall mass of chemical biotransformed.

The observation that gastro-intestinal biotransformation exceeds somatic (including hepatic) biotransformation challenges the common presumption that the liver is the main site of biotransformation in fish. While indeed the liver is the main site for biotransformation of many pharmaceutical drugs dosed via the blood or respiratory route (e.g. gills in fish), this may not be the case for many bioaccumulative substances that are primarily absorbed via the diet.

The apparent dominant role of gastro-intestinal biotransformation in the biotransformation of most of the hydrophobic test substances in the present study may point to the different roles that lipids play in the gastro-intestinal tract and in the body of the fish. In the body of the fish, lipids function as storage compartments of very hydrophobic substances that reduce the bioavailability of very hydrophobic compounds

to biotransforming enzymes in the body of the fish. In the gastro-intestinal tract, lipids increase the availability of compounds dissolved in the lipids to biotransforming enzymes and micro-organisms due to their high degree of digestion and absorption [18], which makes hydrophobic chemicals present in dietary lipids available to gastro-intestinal microflora and digestive enzymes.

Biotransformation in the gastro-intestinal tract lowers the gastro-intestinal magnification that can occur. Equation 3.28 illustrates that under the experimental conditions of the test, gastro-intestinal magnification can be prevented if k^{*}_{GM} exceeds a value of approximately $(0.0116 \times 37/0.20)(2.85 - 0.47) = 5.1\ d^{-1}$, which in the present study corresponds with a dietary uptake efficiency lower than approximately 13% for substances with a log K_{OW} up to 7. The measurement of the dietary uptake efficiency can be used to identify substances which lack the ability to biomagnify in fish due to biotransformation in the gastro-intestinal tract. The measurement of k_{BT} identifies substances that lack the ability to biomagnify in fish due to their ability to be biotransformed in the body of the fish. Substances that are resistant to both gastro-intestinal and somatic biotransformation and also eliminate and excrete slowly (e.g. substances with a log $K_{OW} > 5$) can be expected to have biomagnification potential.

3.5.4. Biomagnification factors

Figure 3.6 illustrates that the BMFs of the reference chemicals increase with increasing log K_{OW} from approximately 0.47 for 1,2,4,5-tetrachlorobenzene to values as high as 22 for PCB 153. The BMFs for the highest K_{OW} chemicals, in particular PCB 52, PCB 153 and PCB 155 are subject to large errors due to the large error in the measurement of the depuration rate constant in fish body resulting from the very slow depuration rate. The BMFs of the test chemicals are all far below 1, illustrating that both somatic and intestinal biotransformation can prevent biomagnification of the parent substance.

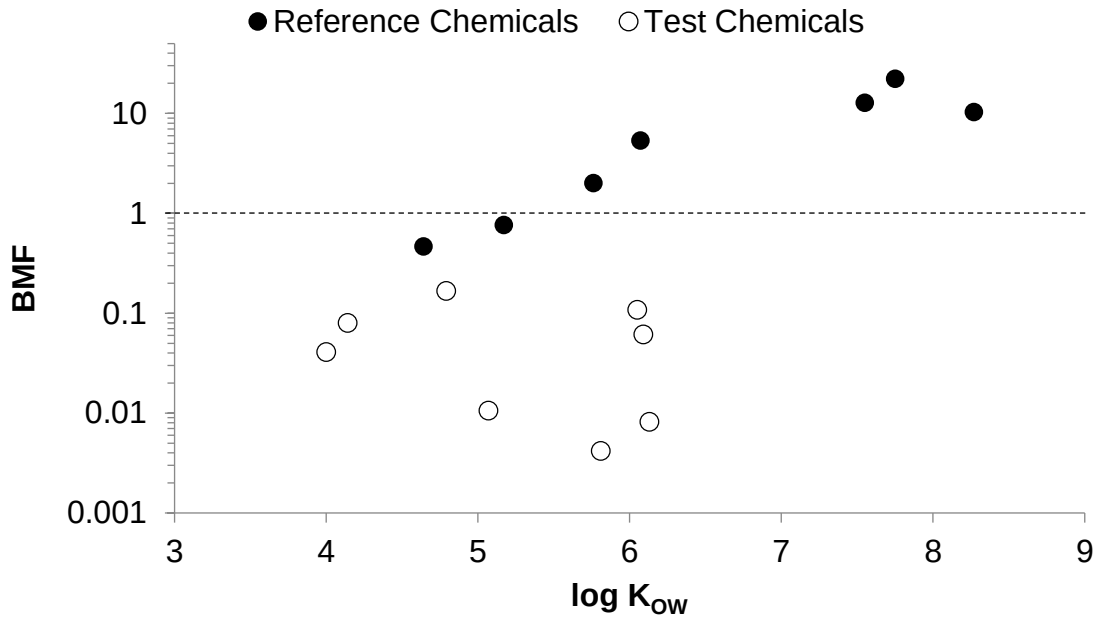

Figure 3.6 Lipid normalized log BMF versus log K_{OW} for reference and test chemicals. The BMF was calculated as $(0.0116 \times E_D \times \phi_{LD}) / (k_{BT} \times \phi_{LF})$.

3.5.5. Regulatory implications

The large contribution of gastro-intestinal biotransformation to the overall biotransformation of the majority of the test chemicals in the present study has implications for regulatory bioaccumulation screening of commercial chemicals. First, the results show that current bioconcentration tests are not able to account for the full degree of biotransformation that chemical substances experience in fish. This is because gastro-intestinal biotransformation does not occur in bioconcentration tests to the same degree as in biomagnification tests. In the environment, however, many hydrophobic, potentially bioaccumulative substances, are predominantly absorbed via the diet [34,35]. Bioconcentration tests may provide inaccurate estimates of the biomagnification potential of a chemical if the chemical is subject to significant biotransformation in the gastro-intestinal tract. For example, in the present study, trans-decalin appeared not to be biotransformed in the fish's body while a high degree of gastro-intestinal biotransformation was observed. The gastro-intestinal biotransformation rate constant of trans-decalin was not significantly different from the rate constant required to prevent gastro-intestinal magnification in the fish and hence avoid

biomagnification. High K_{OW} chemicals (e.g. log K_{OW} > 5), which eliminate and excrete slowly from the fish's body but that are rapidly biotransformed in the gastro-intestinal tract but not in the body of the fish may therefore produce high BCFs in bioconcentration tests but cannot biomagnify. The application of a dietary bioaccumulation test using reference chemicals as described in the present study may provide the additional insights to distinguish between chemicals that can and cannot biomagnify. The recently revamped OECD 305 protocol for a bioconcentration test already includes a dietary study protocol that is similar in design as the present study and also recommends the use of reference substances. In the OECD 305 protocol, reference substances are primarily used to confirm that the method used for spiking food with test chemicals is adequate to achieve homogeneity and bioavailability of the test substances. As the present study illustrates, reference substances can also aid in the measurement of in vivo biotransformation rates of the test chemicals. The broader application of reference chemicals may provide a feasible extension of the existing protocol, which can yield in vivo biotransformation rate data that are invaluable for the development of QSARs for biotransformation and the testing of in vitro-in vivo biotransformation rate extrapolation methods.

Secondly, the methodology used to derive biotransformation rate QSARs from bioconcentration factors derived from bioconcentration tests (which do not involve dietary exposure) may be appropriate for the estimation of BCFs, but may underestimate the contribution of biotransformation to the biomagnification process. Perhaps, QSARs for gastro-intestinal biotransformation can be developed based on measured dietary absorption efficiencies. These QSARs can be useful to identify potentially biomagnifying substances.

Thirdly, the application of in vitro bioassays using hepatic media such as liver S9 homogenates and hepatocytes may be appropriate for the estimation of BCFs, but they are likely inadequate for the estimation of the BMFs for many chemicals, especially those that are biotransformed in gastro-intestinal tract. The development of methods to measure in vitro gastro-intestinal biotransformation rates can be suggested as an important area of research to further strengthen bioaccumulation screening.

3.6. Acknowledgement

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Chapter 4.

Deriving Bioconcentration Factors and Somatic Biotransformation Rates from Dietary Bioaccumulation and Depuration Tests*

Justin C. Lo designed the experiments, conducted the feeding experiments, performed chemical analysis with assistance from Kexin Catherine Rong (SFU Environmental Science Undergraduate) and performed data analysis. Justin C. Lo assisted in writing the chapter in cooperation with Frank A.P.C. Gobas, the lead on this chapter.

4.1. Summary

The present study develops, applies, and tests a method for deriving empirical bioconcentration factors and somatic biotransformation rate constants from dietary bioaccumulation tests and simplified bioaccumulation experiments that measure depuration rates. In this approach, measurement of the chemical concentration in the water is not required. The method aims to improve bioaccumulation assessment, reduce cost and animal use, and shorten experiments.

4.2. Introduction

The Organisation for Economic Co-operation and Development (OECD) recently revised guideline 305 for measuring bioconcentration factors (BCFs) of chemicals in fish by adding a dietary bioaccumulation testing protocol [1]. Several factors precipitated the revision of the long-lived guideline. First, and most importantly, the bioconcentration test is difficult to perform for hydrophobic chemicals. This is because aqueous solutions of hydrophobic chemicals are difficult to generate, maintain at constant values in the presence of fish [2], and measure, as a result of their low concentrations. Also, fish introduce organic matter into the water that can bind very hydrophobic test chemicals, causing a reduction in the bioavailability of the chemical concentration in the water to the fish [3].The freely dissolved chemical concentration in the water that is considered to be bioavailable to the fish is often unknown or difficult to discern experimentally. Second,

bioconcentration tests are expensive and time consuming, and make extensive use of fish. A third reason is the inability of bioconcentration tests to provide direct information of actual magnification of the chemical in ecological systems. Biomagnification of the chemical in a thermodynamic sense (i.e., an increase in chemical potential) is known to occur in living organisms only as a result of dietary uptake [4]. Dietary bioaccumulation tests are often simpler and cheaper than bioconcentration tests because the dietary exposure concentrations of very hydrophobic chemicals in dietary food items are easy to generate, control, and interpret in terms of their bioavailability. Also, dietary bioaccumulation tests can measure a chemical's potential for biomagnification in food webs. While dietary bioaccumulation metrics are generally not considered in regulatory evaluations, there is an emerging trend by regulatory agencies to include information from dietary bioaccumulation tests and field trophic magnification studies in bioaccumulation assessments [5], [6].

Recently, other benefits of dietary bioaccumulation tests have become apparent. Lo et al. 2015 [7] showed that dietary bioaccumulation tests can reveal biotransformation rates of chemicals both in the intestinal tract and in the body of the fish. The latter is useful in on-going research to refine the estimation of biotransformation rates in fish and other organisms and to improve bioaccumulation modeling. However, dietary bioaccumulation tests do not provide the aqueous exposure-based BCF endpoint required by regulations.

Efforts have been made to calculate a bioconcentration factor from a dietary bioaccumulation test, notably the OECD 305 dietary exposure fish test, which estimates BCFs by using various estimation techniques based on fish weight, the substance's octanol–water partition coefficient (K_{OW}), and several other factors including a bioavailability fraction [1]. However, these estimation techniques require the use of theoretical models, which adds substantial error to the estimation of the BCF and takes away from the main purpose of using an empirical approach. In this short communication, we describe a method for deriving a BCF from the results of dietary bioaccumulation and depuration tests. The aim of the present study is to extend the usefulness of dietary bioaccumulation tests for providing information that is of regulatory relevance. We first describe the theoretical framework for the method; then apply the method to the results from a dietary bioaccumulation study to illustrate its potential use; and finally test the performance of the method.

4.3. Theory

The derivation of the bioconcentration factor from a dietary fish bioaccumulation test or depuration study is predicated on two experimental requirements that need to be met. The first requirement is that chemical mass in the lumen of the intestinal tract of the test fish is not included in the measurement of the concentration of the test chemical in the fish. This is common practice in many studies and considered in the OECD 305 guideline.

The second requirement is the use of non-biotransformable reference chemicals in the test. The reference chemicals are to include chemicals of varying K_{OW}, encompassing the K_{OW} of the test chemical(s). The OECD 305 test protocol also recommends the use of reference chemicals. However, in the OECD 305 protocol, reference substances are primarily used to confirm that the method used for spiking food with test chemicals is adequate to achieve homogeneity and bioavailability of the test substances in the food. In the approach described here, the reference substances are used to derive the bioconcentration factor and the somatic biotransformation rate constants of the test chemical(s) in fish.

The method for deriving the bioconcentration factor and somatic biotransformation rate constant from the results of dietary bioaccumulation test or depuration studies is based on the following model for the bioaccumulation of chemicals in fish [8]:

$$dC_F/dt = k_1 C_{WD} + E_D .F_D.C_D – (k_2 + k_E + k_G + k_M) C_F \quad (4.1)$$

where C_F is the concentration of the chemical in the fish on a wet weight (ww) basis (mol chemical.kg ww fish^{-1}), excluding intestinal content; C_{WD} is the concentration of the freely dissolved chemical in the water (mol chemical.L^{-1}); k_1 is the uptake clearance rate for respiratory uptake (L water.kg ww fish^{-1}.d^{-1}); E_D is the dietary assimilation efficiency of the chemical by the fish (unitless); F_D (kg food.kg fish^{-1}.d^{-1}) is the proportional feeding rate of the fish expressed as fraction of the fish's body weight per unit of time; C_D (mol chemical.kg food^{-1}) is the concentration of the chemical in the diet; k_2 (d^{-1}) is the rate constant for respiratory elimination, k_E (d^{-1}) is the rate constant for elimination via fecal egestion; k_G (d^{-1}) is the rate constant for pseudo elimination via growth dilution, and k_M (d^{-1}) is the rate constant for biotransformation of the chemical in the body of the fish and t

is time (d) (Figure 4.1). The binding of the chemical to dissolved organic carbon in water as well as the biotransformation of the dissolved chemical in the gills of the fish, which reduce the concentration of the parent chemical that can be absorbed via the gills, is not represented in k_1. This model can represent chemical bioconcentration (i.e. $C_D = 0$) by the steady-state wet-weight based bioconcentration factor ($BCF_{ww,fd}$) (L water.kg fish^{-1}):

$$BCF_{ww,fd} = C_F/C_{WD} = k_1/(k_2 + k_E + k_G + k_M) = k_1/k_T \quad (4.2)$$

where the total depuration rate constant k_T (d^{-1}) represents the sum of k_2, k_E, k_M and k_G. Equation 4.2 illustrates that the bioconcentration factor can be defined as the steady-state ratio of the concentrations of the chemical in fish and freely dissolved chemical in water (C_F/C_{WD}) as well as the ratio of the uptake and depuration rate constants k_1 and k_T, which is sometimes referred to as the kinetic bioconcentration factor. Equation 4.2 provides the foundation for deriving the bioconcentration factor of chemicals in fish as a function of rate constants without the need to measure the chemical concentration in the water.

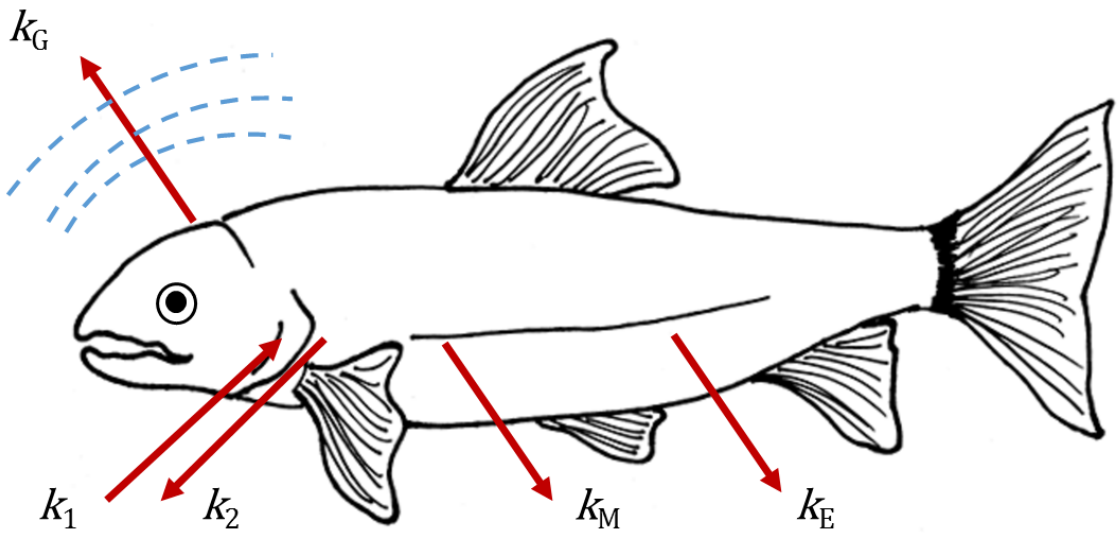

Figure 4.1 Conceptual diagram of the transport and transformation kinetics of hydrophobic organic chemicals in a single compartment fish illustrating the roles of respiratory uptake (k_1) and elimination (k_2) via gills and skin; fecal excretion (k_E), growth dilution (k_G); and somatic biotransformation (k_M).

It is important to emphasize that the $BCF_{ww,fd}$ is not identical to the bioconcentration factor referred to in the OECD 305 test guidelines. The

bioconcentration factor in the OECD 305 guideline is derived using the total concentration of the chemical in the water (not the freely dissolved chemical concentration in the water) and will be referred to as the $BCF_{ww,t}$ in this study. The total concentration of the chemical in the water (C_{WT}) includes freely dissolved chemical in the water and chemical associated with dissolved organic matter in the water. The $BCF_{ww,t}$ (referred to in the OECD305 guideline) can be found from the $BCF_{ww,fd}$ by multiplying $BCF_{ww,fd}$ by the fraction of freely dissolved chemical in the water (ϕ_{DW}) according to methods described in [9]:

$$BCF_{ww,t} = \phi_{DW}.\ BCF_{ww,fd} = BCF_{ww,fd}.(1 + \chi_{DOC}.K_{OC})^{-1} \quad (4.3)$$

where χ_{DOC} is the concentration of dissolved organic carbon in the water (kg oc/L), K_{OC} is the equilibrium partition coefficient (L/kg oc) of the chemical between dissolved organic carbon and water. If the somatic lipid content (ϕ_{BL} in kg lipid/kg ww) is known, then the $BCF_{ww,t}$ and $BCF_{ww,fd}$ can be expressed on a lipid normalized basis as $BCF_{L,t}$, i.e. $BCF_{L,t} = BCF_{ww,t} / \phi_{BL}$ and $BCF_{L,fd} = BCF_{ww,fd} / \phi_{BL}$ respectively; or expressed as a BCF for fish with a somatic lipid content of 5%, i.e. $BCF_{5\%,t} = 0.05 \cdot BCF_{L,t}$ and $BCF_{5\%,fd} = 0.05 \cdot BCF_{L,fd}$ respectively.

The model can also represent dietary bioaccumulation (i.e. $C_W=0$) in the form of the steady-state biomagnification factor (BMF) (kg food.kg ww $fish^{-1}$), as measured in dietary bioaccumulation tests such as the new OECD 305 test guideline [1].

$$BMF = C_F/C_D = E_D.F_D/(k_2 + k_E + k_G + k_M) = E_D.F_D/k_T \quad (4.4)$$

The main measurements in a typical dietary bioaccumulation test are k_T and E_D. The total depuration rate constants (k_T in d^{-1}) is normally derived through linear regression of the natural logarithm of chemical concentrations in fish during the depuration phase versus time, using the equation:

$$k_T = \ln[C_{F,t=0}/C_F(t)]/t \quad (4.5)$$

The dietary uptake efficiency can be determined from the initial slope of the concentrations of the chemical in the fish during the uptake phase or from the initial concentration of the chemical fish during the depuration phase (in tests that do not include concentration measurements during the uptake phase to reduce animal use).

This measurement is useful in determining the gastro-intestinal biotransformation rate constant in the fish [7]. The dietary uptake efficiency is not needed to derive the BCF from the results of a dietary bioaccumulation test. The BCF can be derived from the concentration decline of reference chemicals during the depuration phase. The method of uptake is irrelevant within the limitations of the model where internal distribution of the chemical in the fish is assumed to be fast. This means that the method described here is not limited to dietary bioaccumulation tests but can also be applied to depuration rates from aqueous bioconcentration tests with the convenient attribute that the sometimes difficult to measure concentration of the chemical in the water does not need to be known.

The method proposed here to derive a bioconcentration factor from a dietary bioaccumulation test is based on the consideration that:

$$k_T = k_2 + k_E + k_G + k_M \qquad (4.6)$$

and that for non-biotransformable reference chemicals k_M is 0; k_G is a constant value for all test and reference chemical (hence independent on the chemical), which is typically measured by monitoring fish weights over the length of the test; and k_E, though rarely measured directly, is expected to be constant for reference or test chemicals of varying K_{OW}, only to fall to very low values for extremely high K_{OW} chemicals (log K_{OW} > 8) [10], at which point k_E becomes negligible compared to the growth dilution rate constant k_G; and that k_2 can be expressed in terms of a water-lipid two-phase aqueous uptake and elimination model as [11]:

$$1/k_2 = \omega \,.\, K_{OW} + \lambda \qquad (4.7)$$

where ω and λ are regression coefficients in units of time (d) and K_{OW} represents the lipid-water partition coefficient of the chemical in the fish. The coefficient ω describes the contribution of aqueous controlled transport processes including gill ventilation and diffusion through aqueous boundary layers of membranes to the respiratory elimination rate [11]. The coefficient λ describes the contribution of lipid phase controlled transport processes including membrane lipid bilayer transport. For substances with a log $K_{OW} \geq 3$, λ is very small compared to $\omega \,.K_{OW}$ and can often be ignored [11], simplifying equation 4.7 to:

$$1/k_2 = \omega . K_{OW} \text{ or } k_2 = (1/\omega).(1/K_{OW}) \quad (4.8)$$

illustrating the importance of gill ventilation, aqueous diffusion [11] and lipid-water partitioning as represented by the octanol-water partition coefficient [12]. As a result, the total depuration rate constant of non-biotransformable reference chemicals $k_{T,R}$ (d^{-1}) equals

$$k_{T,R} = k_2 + k_E + k_G \approx (1/\omega).(1/K_{OW}) + \beta \quad (4.9)$$

where β is the total depuration rate constant as K_{OW} approaches an infinite value, i.e. $k_E + k_G$. Equation 4.9 illustrates that the depuration rate constant of the reference chemicals ($k_{T,R}$) is expected to follow a linear relationship with the reciprocal of K_{OW}. This equation provides a relatively simple method for developing a relationship between the depuration rate constant ($k_{T,R}$) and K_{OW} that can be used as a reference point to derive somatic biotransformation rate constants (k_M) for test chemicals of varying log K_{OW}. The slope of the linear relationship between $k_{T,R}$ and $1/K_{OW}$ is $1/\omega$ and represents the reduction in respiratory elimination of hydrophobic organic chemicals from the fish to the water with increasing K_{OW}. The regression coefficient β describes the contribution of depuration processes other than respiratory elimination as K_{OW} approaches infinity. As K_{OW} approaches very large values, actual depuration of non biotransformable substances becomes very small causing growth dilution (k_G) to be the ultimate mechanism of apparent depuration of the chemical from the fish. A linear regression of the depuration rate constants for the non-biotransformable reference chemicals versus $1/K_{OW}$ produces both $1/\omega$ (the slope) and β (the intercept) (Figure 4.2). The somatic biotransformation rate constant of the test chemical can then be found as the difference between the experimental depuration rate constant of the test chemical (k_T) and the corresponding depuration rate constant of the reference chemical ($k_{T,R}$) derived from the regression of equation 4.9 using the K_{OW} of the test chemical:

$$k_M = k_T - k_{T,R} \quad (4.10)$$

An estimate of the standard error (SE) in k_M (SE_{kM}) can be derived from the standard errors of k_T (SE_{kT}) and $k_{T,R}$ ($SE_{kT,R}$) which in turn are determined in the regressions of equations 4.5 and 4.9, using the equation:

$$SE_{kM} = \sqrt{(SE_{kT}^2 + SE_{kT,R}^2)} \quad (4.11)$$

Equation 4.11 is based on the assumption that the errors are random and uncorrelated, which may not be the case in many bioaccumulation experiments. Hence, we refer to the error calculated as an estimate. Whether a substance is subject to somatic biotransformation can be determined by testing whether k_T for the test chemical (derived as the slope of the regression of natural logarithm of the concentration of the test chemical in the fish over the length of the depuration phase) is significantly greater than $k_{T,R}$ (derived from the $k_{T,R}$-$1/K_{ow}$ relationship).

The determination of ω from depuration rate constant data following equation 4.9, provides a way to derive the uptake rate constant k_1 as k_1 and k_2 are related according to [11]:

$$k_1/k_2 = \phi_{LB}\, K_{OW} /d_L \qquad (4.12)$$

Substituting equation 4.9 in 4.12 provides the following relationship between the uptake rate constant k_1 with K_{OW} [11]:

$$1/k_1 = (\omega + \lambda/K_{OW}).(d_L/\phi_{LB}) \qquad (4.13)$$

where λ describes lipid phase transport processes (d^{-1}), d_L is the density of the fish lipids (kg/L) and ϕ_{LB} is somatic lipid content of the fish (kg lipid/kg ww fish). For substances with a log K_{OW} greater than about 3, equation 4.13 can be simplified to:

$$1/k_1 = \omega .d_L/\phi_{LB} \qquad (4.14)$$

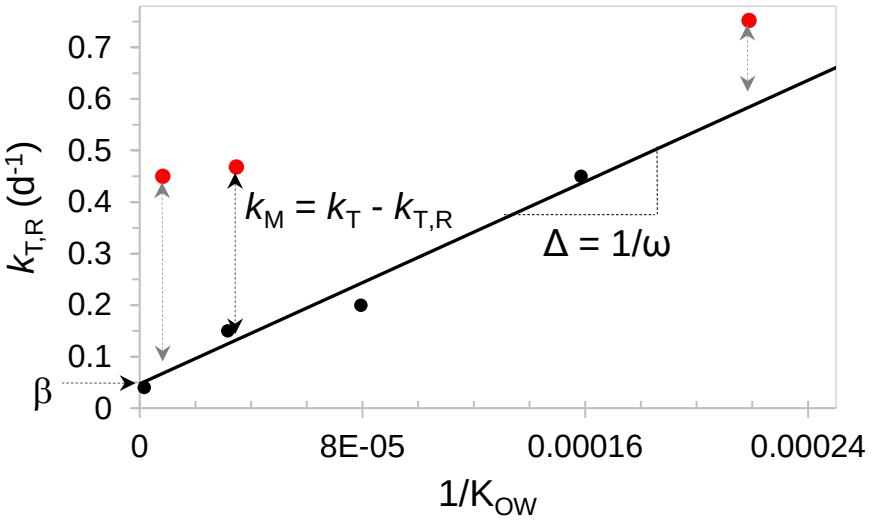

Figure 4.2 **Illustrative diagram of the relationship between the total depuration rate constants ($k_{T,R}$) and the reciprocal of the octanol–water partition coefficient ($1/K_{ow}$) for the reference (black dots) and test chemicals (red dots). The line (Equation 4.9) represents a linear regression fit of the total depuration rate constants of the reference chemical data, where the intercept is the regression coefficient β and the slope is 1/ω. The difference between the elimination rate constant of the test chemicals and the regression line represents the somatic biotransformation rate constant, k_M.**

The coefficient ω derived from the linear regression of equation 4.8 can thus be used to find k_1 (L.kg^{-1}.d^{-1}) for the test chemicals. Equation 4.14 illustrates that for chemicals with a log K_{OW} ≥ 3, the uptake rate constant k_1 is the same for all chemicals. This is because the uptake rate constant for these chemicals is largely controlled by the fish's gill ventilation rate, which is the same for all chemicals. It should be emphasized that in most empirical studies, the uptake rate constant is determined as $k_1.\phi_{DW}$ where ϕ_{DW} is the fraction of freely dissolved chemical in the water. Because ϕ_{DW} is a function of K_{OW}, the uptake rate constant often appears to depend on K_{OW}. The bioconcentration factor $BCF_{ww,fd}$ in units of L.kg ww fish^{-1} of the test chemical can then be determined as:

$$BCF_{ww,fd} = k_1/k_T \qquad (4.15)$$

The $BCF_{ww,fd}$ represents the bioconcentration factor for the freely dissolved chemical in water and is therefore independent on the bioavailability of the chemical in the water. The concentration of the test chemical in the water is not used in the determination of the BCF. The $BCF_{ww,fd}$ represents steady-state conditions; is specific to

the lipid content of the fish for which the BCF is determined; and includes the effect of growth dilution. Some authors have calculated bioconcentration factors for non-growing fish as conservative estimate of the bioconcentration factor [13]. The BCF for a non-growing fish can be approximated (e.g. in absence of fish growth measurements) from the intercept of equation 4.9 as;

$$BCF_{ww,fd} = k_1/(k_T - \beta) \quad (4.16)$$

or alternatively (if fish growth data are available) as:

$$BCF_{ww,fd} = k_1/(k_T - k_G) \quad (4.17)$$

Equations 4.16 and 4.17 should be applied with great caution as errors in k_T and β or estimates of growth dilution rate constant k_G (which may be represented by β) can be so large that the difference between k_T and β (or k_G) is meaningless. It is further recommended that growth dilution rate constants are compared to proportional feeding rates to ensure that growth dilution rate estimates are plausible, i.e. less than the proportional feeding rates. For example, using a conservative tracer (chromic oxide) in the diet of rainbow trout in a typical dietary bioaccumulation study, it was found that rainbow trout assimilate approximately half the mass of the ingested diet [4]. Since only a portion of the assimilated food is converted into fish growth, it can be expected that rainbow trout fed at a rate of 1.5% of their body weight per day grow at a rate less than 0.5 x 1.5% or 0.75% per day. Sampling bias (e.g. sampling small fish at the beginning of the test and larger fish at the end of the test) can produce substantial error in growth dilution rate constant estimates.

4.4. Materials and Methods

The method for deriving the BCF and somatic biotransformation rate constant was applied to a study by Lo et al. 2015 [7], who conducted dietary bioaccumulation studies in rainbow trout (Oncorhynchus mykiss). The initial body weight of the fish was 32 $\pm$ 2 (SE) g, which increased over time to 42 $\pm$ 4 (SE) g at the end of the 14-d uptake period, and to 81 $\pm$ 39 (SE) g at the end of the 128-d experiment. The average body weight of the fish during the uptake and depuration periods were 37 $\pm$ 4 (SE) g and 62 $\pm$ 4 (SE) respectively. The lipid content of the fish was 6.7 $\pm$ 1.0 (SE) %. The average

feeding rate was 0.012 ± 0.002 (SE) g dry food/g wet fish /day and the growth dilution rate constant for the test fish was 0.0066 ± 0.0012 (SE) d^{-1} and 0.0066 ± 0.0015 (SE) d^{-1} for the control fish. The study involved the simultaneous dietary exposure of 7 reference chemicals (i.e. 1,2,4,5-tetrachlorobenzene; pentachlorobenzene (PCBz); hexachlorobenzene (HCBz): 2,2',5,5'-PCB (PCB 52); 2,2',4,4',6,6'-PCB (PCB 155); 2,2',4,4',5,5'-PCB (PCB 153): 2,2',3,3',4,4',5',5',6,6'-PCB (PCB 209)), which were assumed not be biotransformed significantly, and 8 test chemicals (i.e. 1,2,3,4-tetramethyl benzene;β-hexachlorocyclohexane (β-HCH); trans-decalin; 9-methylanthracene; chrysene, hexylcyclohexane; 2,6-dimethyldecane; benzo[a]pyrene), which were expected to be biotransformed. The authors measured depuration rate constants over a period of 114 days and used linear regression of the natural logarithm of the concentrations of the test and reference chemicals versus time to determine the depuration rate constant for the reference and test chemicals. The authors also made measurements of the dietary uptake efficiency and several other bioaccumulation metrics. We refer to the original work of the authors for other information not addressed in this study. One of the limitations of the study for the purpose of testing the proposed methodology for deriving the BCF is that the range of log K_{OW} values of the reference chemicals did not cover the entire log K_{OW} range of the test chemicals. Future applications of the method described here best include reference chemicals with a log K_{OW} range that includes the log K_{OW} of the test chemicals.

As part of this study, a linear least squares weighted regression of $k_{T,R}$ versus the reciprocal of the chemical's K_{OW}, i.e. 1/ K_{OW}, was conducted (equation 4.9). A weighted regression was conducted to account for differences in precision among the depuration rate constant estimates because residuals of $k_{T,R}$ carried unequal variances. The regression weights were equal to the reciprocal of the standard error of the depuration rate constants of the reference chemicals (n=7). The regression coefficient for the slope ω was then used to determine k_1 and the $BCF_{ww,fd}$ following equations 4.14 and 4.15.

Somatic biotransformation rate constants for the reference chemicals were determined as the difference between the experimental depuration rate constants of the test chemical (k_T) and the corresponding depuration rate constants of the reference chemical ($k_{T,R}$) using equation 4.10 with an error calculated according to equation 4.11.

The $BCF_{ww,fd}$ was converted into the $BCF_{ww,t}$ following equation 4.3 using a dissolved organic carbon concentration of 2 mg/L (i.e., equal to the maximum allowable concentration in a OECD 305 aqueous exposure test) and a dissolved organic carbon/water partition coefficient (K_{OC}) calculated from log K_{OW} according to log K_{OC} = 0.97·log K_{OW} – 1.27 [14]. Derived $BCF_{ww,t}$ in this study were compared to literature reported empirical $BCF_{ww,t}$ in fish where possible. Empirical bioconcentration factors were taken from the Arnot and Gobas data base [15] and EpiSuite 4.11 [16]. Empirical bioconcentration factors were available for pentachlorobenzene (n=48 studies); hexachlorobenzene (n=178 studies) and PCB52 (n=12 studies); hexachlorocyclohexane (n=3 studies) and chrysene (n=1 study). The bioconcentration factor of 1,2,4,5-tetrachlorobenzene, which was not available, was represented by the geometric mean of the bioconcentration factors of all tetrachlorobenzene congeners (n=38 studies). Similarly, the bioconcentration factors of PCB 155 and PCB 153 were represented by the geometric mean bioconcentration factors of all available hexachlorobiphenyl congeners (n=12 studies). Table 4.1 lists the derived and empirical $BCF_{ww,t}$ and also BCFBAF QSAR estimates for the $BCF_{ww,t,}$ which incorporate an estimation of the biotransformation rate of the chemicals in fish with a body weight of 62 g (i.e. equal to the mean body weight of the fish during the depuration phase of the experiment [7]) at a temperature of 12.6 °C.

Table 4.1 Summary data for reference and test chemicals[a]

Reference Chemicals	log K_{OW}	$k_{T,R}$ ± SE (d^{-1})	k_1 ± SE ($L.kg^{-1}.d^{-1}$)	$BCF_{ww,t}$ ± SE ($L.kg^{-1}$)	$BCF_{ww,t}$ [95% CI] ($L.kg^{-1}$) other studies	BCF QSAR [b]
1,2,4,5-tetrachlorobenzene	4.64 [23]	0.045 ± 0.016	183 ± 27	4000 ± 1500	2775[c] 4390 [3699-5732][d]	2600
pentachlorobenzene (PCBz)	5.17 [23]	0.024 ± 0.002	183 ± 27	7600 ± 1300	5656[c] 6640 [5682-8557][d]	6934
hexachlorobenzene (HCBz)	5.73 [24]	0.0088 ± 0.0019	183 ± 27	20000 ± 5000	21429[c] 7480 [6488-13027][d]	17338
2,2',5,5'-PCB (PCB 52)	6.09 [23]	0.0028 ± 0.0020	183 ± 27	61000 ± 45000	18001[c] 9400 [5878-19561][d]	19011
2,2',4,4',6,6'-PCB (PCB 155)	7.55 [23]	0.0012 ± 0.0022	183 ± 27	48000	14200 [7024-59427][d]	7413
2,2',4,4',5,5'-PCB (PCB 153)	7.75 [23]	0.00069 ± 0.0021	183 ± 27	58000	5922[c] 14200 [7024-59427][d]	5140
2,2',3,3',4,4',5',5',6,6'-PCB (PCB 209)	8.27 [25]	0.0011 ± 0.0021	183 ± 27	14000	17378[c]	2824
Test Chemicals	**log K_{OW}**	**k_T ± SE (d^{-1})**	**k_1 ± SE ($L.kg^{-1}.d^{-1}$)**	**$BCF_{ww,t}$ ± SE ($L.kg^{-1}$)**	**$BCF_{ww,t}$ [95% CI] ($L.kg^{-1}$) other studies**	**BCF QSAR [g]**
1,2,3,4-tetramethyl benzene	4.00 [26]	0.36 ± 0.10	183 ± 27	510 ± 160		627
β-hexachlorocyclohexane (β -HCH)	4.14 [23]	0.23 ± 0.079	183 ± 27	810 ± 300	1450[c] 620 [190-1695][d]	899
trans-decalin	4.79 [25]	0.038 ± 0.0025	183 ± 27	4800 ± 800	1905[c]	995
9-methylanthracene	5.07 [26]	0.41 ± 0.10	183 ± 27	450 ± 70		845
chrysene	5.81 [26]	0.39 ± 0.12	183 ± 27	450 ± 150		2182
hexylcyclohexane	6.05 [24]	0.043 ± 0.020	183 ± 27	4000 ± 2000		4549
2,6-dimethyldecane	6.09 [24]	0.18 ± 0.043	183 ± 27	910 ± 260		931
benzo[*a*]pyrene	6.13 [27]	0.094 ± 0.031	183 ± 27	1800 ± 700		500

[a] Data are given for the log K_{OW}; the measured depuration rate constants for the reference chemicals $k_{T,R}$ (d^{-1}) (derived from Equation 4.5) and test chemicals k_T (d^{-1}) (derived from Equation 4.5) with their standard errors (SE) from Lo et al. [7]; the respiratory uptake rate constant k_1 ($L\ kg^{-1}\ d^{-1}$) (derived from Equation 4.14); the steady-state wet weight-based bioconcentration factor $BCF_{ww,t}$ ($L\ kg^{-1}$) (derived from Equations 4.3 and 4.15) and its SE derived from the results of the dietary bioaccumulation test by Lo et al. [7]; the $BCF_{ww,t}$ ($L\ kg^{-1}$) reported in the Arnot and Gobas and EpiSuite 4.11 databases [15-16]; and model calculations by the Arnot–Gobas BCF bioaccumulation factor (BAF) quantitative

structure–activity relationship (BCFBAF QSAR) program in Epi Suite 4.11 [16].
[b] Calculated BCF using BCFBAF-predicted k_M and food-web model for mid-trophic-level fish (EPI Suite, Ver 4.11) [16].
[c] Experimental database match from EPI Suite, Ver 4.11 [16].
[d] Experimental values for fish in Arnot and Gobas 2006 database [15] based on total water concentrations.

HCH = hexachlorocyclohexane; HCBz = hexachlorobenzene; PCB = polychlorinated biphenyl; SE = standard error

4.5. Results and Discussion

Figure 4.3 illustrates that the depuration rate constants of the reference chemicals appear to follow a linear relationship with $1/K_{OW}$ according to:

$$k_{T,R} = 2455\ (\pm\ 358\ SE).(1/K_{OW}) + 0.0024\ (\pm\ 0.0016\ SE)\ ,\ n=7,\ r^2 = 0.90 \quad (4.18)$$

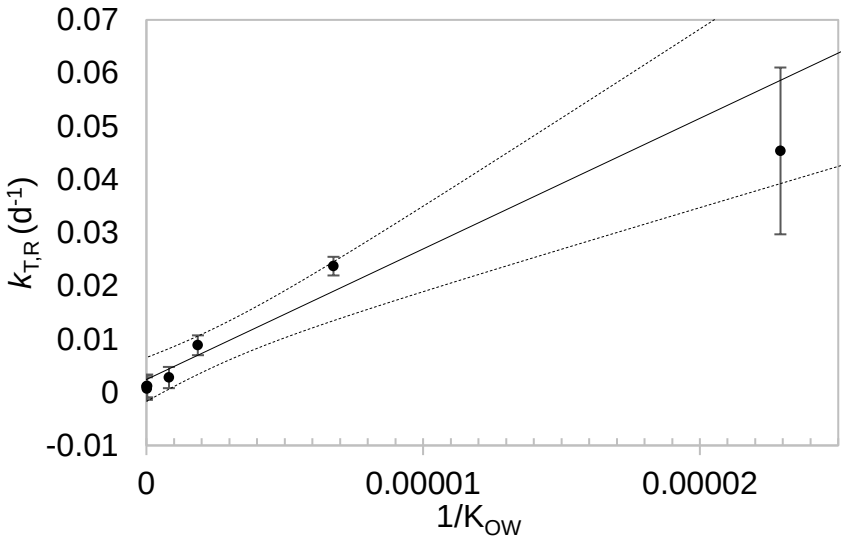

Figure 4.3 Measured total depuration rate constants ($k_{T,R}$) of 7 reference chemicals as a function of the reciprocal of the octanol–water partition coefficient ($1/K_{ow}$) for the 7 reference chemicals (black dots). Error bars are standard errors. The line (Equation 4.9) represents a linear weighted regression of $k_T$for the reference chemicals. Dotted lines respresent 95% confidence intervals for the predicted mean.

The fit of the simple linear model (i.e. equation 4.9) to the experimental data, as measured by the root mean squared error (RMSE), i.e. 0.078, is approximately equal to that presented in [7], i.e., a RMSE of 0.076, using a mechanistic bioaccumulation model. This may reflect the fact that the relationship between $k_{T,R}$ and K_{OW} is dominated by only

a few key parameters, i.e. ω and k_G. The slope of the regression equation ω^{-1} represents the respiratory exchange rate in the fish. The value for ω^{-1} is 2455 $\pm$ 358 (SE) d^{-1}. Hence, ω is 0.000407 $\pm$ 0.000058 (SE) d. The intercept β represents depuration of substances with infinite K_{OW}, which do not depurate other than through growth dilution. Its value of 0.0024 $\pm$ 0.0016 (SE) is reasonably close to the measured growth dilution rate constant k_G of 0.0066 $\pm$ 0.0012 (SE) d^{-1} in [7]. The apparent difference between β and k_G may be the result of errors in K_{OW} of the reference chemicals (which are not fully accounted for in the error analysis) and sampling bias in the growth rate determination. Despite this difference, it may in some cases be advantageous and reasonable to use the growth dilution rate constant as an approximation of the intercept β in equation 4.9.

Table 4.1 summarizes the respiratory uptake rate constants k_1 which were determined from ω of 0.000407 $\pm$ 0.000058 (SE) d; the somatic lipid content ϕ_{LB} of 6.7 % and the density of lipids d_L of 0.90 kg/L according to equation 4.14. The respiratory uptake rate constant k_1 is (2455 ($\pm$ 358 SE) x 0.067)/0.90 = 183 $\pm$ 27 (SE) d^{-1} and is the same for all chemicals because it is largely controlled by the fish gill ventilation rate, which is the same for all the study chemicals. In the lipid-water two-phase resistance model [11], ω represents the ratio of the lipid volume of the fish (V_L in L) and the water phase transport conductivity (Q_W in L/d) for chemicals with a log $K_{OW} \geq 3$. Since V_L is ((37 $\pm$ 4 (SE) g ww fish) x (0.067 $\pm$ 0.01 (SE)) g lipid/g ww fish)) / (0.90 g lipid/mL lipid) = 2.8 $\pm$ 0.3 (SE) mL lipid, it follows that Q_W is (2.8 $\pm$ 0.3 (SE) mL) x (2455 $\pm$ 358 (SE) d^{-1}) = 6874 $\pm$ 1243 (SE) mL/d or approximately 6.9 $\pm$ 1.2 (SE) L/d, which is within the 95% confidence interval of Q_W estimates of 1.4 x $(37)^{0.4}$ or 5.9 L/d and 1.4 x $(37)^{0.8}$ or 25 L/d for fish with wet weights between 0.35 and 900 g [11]. If Q_L in rainbow trout is indeed approximately 1% of Q_W [11], then for the 37 g rainbow trout in this study, Q_L is approximately 0.069 L/d and λ is 2.8 mL/69 mL.d^{-1} = 0.041 d. The chemical with the lowest K_{OW} (hence most sensitive to the assumption that lipid phase transport can be ignored) is 1,2,3,4-tetramethylbenzene with a log Kow of 4.00 and a $1/k_2$ equal to ω. K_{OW} + λ or 0.000407 x $10^{4.00}$ + 0.041 = 4.07 + 0.041 where the term 0.041 (representing lipid phase transport) can be safely ignored. For the other substances with higher K_{OW}, lipid phase transport plays an even less important role. This illustrates that ignoring λ in equation 4.7 is a reasonable assumption for substances with a log K_{OW} greater than approximately 3.

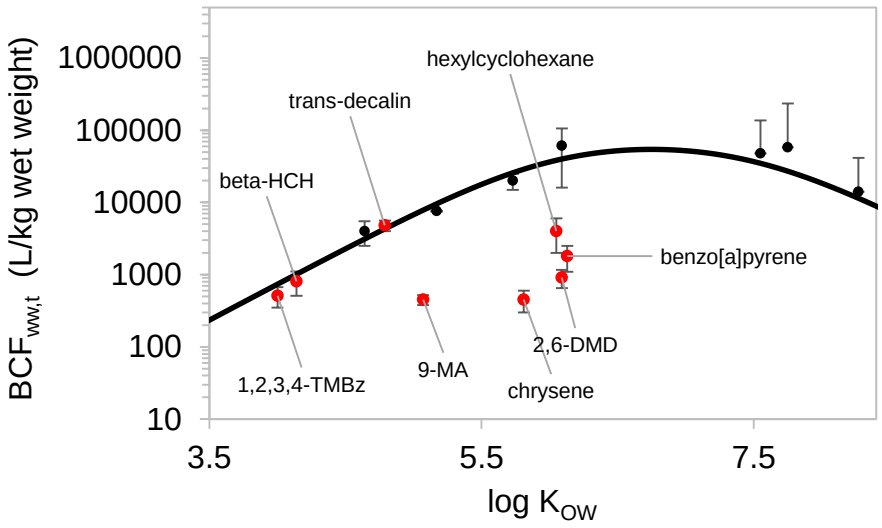

Figure 4.4 Bioconcentration factors ($BCF_{ww,t}$ in units of L/kg wet wt fish) versus log K_{ow} for the reference (black dots) and test chemicals (red dots). Error bars represent standard errors.

The $BCF_{ww,t}$ of the reference chemicals (Table 4.1) increased with increasing K_{OW} from 4,000 L.kg ww $fish^{-1}$, for 1,2,4,5-tetrachlorobenzene to 61,000 L.kg ww $fish^{-1}$ for PCB 52 and then fell to 14,000 for PCB 209 (Figure 4.4). The $BCF_{ww,t}$ of the test chemicals (Table 4.1) varied from 450 to 4,800 L.kg ww $fish^{-1}$and was lower than their corresponding reference chemicals of similar K_{OW} in all cases (Figure 4.4). The somatic biotransformation rate constant k_M of the test chemicals varied from essentially 0 for trans-decalin to 0.39 d^{-1} for chrysene (Table 4.2). These somatic biotransformation rate constants are essentially equal to those derived in [7] using a bioaccumulation model. The gastro intestinal biotransformation rate constants of the test chemicals can also be found from the data of the dietary bioaccumulation test as discussed in [7].

Table 4.2 Summary of rate constants for 62-g fish in water[a]

Test chemical	$k_{T,R}$ ± SE (d^{-1})	k_M ± SE (d^{-1})	$k_{M,BCFBAF}$ (d^{-1})
1,2,3,4-tetramethylbenzene	0.24 ± 0.04	0.11 ± 0.03	0.66
β -HCH	0.18 ±0.03	0.047 ± 0.025	0.025
trans-decalin	0.042 ±0.007	-0.004 ± 0.003	0.06
9-methylanthracene	0.023 ±0.003	0.38 ± 0.03	0.22
chrysene	0.006 ±0.002	0.38 ± 0.04	0.14
hexylcyclohexane	0.005 ±0.002	0.0378 ± 0.005	0.07
2,6-dimethyldecane	0.005 ±0.002	0.18 ± 0.01	0.09
benzo[*a*]pyrene	0.004 ±0.002	0.09 ± 0.01	0.48

[a] Depuration rate constants of the nonbiotransformed chemical $k_{T,R}$ (derived from Equation 4.18); somatic biotransformation rate constants (k_M); and biotransformation rate constants calculated by the bioconcentration factor (BCF), bioaccumulation factor (BAF) quantitative structure–activity relationship (BCFBAF QSAR) program (Ver 3.00) [16] ($k_{M,BCFBAF}$) for 62-g fish in water at a temperature of 12.6 °C.

SE = standard error.

Figure 4.5a illustrates that bioconcentration factors derived by the method described above are in reasonable agreement with other bioconcentration factor measurements in fish reported in the Arnot and Gobas [15] and EpiSuite 4.11 [16] data bases. The comparison of derived and empirical bioconcentration factors for the the substances with the highest octanol-water partition coefficients (i.e., PCB 155, PCB 153 and PCB 209) are highly sensitive to the selection of the concentration of dissolved organic matter in the water, which was not measured or reported for the bioconcentration factors listed in the data bases [15,16]. Figure 4.5b shows that for the biotransformable test chemicals, the agreement between the BCFs determined in this study and those derived in other tests or through estimation are also in most cases reasonable. For benzo[a]pyrene, which has the largest octanol-water partition coefficients among the test chemicals, the discrepancy between the EPI Suite QSAR BCF and the BCF in this study is the greatest, due to possible differences in the empirical (current study) and the QSAR biotransformation estimates. Sorption of the chemical in the water to organic matter in the water column is expected to be a significant contributor to this apparent discrepancy. Differences in biotransformation capacities among fish species, fish weights, dosing concentrations and possible mixture effects may play a role as well.

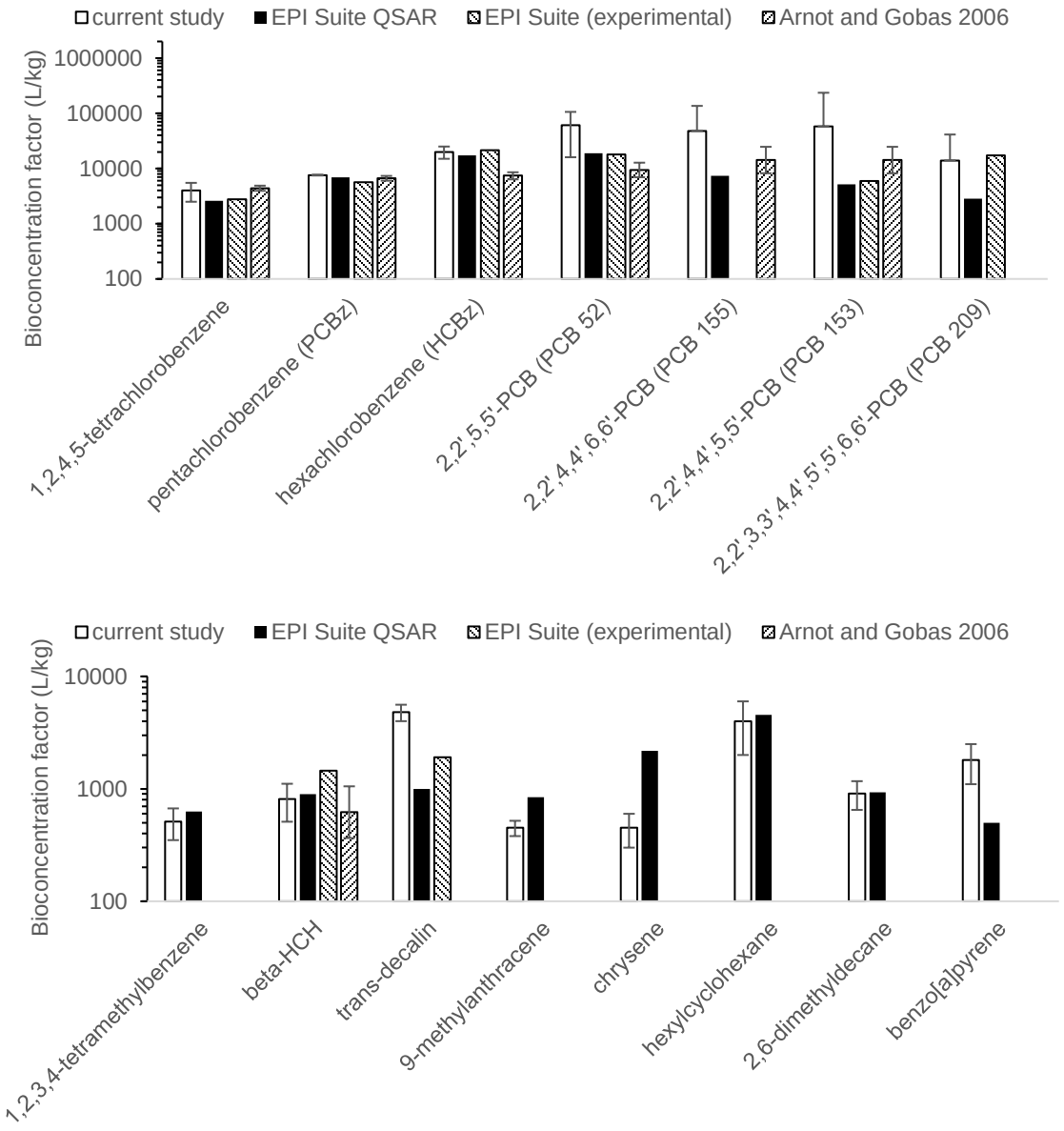

Figure 4.5 Bioconcentration factors of reference (A) and test chemicals (B) in fish determined as part of the present study (open bars) and those measured and compiled in Arnot and Gobas [15] and the US Environmental Protection Agency [16] and calculated by the bioconcentration factor/bioaccumulation factor quantitative structure–activity relationship (BCFBAF QSAR) model of EPI Suite, Ver 4.11 [16]. Error bars represent the standard error of the mean estimates. HCH = hexachlorocyclohexane; HCBz = hexachlorobenzene; PCB = polychlorinated biphenyl.

The analysis of the data from the dietary bioaccumulation shows that it is possible to derive bioconcentration factors and somatic biotransformation rate constants from dietary bioaccumulation tests. The same approach can also be applied to the

results from bioconcentration and depuration tests. A measurement of the chemical concentration in the water is not required. This provides the opportunity to conduct simplified or “stream-lined” bioaccumulation tests, where the fish are provided a dose, either through aqueous or dietary exposure for a certain period of time, followed by a depuration phase where the depuration rate constant for the test and reference chemicals are the only measurements made. It has been suggested that the depuration rate constant can be used as a metric to determine whether a chemical is bioaccumulative [17]. While there is merit to using the depuration rate constant as a bioaccumulation metric, the application of this metric is often unpractical as depuration rate constants are a function of the weight of the organism, the lipid content of the organisms, temperature, fish type, condition and metabolic capacity and other factors [9, 18, 19]. Also, regulatory criteria for depuration rate constants do not exist at this time. However, with the use of reference chemicals, which are subject to the same exposure conditions and fish characteristics as the test chemicals, the effect of organism and test specific attributes can be eliminated to a large extent. The application of reference chemicals provides a way to take advantage of the depuration rate constant as a bioaccumulation metric. For example, one reference chemical, pentachlorobenzene (PCBz) we suggest, may be used as a bright line or benchmark to distinguish between chemicals that meet the often used regulatory criterion for the bioconcentration factor of 5,000 or not. Pentachlorobenzene is a very stable chemical, hence an appropriate reference chemical with an insignificant rate of biotransformation and not significantly affected by a reduced bioavailability in the water with concentrations of dissolved organic matter in the water up to 2 mg/L (i.e. the calculated fraction of freely dissolved pentachlorobenzene in water is 0.99 at 2mg organic matter/L). Pentachlorobenzene has a log K_{OW} of approximately 5, which is the same log K_{OW} that is specified in many bioaccumulation regulations. It has a $BCF_{ww,t}$ of , 7600 ± 1700 L/kg-ww and a corresponding $BCF_{5\%,t}$ for a fish with a lipid content of 5% of approximately7600 x 5.0/6.7 = 5,700 ± 1,300 L/kg-ww which is approximately equal to the regulatory criterion value of 5,000 given the margins of error. Pentachlorobenzene also has a lipid normalized biomagnification factor close to 1 [7]. Following this approach, a test chemical can be considered to exhibit a $BCF_{ww,t}$ less than 5,000 if the depuration rate constant is significantly greater than that of pentachlorobenzene in the test. For example, in this study, chrysene has a k_T of 0.39 ± 0.12 d^{-1}, which is significantly greater than the $k_{T,R}$ of pentachlorobenzene which of 0.024 ± 0.002 d^{-1} (Table 4.1) or 0.027 ±

0.003 d^{-1} when using equation 4.9 for a chemical with a log K_{OW} of 5. In this example, chrysene, which has a log K_{OW} of 5.81, would not meet the bioconcentration criterion of 5,000. The BCF of chrysene in this study was 450 ± 170 (SE) L/kg-ww (Table 4.1), which is less than 5,000. In studies with other fish, the depuration rate constants may be quite different from those measured in this test, but the relative depuration rates between test and reference chemicals is expected to be the same. One advantage of this approach is that decisions whether a substance meets the regulatory criterion or not can be based on depuration rates which can be measured with greater precision than conventional bioconcentration factors. If pentachlorobenzene is not a good or practical reference chemical, then a more appropriate reference chemical(s) can be chosen and the relationship between k_T and K_{OW} can be used to find the k_T for a substance with a log K_{OW} of 5. The selection of reference chemicals is also important in minimizing the error in $k_{T,R}$ estimates produced by equation 4.9. Reference chemicals in the log Kow range between 3 and 5 have the greatest effect on the error of $k_{T,R}$ estimates. Reference chemicals with a very high log K_{OW} (i.e. greater than 5) tend to contribute less uncertainty. The latter is advantageous as the uncertainty in log K_{OW} values is often greatest for substances with the highest log K_{OW}. A sufficient number of reference chemicals should be selected to obtain the required level of error. Also, the reference chemicals should cover a log K_{OW} range that includes the log K_{OW} of the test chemicals. Another suggestion for reducing error is the use of a common method for characterizing log K_{OW} of test and reference chemicals because this may reduce error in the differences between K_{OW} values of test and reference chemicals. Such methods may include high pressure liquid chromatography measured retention time or a specific computational method.

One of the factors that is not captured by this approach is the contribution of gastro-intestinal transformation rates to the bioaccumulation behavior of chemicals. Gastro-intestinal biotransformation, which take place in the lumen of the intestinal tract as a result of intestinal micro-flora and gastric enzymes, has no significant effect on the determination of the BCF. However, it does have a significant effect on the biomagnification factor [7]. Biotransformation in the gastro-intestinal tract lowers the dietary uptake efficiency and reduces the BMF [7]. This means that chemicals can have a BCF of 5,000 or greater but a BMF less than 1. The occurrence of intestinal biotransformation means that the BCF and BMF are not related through a common

biotransformation rate as laid out in the bioaccumulation theory described in Mackay et al. [20]. Hence, a BCF cannot serve as an accurate proxy of the BMF if intestinal biotransformation is significant.

Another limitation of this approach is that while the lipid-water two phase resistance model has generally shown to be applicable to neutral hydrophobic chemicals that have a high affinity for lipids, it is not adequate for ionic chemicals [21,22]. Membrane transfer of ionic chemicals involves processes other than lipid and aqueous boundary layer diffusion and proteins can play an important role in the partitioning of the chemical between water and fish.

We conclude that it is possible to derive bioconcentration factors and in-vivo somatic biotransformation rate constants from in-vivo dietary bioaccumulation experiments and “simplified” or “stream lined” bioaccumulation experiments that only measure depuration rates. Such tests can provide considerable cost savings over traditional bioconcentration tests, reduce test completion times and reduce animal use. The approach can also be applied retrospectively to derive bioconcentration factors from dietary bioaccumulation and bioconcentration tests that have been performed in the past to derive bioconcentration factors that are not compromised by errors in the measurement of the chemical concentration in the water. Many bioaccumulation studies have involved multiple analytes, which may provide adequate information to derive a relationship between $k_{T,R}$ vs. $1/K_{OW}$ relationship for a set of reference chemicals. We hope that the method will be useful in the improvement of the bioaccumulation assessment for neutral lipophilic organic commercial chemicals and that is application to ionic and proteinophilic substances will be investigated further.

4.6. Acknowledgement

The authors would like to acknowledge the National Science and Engineering Research Council of Canada for support.

4.7. Data availability

Data, associated metadata, and calculation tools are available in the Supplemental Data of Lo JC, Campbell DA, Kennedy CJ, Gobas FAPC. 2015. Somatic

and gastrointestinal in vivo biotransformation rates of hydrophobic chemicals in fish. *Environ Toxicol Chem* 34:2282–2294.

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Chapter 5.

In Vivo Biotransformation Rates of Organic Chemicals in Fish: Relationship with Bioconcentration and Biomagnification Factors*

Justin C. Lo performed the data analysis and wrote the paper with assistance from Frank A.P.C. Gobas. Daniel J. Letinski and Thomas F Parkerton conducted the feeding experiments, performed the chemical analysis, and provided the raw experimental data to Justin C. Lo.

5.1. Summary

In vivo dietary bioaccumulation experiments for 85 hydrophobic organic substances were conducted to derive in-vivo gastro-intestinal biotransformation rates, somatic biotransformation rates, bioconcentration factors (BCF) and biomagnification factors (BMF) for improving methods for bioaccumulation assessment and to develop an in-vivo biotransformation rate data base for QSAR development and in-vitro to in-vivo biotransformation rate extrapolation. The capacity of chemicals to be biotransformed in fish was found to be highly dependent on the route of exposure. Somatic biotransformation was the dominant pathway for most chemicals absorbed via the respiratory route. Intestinal biotransformation was the dominant metabolic pathway for most chemicals absorbed via the diet. For substances not biotransformed or transformed exclusively in the body of the fish, the BCF and BMF appeared to be closely correlated. For substances subject to intestinal biotransformation, the same correlation did not apply. We conclude that intestinal biotransformation and bioavailability in water can modulate the relationship between the BCF and BMF. However, this study also supports a fairly simple rule of thumb that may be useful in the interpretation of dietary bioaccumulation tests, i.e., chemicals with a BMFL < 1 tend to exhibit BCFs based on either freely dissolved (BCFww,fd) or total concentration (BCFww,t) of the chemical in the water that are less than 5,000.

5.2. Introduction

The capacity of chemicals to bioaccumulate in biota is recognized to be an important property that contributes to a substances' potential to harm wildlife. Bioaccumulation is therefore widely considered in international and national chemical management programs (*1-5*). The bioconcentration factor (BCF) is a common metric used in regulations to express the extent of chemical bioaccumulation. The chemical's octanol-water partition coefficient (K_{OW}; $C_{Octanol}/C_{Water}$) is a surrogate used to predict the extent of bioaccumulation. The field-derived bioaccumulation factor (BAF; $C_{Organism}/C_{Water}$) may also be used. Recent guidance also includes the biomagnification factor (BMF; $C_{Organism}/C_{Diet}$) and the trophic magnification factor (TMF; the antilog of the log-linear regression slope of $C_{Organism}$ vs. trophic level) and recommends a weight of evidence approach in bioaccumulation assessments (*6-8*). However, to date, the BCF often remains the preferred metric used in regulatory evaluations. The BCF is typically measured in laboratory bioconcentration tests, where organisms (e.g. fish) are exposed to the chemical via water. The preferred method for the determination of the BCF conforms with guidelines developed by the OECD (*9*). Current OECD protocols for bioaccumulation testing provide options for tests involving both aqueous and dietary exposure. Bioaccumulation tests are typically costly; time consuming; and require substantial animal use. An alternative to such testing, is the use of bioaccumulation models. These models have shown to be successful at estimating the BCF and BAF for chemicals that are not biotransformed considerably in the organism (*10-11*) but overestimate the extent of bioaccumulation for chemicals that are biotransformed (*12*). This bias is due to the fact that the models do not *a priori* incorporate predictions of the biotransformation rates of chemicals in organisms. To develop methods for improving BCF estimates of the many thousands of chemicals in commerce requiring evaluation, several research initiatives have developed. One initiative involves the back calculation of biotransformation rates from BCFs using the AQUAWEB model (*10*) and the subsequent development of a quantitative structure activity relationship (QSAR) for biotransformation that is incorporated in the US EPA EPI Suite program for estimating BCFs (*13-16*). A second initiative uses *in-vitro* measurements of chemical depletion rates in liver homogenates and hepatocytes, which are then extrapolated to make estimates of whole organism biotransformation rates, and then used as input to extrapolation models to estimate BCF values (*17-23*). This initiative aims to make BCF

determinations less labor intensive, cheaper, and less animal-use intensive. A third initiative, explored in this study, involves the development of simplified test designs (involving less animals, labor and costs than typical OECD bioconcentration tests) to measure *in-vivo* biotransformation rates and corresponding BCFs of chemicals. This research serves to fill an important data gap for biotransformable substances because it provides actual measurements of biotransformation rates of chemicals in whole animals. The biotransformation rate data can be used to test the ability of *in-vitro* and QSAR-based methods to reliably estimate *in-vivo* biotransformation rates and BCFs.

Generally accepted bioassays for the measurement of *in-vivo* biotransformation rates do not exist to date. Previous work on experimentally deriving *in-vivo* biotransformation rates revealed that *in-vivo* biotransformation involve both hepatic and gastro-intestinal biotransformation rates and that the contribution of somatic and gastro-intestinal biotransformation to bioaccumulation is dependent on the route of chemical exposure (*24*). This means that BCFs and BMFs may be affected by biotransformation in different ways. However, in most bioaccumulation models, *in-vivo* biotransformation is viewed as depuration from the body (i.e. somatic) only (*25*), hence affecting BCF and BMFs in a similar way.

The objective of the present study is to develop and apply a method for simultaneously determining *in-vivo* gastro-intestinal biotransformation rates, somatic biotransformation rates, BCFs and BMFs. Such a test methodology does currently not exist. A second goal is to provide measurements of *in-vivo* biotransformation rates for a number of structurally diverse chemicals to allow the testing and further development of quantitative structure activity relationships for predicting biotransformation rates and the testing of extrapolation methods for estimating *in-vivo* rates from *in-vitro* biotransformation rate data. Such a biotransformation data base is also not available to date. A third objective is to investigate the relationship between the BCFs and BMFs for substances subject to somatic and gastrointestinal biotransformation. This information is also not available. The main purpose of the study is to make bioaccumulation determinations for substances more accurate, efficient, and less costly, while reducing animal use.

5.3. Theory

5.3.1. Bioaccumulation model for in vivo biotransformation studies

To describe the contribution of biotransformation of chemicals in the soma (i.e. somatic biotransformation including hepatic metabolism) and in the gut of the fish (i.e. gastro-intestinal biotransformation in the lumen of the intestines due to intestinal microflora and gastric enzymes), the fish is divided into two compartments, i.e., the body (B) and the gastrointestinal content or digesta (G). The following mass balance for the body of the fish describes this process (*24*):

$$dC_B/dt = k_{B1}\cdot C_{WD} + (k_{GB}/(k_{GB} + k_{GE} + k_{GM}))\cdot(G_I / W_B)\cdot C_D - (k_{B2} + k_{BG}\cdot((k_{GE} + k_{GM})/(k_{GB} + k_{GE} + k_{GM})) + k_{GD} + k_{BM})\cdot C_B \quad (5.1)$$

where C_B is the concentration of the chemical in the body of the fish (mol/kg fish); G_I is the food ingestion rate (kg food$\cdot d^{-1}$), C_{WD} is the freely dissolved concentration of the chemical in the water (mol chemical$\cdot L^{-1}$); C_D is the concentration of the chemical in ingested diet (mol chemical$\cdot$kg food^{-1}); W_B is the weight of the fish (kg) on a wet weight (ww) basis; k_{B1} is the uptake clearance rate for respiratory uptake (L water$\cdot$kg ww fish$^{-1}\cdot d^{-1}$); k_{B2}, k_{GB}, k_{BG}, k_{GD}, k_{BM}, k_{GE}, and k_{GM} are the rate constants (d^{-1}) for respiratory elimination, chemical transfer from the gastrointestinal content to the fish body; chemical transfer from the fish body to the gastrointestinal content, growth dilution, biotransformation of the chemical in the body of the fish (i.e. somatic biotransformation), fecal egestion of the gastrointestinal content, and biotransformation of the chemical in the gastrointestinal content, respectively; and t is time (d).

This equation can be simplified by recognizing that $k_{GB} / (k_{GB} + k_{GE} + k_{GM})$ in Equation 5.1 is the dietary uptake efficiency for a substance that is biotransformed in the gastro-intestinal tract ($E_{D,M}$) and that (G_I/W_B) is the proportional feeding rate F_D expressed as the fraction of the fish's body weight consumed in food per day:

$$dC_B/dt = k_{B1}\cdot C_{WD} + E_{D,M}\cdot F_D\cdot C_D - (k_{B2} + k_{BG}\cdot(1 - E_{D,M}) + k_{GD} + k_{BM})\cdot C_B \quad (5.2)$$

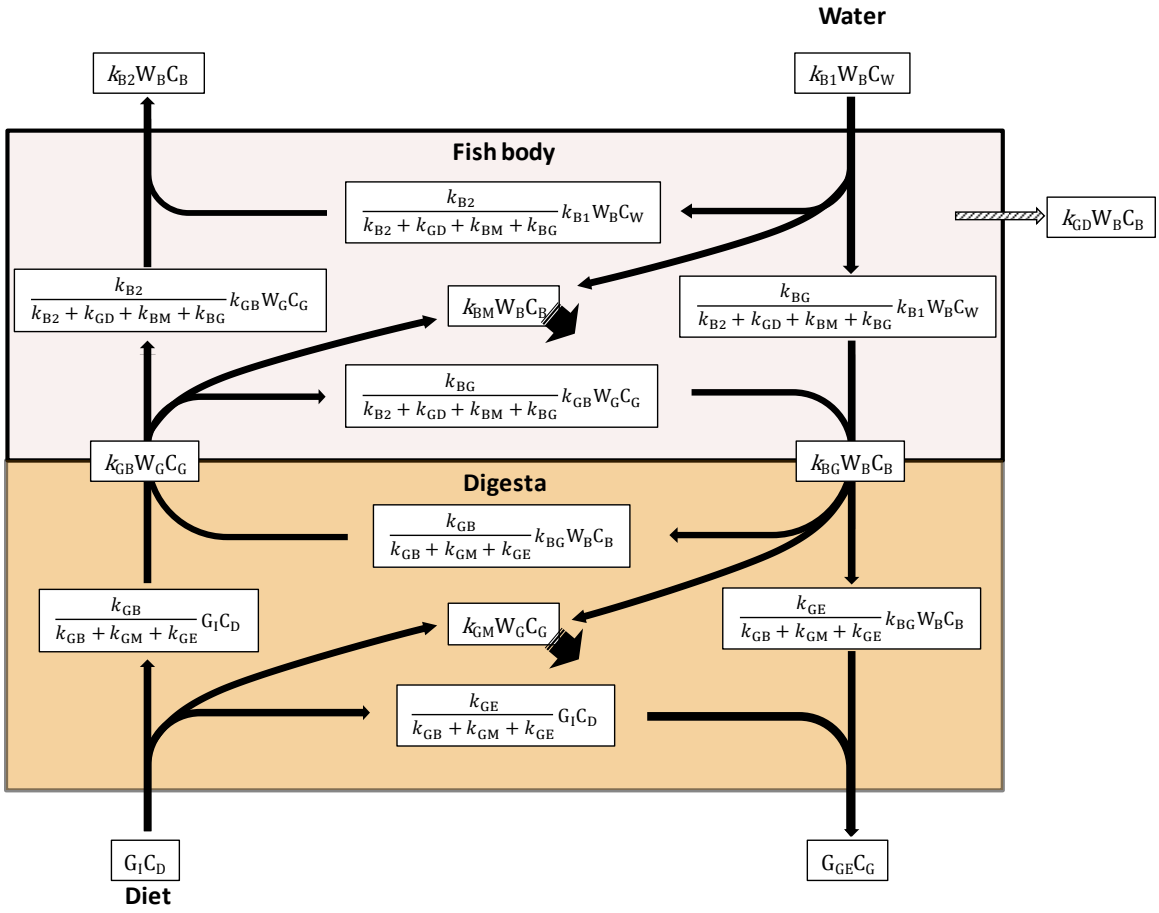


Figure 5.1 Detailed schematic diagram of the chemical fluxes in a 2 compartment model separating the fish body from the contents of the digestive tract illustrating the role biotransformation (represented by the ➘ arrow) in the body (k_{BM}) and the gastrointestinal tract (k_{GM}) of the fish.

A detailed derivation of the model can be found in Lo et al. (*24*). Figure 5.1 shows that intestinal biotransformation includes both (i) chemical transformation upon ingestion and (ii) chemical transformation upon chemical elimination from the body of the fish into the lumen. Likewise, the chemical flux biotransformed in the soma also consists of dual contributions, i.e. (i) chemical transformation upon respiratory uptake and (ii) chemical transformation upon chemical transport from the lumen into the fish body. The total chemical flux biotransformed due to gastrointestinal biotransformation (i.e. $k_{GM}\cdot M_G$) or somatic biotransformation (i.e. $k_{BM}\cdot M_B$) is therefore dependent on the route of chemical intake. The application of the mass balance approach to determine biotransformation rates is a frequently used strategy in biotransformation research. It is based on the assumption that loss of mass of the test chemicals relative to non-

biotransformable reference chemicals is due to biotransformation. This research strategy can derive overall biotransformation rates of chemicals, but lacks the capacity to detect individual biotransformation products. It complements research focused on detection of specific metabolites.

5.3.2. Somatic biotransformation rate constant (k_{BM})

Under conditions of first order kinetics of biotransformation and transport kinetics, the somatic biotransformation rate constant (k_{BM}) can be determined from measurements of the depuration rate constants when using non-biotransformable reference chemicals (*24*) since, for biotransformed chemicals, the total elimination rate constant in the body (k_{BT}) is:

$$k_{BT} = k_{B2} + k_{BG}\cdot(1 - E_{D,M}) + k_{GD} + k_{BM} \quad (5.3)$$

while for the non-biotransformed reference chemicals, the total depuration rate constant of the chemical from the body of the fish ($k_{BT,R}$) is

$$k_{BT,R} = k_{B2} + k_{BG}\cdot(1 - E_{D,N}) + k_{GD} \quad (5.4)$$

where $E_{D,N}$ is the dietary uptake efficiency for a non-biotransformed substance, i.e. $E_{D,M}$ but with a k_{BM} of 0. The somatic biotransformation rate constant in the body of the fish can therefore be determined as:

$$k_{BM} = k_{BT,R} - k_{BT} \quad (5.5)$$

where $k_{BT,R}$ is depuration rate constant of a reference chemical with the same K_{OW} as that of the test chemical. For substances with a log Kow > 3, the following linear regression model (*26*) can be used to determine $k_{BT,R}$:

$$k_{BT,R} = (1/\omega)\cdot(1/K_{OW}) + \beta \quad (5.6)$$

where $1/\omega$ and β are regression coefficients in units of d^{-1}. The intercept β represents the $k_{BT,R}$ for a substance with an infinite K_{OW} and hence can be approximated with k_{GD}. As described in Gobas and Lo (26), $1/\omega$ represents the increase in resistance to chemical transport from the fish to the water with increasing $K_{OW,}$ and is a function of the lipid content fish body Φ_{BL} and the body weight of the fish (*27*). To derive a relationship

between $k_{BT,R}$ and $1/K_{OW}$ that can account for the differences in growth rates, lipid contents, and body weights between the multiple bioaccumulation tests of the present study, Equation 5.6 was rewritten as:

$$k_{BT,R} = (\alpha \cdot W_B^b / \Phi_{BL}) \cdot (1/K_{OW}) + k_{GD} \quad (5.7)$$

where α and b are allometric coefficients, describing the fish's body weight dependence of the water phase transport parameter. It should be stressed that when following this method for deriving biotransformation using structurally different test and reference chemicals, it is inherently assumed that K_{OW} is the most important chemical-specific factor controlling the non-biotransformation related depuration kinetics of non-ionic hydrophobic substances.

5.3.3. Respiratory uptake and elimination rate constants and the Bioconcentration Factor (BCF)

As detailed Appendix C, the wet weight based BCF based on the freely dissolved concentration of the chemical in the water ($BCF_{WW,fd}$) can also be derived as:

$$BCF_{WW,fd} = k_{B1}/k_{BT} = (\alpha \cdot W_B^b/d_L)/k_{BT} \quad (5.8)$$

BCFs calculated in this fashion are kinetic BCFs at steady-state based on freely dissolved concentrations of the chemical. BCFs based on the total concentration of the chemical in the water ($BCF_{WW,t}$) measured in OECD 305 style aqueous exposure tests and considered in most regulations are based on a total chemical concentration in the water and are lower than those calculated here, especially for very hydrophobic chemicals due to their high binding affinity to organic matter in the water. The $BCF_{WW,fd}$ based on freely dissolved concentrations of the chemical in the water can be converted into the $BCF_{WW,t}$ following equations by Burkhard (*28*) or Arnot and Gobas (*10*) based on equilibrium partitioning of the chemical between the water and dissolved organic matter:

$$BCF_{ww,t} = BCF_{ww,fd}.(1 + \chi_{OC}.K_{OC})^{-1} \quad (5.9)$$

where χ_{OC} is the concentration of organic carbon in the water (kg/L), respectively. K_{OC} is the equilibrium partition coefficient of the chemical between organic carbon and water. The $BCF_{ww,t}$ and $BCF_{ww,fd}$ can be expressed on a lipid normalized basis as $BCF_{L,t}$, i.e.

$BCF_{L,t} = BCF_{WW,t} / \Phi_{BL}$ and $BCF_{L,fd} = BCF_{WW,fd} / \Phi_{BL}$ respectively, or expressed as a BCF for fish with a lipid content of 5%, i.e. $BCF_{5\%,t} = 0.05 \cdot BCF_{L,t}$ and $BCF_{5\%,fd} = 0.05 \cdot BCF_{L,fd}$ respectively, if the BCF follows a linear relationship with the lipid content of the fish (e.g. lipophilic chemicals).

5.3.4. Gastrointestinal biotransformation (k_{GM})

Under conditions of first order kinetics of biotransformation and transport kinetics, the intestinal biotransformation rate constant (k_{GM}) can be determined from measurements of the dietary uptake efficiencies for biotransformable test chemicals ($E_{D,M}$) and non-biotransformable reference ($E_{D,N}$) chemicals (*24*) as:

$$k_{GM} = (E_{D,M}^{-1} - E_{D,N}^{-1}) \cdot (E_{D,N} / (1-E_{D,N})) \cdot (G_{GE} / W_G) \quad (5.10)$$

where G_{GE} (kg digesta·d^{-1}) is the fecal egestion rate and W_G (kg) is the steady state amount of digesta in the gastrointestinal tract. As described in Lo et al. (*24*), G_{GE} can be estimated from the dietary ingestion rate G_I, i.e. the product of the proportional feeding rate F_D and the weight of the body of the fish W_B, and the food assimilation efficiency γ_{GI} as $\gamma_{GI} \cdot G_I$. W_G can be estimated as the ratio G_I/δ, where δ is the digesta evacuation rate constant (d^{-1}), which can be approximated by the 95% digesta evacuation time ($t_{E,95}$) as $3/t_{E,95}$, as explained in the Appendix C.

5.3.5. Biomagnification factors (BMF)

BMFs can be determined from the dietary uptake efficiency and the depuration rate constant as:

$$BMF_{WW} = k_{BD} / k_{BT} = E_{D,M} \cdot F_D / k_{BT} \quad (5.11)$$

The BMF_{WW} can be expressed on a lipid normalized basis as BMF_L, i.e. $BMF_L = BMF_{WW} \cdot (\Phi_{DL} / \Phi_{BL})$. The BMF_L expresses true chemical magnification, i.e. an increase in chemical potential (or activity) that occurs as a result dietary bioaccumulation. The BCF_L can also be expressed in a form that approximates the maximum theoretical bioconcentration at equilibrium by dividing BCF_L by K_{OW}, for substances for which octanol adequately represents the partitioning properties of the chemical in the lipids of the fish (*6*).

5.4. Materials and Methods

5.4.1. General

To measure somatic and intestinal biotransformation rates, dietary uptake efficiencies, BCFs and BMFs of a range of neutral hydrophobic organic chemicals in rainbow trout, ten dietary bioaccumulation tests (i.e. 9 studies performed at Exxon Mobil Biomedical Sciences. Inc. (EMBSI) and one study (*24*), conducted at Simon Fraser University (SFU)) following a similar methodology were carried out. The SFU study complements the EMBSI study by providing reference chemicals that cover the range of log K_{OW} of the test chemicals in the EMBSI study. Details of the study at SFU can be found in (*24*). The tests performed at EMBSI are described below. Methods for chemical and lipid content analyses are in the Appendix C.

5.4.2. Fish

Juvenile rainbow trout (*Oncorhynchus mykiss*) were obtained from Thomas Fish Company. Fish were kept in 31 L flow-through aquaria, with a flow through rate of approximately 5-7 replacement volumes per day. An average of 53 (SE 5) test fish were used in each test. Water temperatures were kept at 13.6 (SD 0.3) ºC, approximately the same to the 12.6 ºC in Lo et al. (*24*). Water contained a mean dissolved oxygen content of 9.0 (SD 0.2) mg/L, and the pH was 7.7 (SD 0.2). The mean fish weight from all experiments was 1.5 (SD 0.5, range 0.9 – 2.3) g, and the mean fish lipid content was 3.6 (SD 0.8, range 2.4 – 5.6) % wet weight (Table C1). Fish were fed Finfish Starter, #1 crumble (Zeigler Bros., Inc., Gardners, PA, USA) an average of 3 (SD 1) % bodyweight·d^{-1}. The dietary lipid content in the studies ranged between 15 and 15.6 %, and was slightly lower than the value of 18.6% used in Lo et al. (*24*). In each feeding study, a control fish group was present to monitor for effects and to account for potential background concentrations of the test substances in fish tissues. Effects monitored in both test and control fish groups included mortality, growth rate constants, changes in feeding behavior (any deviations from rapid feeding), and other adverse effects including physical attributes (e.g. pigmentation, etc.), lethargy, and swimming behavior.

5.4.3. Chemicals

Test chemicals included parent and alkylated aromatic hydrocarbons, cycloalkanes, and linear and branched aliphatic hydrocarbons, musk xylene and methoxychlor. The log K_{OW} of the test chemicals were obtained from EpiSuite 4.11 and varied between 3.3 and 8.9 (Table C2). All 9 dietary bioaccumulation tests included the reference chemical hexachlorobenzene which was assumed to undergo no or negligible biotransformation. The test chemical trans-decalin was also considered a reference chemical because previous work found trans-decalin to resist somatic biotransformation in rainbow trout (*24*). In each of 9 tests, 5 to 14 test and reference chemicals (Table C2) were dissolved in corn oil and added to the feed. Individual chemicals in the test mixture were selected to provide diverse hydrocarbon structures of varying hydrophobicity and facilitate use of a common analytical method while avoiding toxicity to exposed fish. Motivations for investigating multiple test compound exposures rather than individual chemicals were to reduce vertebrate animal use, testing costs and time required for *in-vivo* data collection. The content of corn oil spiked to diet was 0.5%. The chemical concentrations in the diet were measured in triplicate at the beginning and end of the uptake period to confirm the stability of the chemical in the food, as described in Appendix C where the mean and standard deviation of diet exposure concentrations are reported in Table C2.

5.4.4. Dietary bioaccumulation studies

Fish were fed a contaminated diet for 10 to 14 days, followed by a 3-24 day depuration phase with no chemical exposure. Diets contained an average of 11 (range 5-14) chemicals per test (Table C2). Fish were sampled throughout the uptake and depuration phase, with 3-10 fish sampled for each time point. The whole fish were homogenized and used for chemical extraction. The methodology for the kinetic analysis of the data is included in Appendix C.

5.5. Results and Discussion

5.5.1. Diet

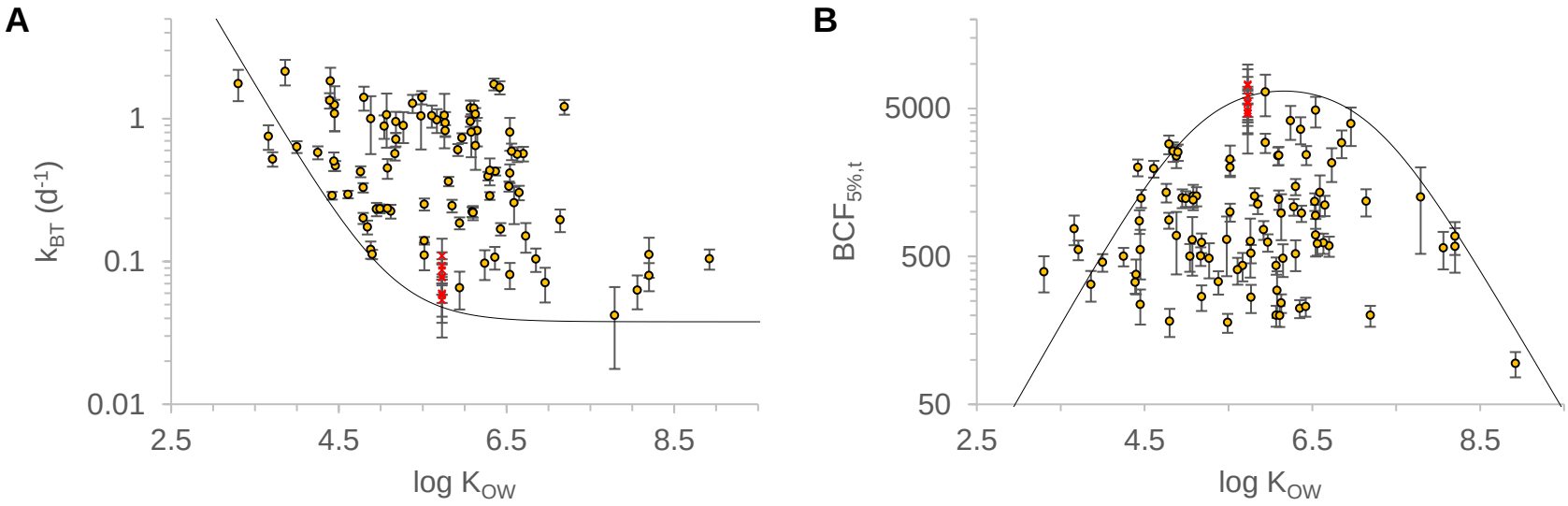
Measured concentrations of the various test chemicals in the diet ranged from 370 to 1171µg/g (Table C2). Concentrations of the test and reference chemicals in the diet did not appear to change significantly throughout the exposure period, as evidenced by the low standard deviations of the dietary concentration measured throughout the exposure period and the associated low coefficient of variation for concentrations in fish foods from the beginning and end of the exposure period.

5.5.2. Fish

No fish mortalities, changes in feeding behavior, or other apparent adverse effects were observed in the exposure and control groups of all experiments. Also, there was no evidence of a difference in growth rates (k_{GD}) between control and test groups (Table C1). Mean fish body weights increased over time. The growth rates, calculated as the slope of the natural logarithm of the fish weight vs. time for each of the 9 experiments varied from 0.027 to 0.047 d^{-1} with a mean of 0.04 (SD 0.01) d^{-1}. Starting fish weights among 9 experiments ranged between 0.88 and 2.3 g with a mean value of 1.5 (SD 0.5) g (Table C1). The lipid content of the fish body among the tests varied between 2.4 and 5.6%. The mean lipid content of the fish's diet was 15.5% (range 15-15.6%) (Table C1).

5.5.3. Concentrations of chemicals in the fish body

Concentrations of the test and reference chemicals in the control fish groups were below their limits of quantitation. In all cases, the mean concentration of the chemicals in the test fish body increased throughout the dietary exposure phase, with certain chemicals approaching an apparent steady state before the end of the exposure period. During the depuration phase, mean concentrations in the fish body decreased in an apparent log-linear fashion, with concentrations of certain chemicals decreasing below detectable levels before the end of the depuration period (Figure C1).


A
1
0.1
0.01
k_{BT} (d^{-1})
2.5
4.5
6.5
8.5
log K_{OW}
B
5000
500
50
$BCF_{5\%,t}$
2.5
4.5
6.5
8.5
log K_{OW}

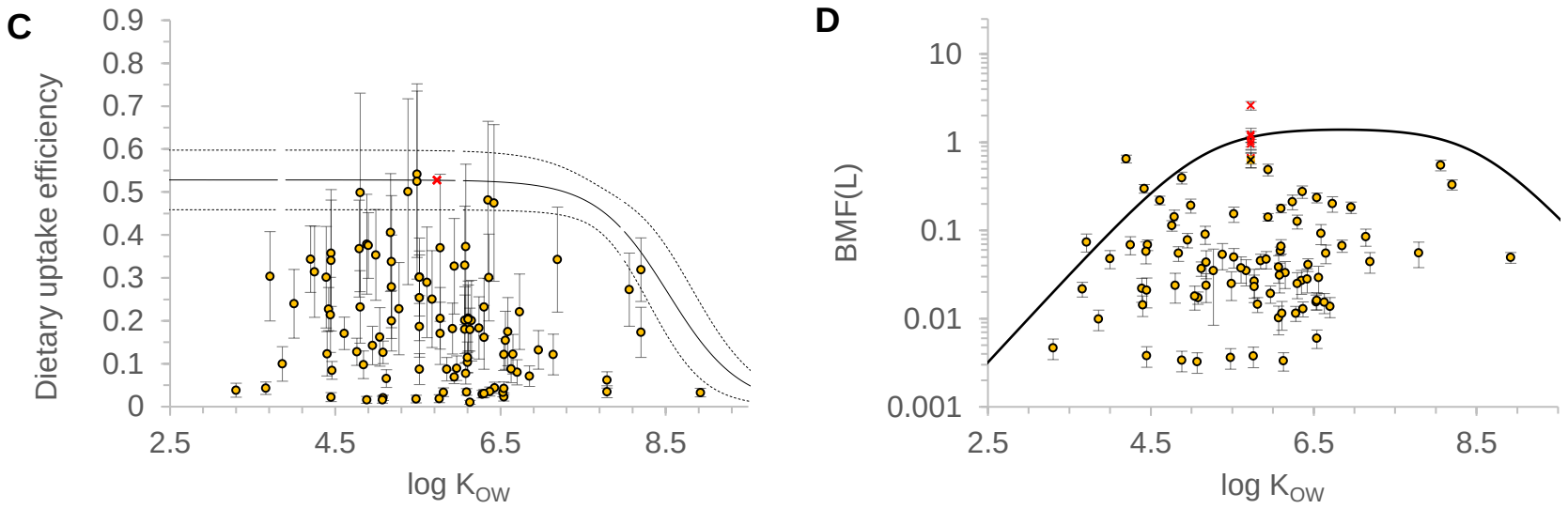


Figure 5.2 **(A) The total depuration rate constant from the fish body, k_{BT}. (B) The bioavailability-corrected bioconcentration factor normalized to 5% lipid, $BCF_{5\%,t}$. (C) The dietary uptake efficiency ($E_{D,M}$ for test chemicals, $E_{D,N}$ for reference chemicals) normalized to hexachlorobenzene in each test. Solid and dashed lines represent predicted 95% confidence intervals of the predicted $E_{D,N}$ (Equation 5.13). (D) The lipid normalized biomagnification factor BMF_L (bottom right) versus log K_{OW}. Solid lines in Figures 5.2A, 5.2C, and 5.2D are parameterized to fish with mean experimental values of W_B = 1.5 g, Φ_{BL} = 5%, k_{GD} = 3.7%·d^{-1}, Φ_{DL} = 15% and F_D = 3.5% ·$kg.kg^{-1}.d^{-1}$. Error bars represent the standard error of the mean values. The red × represent the reference chemicals in each test.**

5.5.4. Depuration rate constants

Total depuration rate constants from the fish body for the reference ($k_{BT,R}$) and test (k_{BT}) chemicals (Table C2) decreased with increasing K_{OW} (Figure 5.2A). This is due in part to the decrease in respiratory elimination with increasing hydrophobicity. A weighted multiple linear regression (regression weights = SE^{-1}) of the depuration rate constants of the reference chemicals ($k_{BT,R}$) in the study by Lo et al. (*24*) (n = 8) and in the present study (n = 10) using Equation 5.7 for the juvenile rainbow trout produced the following relationship:

$$k_{BT,R} = [291(SE\ 34)W_B^{[-0.19(SE\ 0.02)]} / \Phi_{BL}]\cdot(1/K_{OW}) + k_{GD},\ n=18,\ RMSE = 0.15 \quad (5.12)$$

where fish body lipid content (Φ_{BL}), fish body weight (W_B), and the growth rate (k_{GD}) from populations of test fish were specific to each experiment (Table C1). Empirical $k_{BT,R}$ values for each test chemical are reported in Table C2. Figure C3 shows that all test chemicals exhibited depuration rate constants that were equal to or greater than the $k_{BT,R}$, with the exception of naphthalene, 1,3,5-trimethylbenzene, and cis-bicyclo(4,3,0)nonane, which exhibit some of the lowest reported K_{OW} values of the chemicals in the present study. These chemicals may illustrate the limits of the current study design for determining somatic biotransformation rates. For these low Kow chemicals, respiratory elimination rate constants are high, making it difficult to obtain reliable values of relatively low biotransformation rate constants, which are derived as the difference between the high depuration rates of both test and reference chemicals.

5.5.5. Somatic biotransformation rate constants

The somatic biotransformation rate constant (k_{BM}) estimates for the test chemicals are listed in Table C2. Somatic biotransformation rate constants of 6 of the test chemicals in the EMBSI studies were in good agreement with those in Lo et al. (*24*) after the somatic biotransformation rate constants are normalized to the same size fish following Arnot et al. (*14*) (Figure C4). A comparison of empirical k_{BM} and body-weight normalized k_M values estimated by the BCFBAF QSAR (EpiSuite 4.11) illustrates some agreement between k_{BM} and k_M values (Figure 5.3). A regression analysis of the empirical log k_{BM} data and BCFBAF QSAR log k_M estimates indicates a correlation

coefficient (r^2) of 0.23 and that k_M estimates are approximately within 2 orders of magnitude of the empirical k_{BM} data in 95% of cases. It should be stressed that because fish were exposed to multiple chemicals at a single concentration for each chemical, there is the potential that both competitive inhibition and enzyme saturation effects affect the measured biotransformation rates. The in-vivo biotransformation rate constants reported in this study may therefore be more conservative (i.e. lower) than in single compound experiments where dietary chemical concentrations are lower than those used in this study and/or competing substrates are absent.

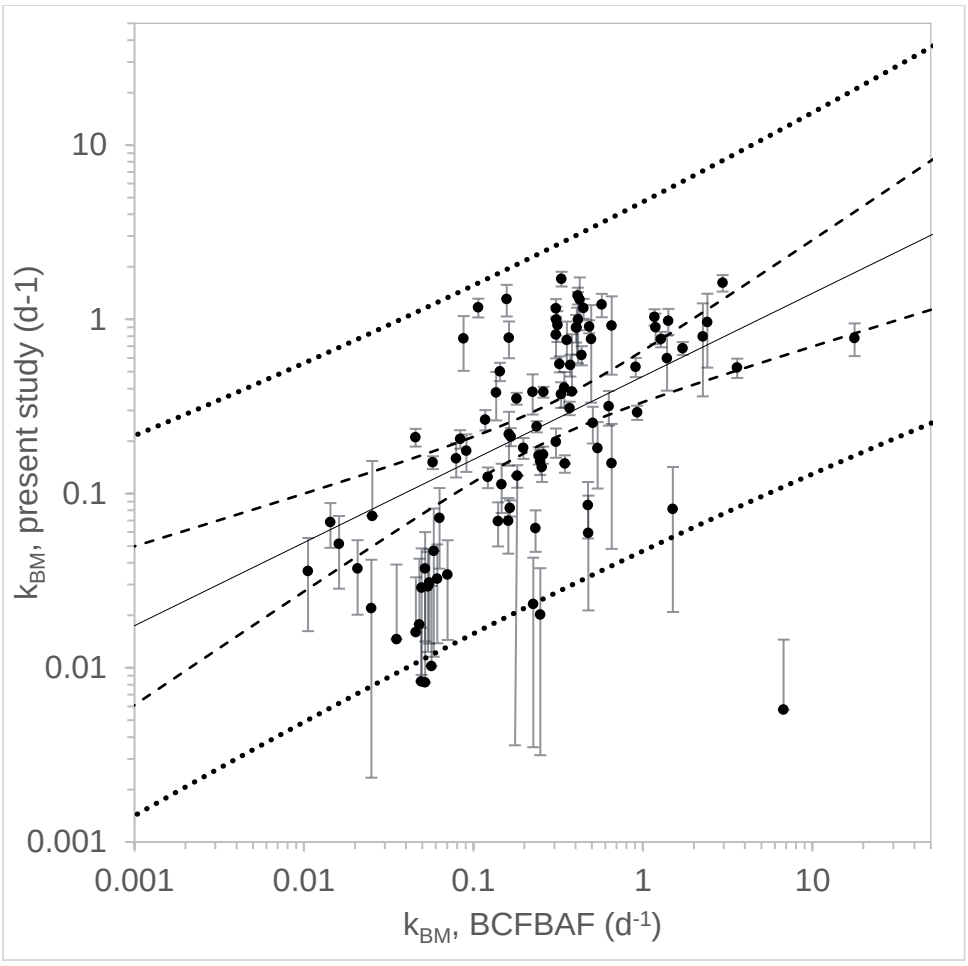


Figure 5.3 Observed somatic biotransformation rate constants (k_{BM}) from the present study as a function of BCFBAF QSAR (EPI Suite v. 4.11) predicted biotransformation rate constants (k_{BM}), normalized to the same fish weights as those corresponding to the observed values. Solid line represents the mean regression fit, dashed lines represent the 95% confidence intervals of the predicted mean and the dotted lines represent the 95% prediction interval of individual chemicals.

5.5.6. Dietary Uptake Efficiency

The following relationship between $E_{D,N}$ and K_{OW} (Figure C5) observed for the reference chemicals from Lo et al. (*24*) (n = 7) was used to determine intestinal biotransformation rate constants:

$$E_{D,N}^{-1} = 5.6 \times 10^{-9}\,(\text{SE } 1.8 \times 10^{-9}) \times K_{OW} + 1.9\,(\text{SE } 0.1) \quad (5.13)$$

In all tests, the dietary assimilation efficiencies of test chemicals were either equal to or less than the dietary assimilation efficiencies of the corresponding reference

chemicals (Figure 5.2C). This indicates that the $E_{D,N}$-K_{OW} relationship serves as a reasonable reference point for deriving intestinal biotransformation rates in fish species.

5.5.7. Gastro-intestinal biotransformation rate constants

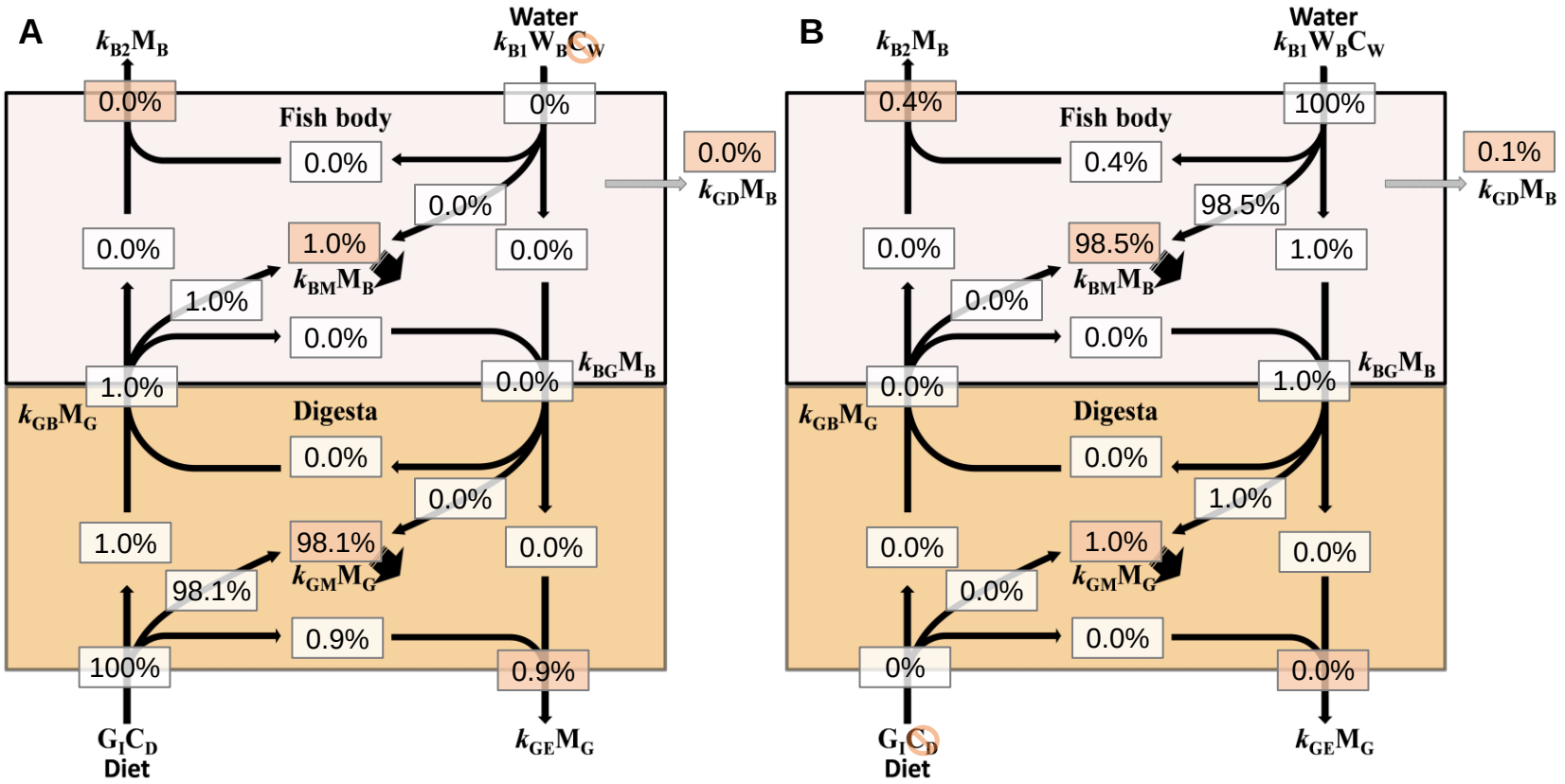
Estimates of kGM are listed in Table C4. For the majority of chemicals tested in this study, there are no gastro-intestinal biotransformation rate data that can be used for comparison. However, benzo[a]pyrene and related polycyclic aromatic hydrocarbons (PAHs) have shown low dietary uptake efficiencies in rainbow trout (30-33) and are significantly biotransformed into water soluble metabolites in the intestines of fish, with 50% (for un-induced animals) and 90% (for induced animals) recovery of benzo[a]pyrene from the portal vein in the form of metabolites (34). These findings are in agreement with the findings from this study and further indicate the importance of gastrointestinal biotransformation on bioaccumulation of many substances in fish.

5.5.8. Respiratory uptake and elimination rate constants and BCF

The bodyweight-scaled respiratory uptake rate constant (k_{B1}) and elimination rate constant (k_{B2}) were derived as $\alpha \cdot W_B^b/d_L$ and $(\alpha \cdot W_B^b/\Phi_{BL}) \cdot (1/K_{OW})$ respectively, based on the regression coefficients α of 291 (SE 34), b of -0.19 (SE 0.02) (Equation 5.12), the density of lipid d_L of 0.9 and the lipid content of the fish (Φ_{BL}) in each experiment and listed in Table C3. $BCF_{WW,fd}$ derived as k_{B1}/k_{BT}, and $BCF_{ww,t}$ and corresponding values that are adjusted to a 5% lipid content ($BCF_{5\%,t}$ t) are listed in Table C3. The mean $BCF_{ww,fd}$ and a $BCF_{ww,t}$ of all test chemicals were less than 5,000. The mean $BCF_{5\%,t}$ was also less than the regulatory criterion of 5,000 for all chemicals except hexadecahydropyrene. No test chemical was found to exhibit a $BCF_{ww,fd}$, $BCF_{ww,t}$ or $BCF_{5\%,t}$ significantly (p=0.05) greater than 5,000. There appears little correlation between the $BCF_{5\%,t}$ and K_{OW} for the test chemicals (Figure 5.2B), caused in large part by the fact that the majority of test chemicals are biotransformed at rates that exceed respiratory elimination rates. The lack of correlation between $BCF_{5\%,t}$ and K_{OW} suggests caution in the derivation of the $BCF_{5\%,t}$ from $BCF_{ww,t}$ as linearity between the $BCF_{ww,t}$ and the fish's lipid content may not exist for biotransforming chemicals.

5.5.9. Dietary uptake and excretion rate constants and BMF

Rate constants for gastro-intestinal exchange (k_{GB}, k_{BG}) and fecal egestion (k_{GE}), derived from the experimental observations according to Lo et al. (*24*), are listed in Table C5. The BMFs, derived as $(F_D \cdot E_{D,M})/k_{BT}$ are reported in Table C4 for all test chemicals and are also expressed on a lipid normalized basis (as BMF_L in units of L dietary lipid/L fish body lipids) in Figure 5.2D. The BMF_L of all test chemicals were less than 1. Only the reference chemical hexachlorobenzene exhibited a BMF_L greater than 1. There appeared to be no relationship between the BMF_L and K_{OW}, likely as a result of the high biotransformation rates of the test chemicals in both the soma and gastro-intestinal contents of the fish.


A
$k_{B2}M_B$
Water
$k_{B1}W_BC_W$
0.0%
Fish body
0%
0.0%
0.0%
0.0%
0.0%
$k_{GD}M_B$
1.0%
$k_{BM}M_B$
0.0%
1.0%
0.0%
$k_{BG}M_B$
1.0%
0.0%
$k_{GB}M_G$
Digesta
0.0%
0.0%
1.0%
98.1%
$k_{GM}M_G$
0.0%
98.1%
100%
0.9%
0.9%
G_IC_D
Diet
$k_{GE}M_G$
B
$k_{B2}M_B$
Water
$k_{B1}W_BC_W$
0.4%
Fish body
100%
0.4%
98.5%
0.1%
$k_{GD}M_B$
0.0%
98.5%
1.0%
$k_{BM}M_B$
0.0%
0.0%
$k_{BG}M_B$
0.0%
1.0%
$k_{GB}M_G$
Digesta
0.0%
1.0%
0.0%
1.0%
$k_{GM}M_G$
0.0%
0.0%
0%
0.0%
G_IC_D
Diet
$k_{GE}M_G$

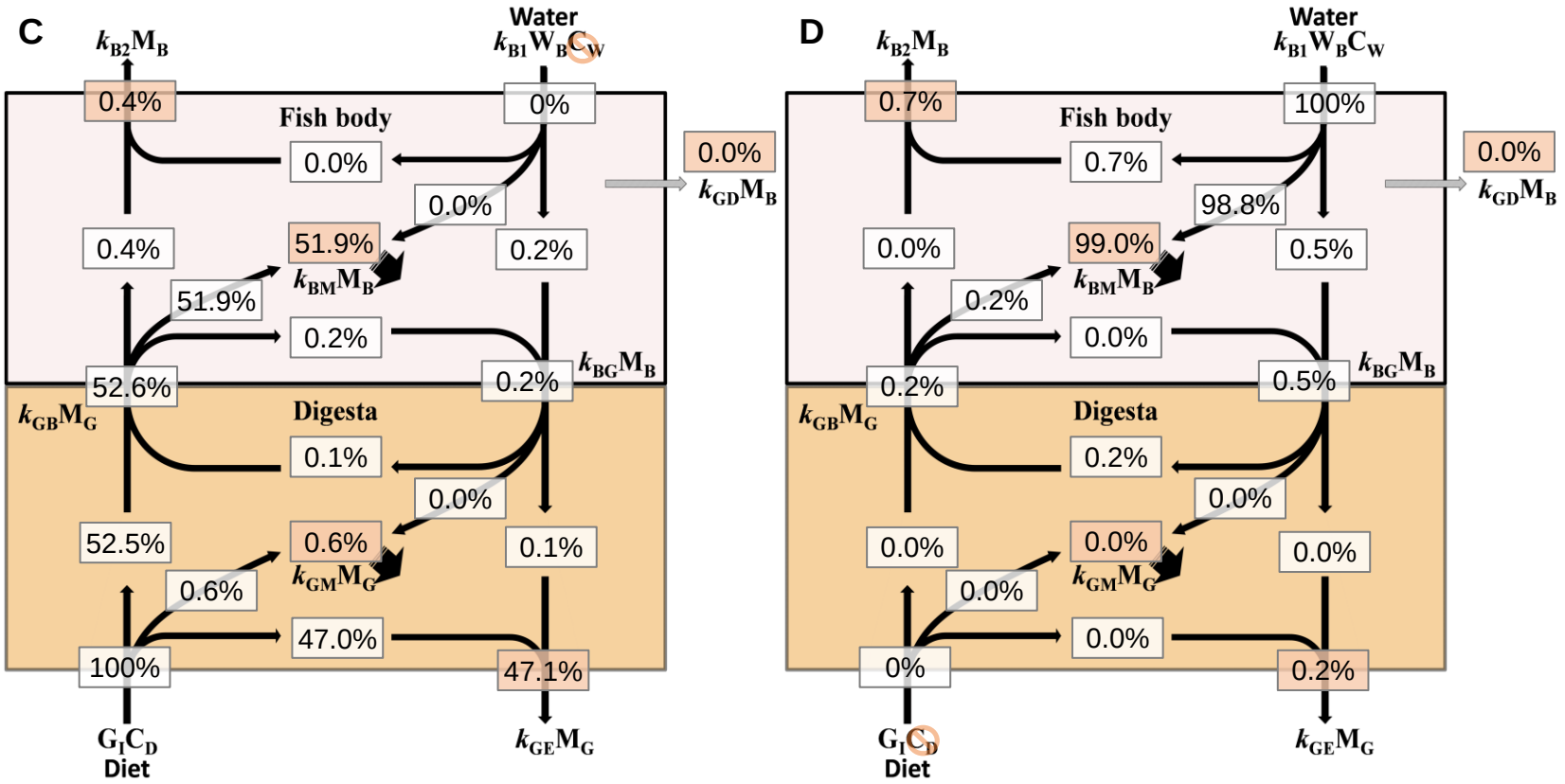
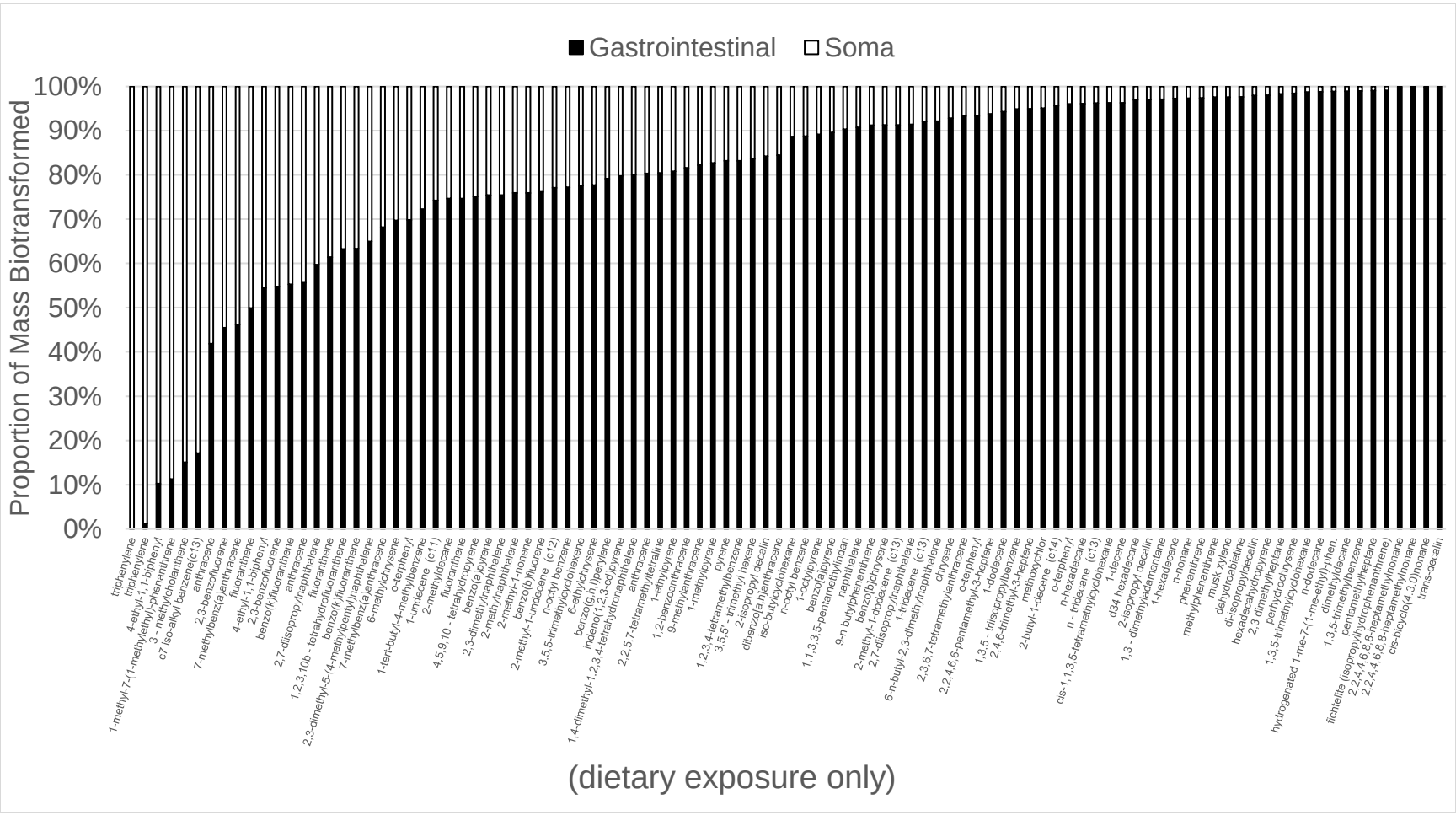


Figure 5.4 **Detailed schematic diagram of the internal distribution dynamics, expressed in terms of the fraction of the administered chemical intake rate in units of grams per day for benzo[*a*]pyrene from test #3 (A,B) and triphenylene test #7 (C,D) in a dietary-only exposure environment (A,C) and in an aqueous-only exposure environment (B,D). Biotransformation (represented by the ↘ arrows) occurs in the body (k_{BM}) and the gastrointestinal tract (k_{GM}) of the fish. Red boxes indicate routes of elimination in the fish model.**

5.5.10. Internal distribution

The ability of the test design to derive the various rate constants (Tables C1-C5) in the bioaccumulation model (Figure 5.1) from the empirical data allows for evaluation of the internal distribution of the test and reference chemicals in fish and to determine the contribution of transport and transformation processes to the bioaccumulation behavior of the chemical under various exposure scenarios. Figure 5.4, which shows chemical transport and transformation fluxes as a fraction of the total intake flux from either a dietary or aqueous exposure, illustrates that benzo[*a*]pyrene is biotransformed in both the digesta and in the body (including the liver) of the fish. When exposed via the diet, the great majority of benzo[*a*]pyrene (i.e. 98.1%) is biotransformed in the intestinal tract. Vetter et al. (*35*) also demonstrated rapid benzo[*a*]pyrene metabolism in the intestines of fish. Bock et al. (*36*) demonstrated that benzo[*a*]pyrene is extensively metabolized during the passage through the gastrointestinal tract of the rat. Figure 5.4 also shows that when fish are exposed via the respiratory route, the great majority of benzo[*a*]pyrene (i.e. 98.5%) is biotransformed in the body of the fish. For benzo[*a*]pyrene, both somatic and intestinal biotransformation appear to play an important role in chemical bioaccumulation and the relative contribution of the soma and intestines as a site for biotransformation is largely controlled by the relative concentrations of benzo[*a*]pyrene in the diet and water of the fish. Figure 5.4 shows that this is not the case for triphenylene. Triphenylene appears to be virtually recalcitrant in the intestinal tract while it is quickly biotransformed in the fish body. Upon ingestion, approximately half the ingested dose of triphenylene is egested in an untransformed state in fecal matter. The other half of the ingested dose is absorbed into the body of the fish and then almost fully biotransformed in fish body. Upon respiratory uptake via water exposure, virtually all triphenylene is biotransformed in the fish body.


Gastrointestinal
Soma
Proportion of Mass Biotransformed
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
triphenylene
triphenylene
4-ethyl-1,1-biphenyl
1-methyl-7-(1-methylethyl)-phenanthrene
3 - methylcholanthrene
c7 iso-alkyl benzene(c13)
anthracene
2,3-benzofluorene
7-methylbenz(a)anthracene
fluoranthene
4-ethyl-1,1-biphenyl
2,3-benzofluorene
benzo(k)fluoranthene
anthracene
2,7-diisopropylnaphthalene
fluoranthene
1,2,3,10b - tetrahydrofluoranthene
benzo(k)fluoranthene
2,3-dimethyl-5-(4-methylphenyl)naphthalene
7-methylbenz(a)anthracene
6-methylchrysene
o-terphenyl
1-tert-butyl-4-methylbenzene
1-undecene (c11)
2-methyldecane
fluoranthene
4,5,9,10 - tetrahydropyrene
benzo(a)pyrene
2,3-dimethylnaphthalene
2-methylnaphthalene
2-methyl-1-nonene
benzo(b)fluorene
2-methyl-1-undecene (c12)
n-octyl benzene
3,5,5-trimethylcyclohexene
6-ethylchrysene
benzo(g,h,i)perylene
1,4-dimethyl-1,2,3,4-tetrahydronaphthalene
indeno(1,2,3-cd)pyrene
anthracene
2,2,5,7-tetramethyltetraline
1-ethylpyrene
1,2-benzoanthracene
9-methylanthracene
1-methylpyrene
pyrene
1,2,3,4-tetramethylbenzene
3,5,5' - trimethyl hexene
2-isopropyl decalin
dibenzo(a,h)anthracene
iso-butylcyclohexane
n-octyl benzene
1-octylpyrene
benzo(a)pyrene
1,1,3,3,5-pentamethylindan
9-n butylphenanthrene
naphthalene
benzo(b)chrysene
2-methyl-1-dodecene (c13)
2,7-diisopropylnaphthalene
1-tridecene (c13)
6-n-butyl-2,3-dimethylnaphthalene
2,3,6,7-tetramethylnaphthalene
chrysene
2,2,4,6,6-pentamethyl-3-heptene
o-terphenyl
1,3,5 - triisopropylbenzene
1-dodecene
2,4,6-trimethyl-3-heptene
methoxychlor
2-butyl-1-decene (c14)
o-terphenyl
n-hexadecane
n - tridecane (c13)
cis-1,1,3,5-tetramethylcyclohexane
1-decene
d34 hexadecane
2-isopropyl decalin
1,3 - dimethyladamantane
1-hexadecene
n-nonane
phenanthrene
methylphenanthrene
musk xylene
dehydroabietine
di-isopropyldecalin
hexadecahydropyrene
2,3 dimethylheptane
perhydrochrysene
1,3,5-trimethylcyclohexane
hydrogenated 1-me-7-(1-me-ethyl)-phen.
n-dodecane
dimethyldecane
1,3,5-trimethylbenzene
pentamethylheptane
fichtelite (isopropylhydrophenanthrene)
2,2,4,4,6,8,8-heptamethylnonane
2,2,4,4,6,8,8-heptamethylnonane
cis-bicyclo[4.3.0]nonane
trans-decalin
(dietary exposure only)

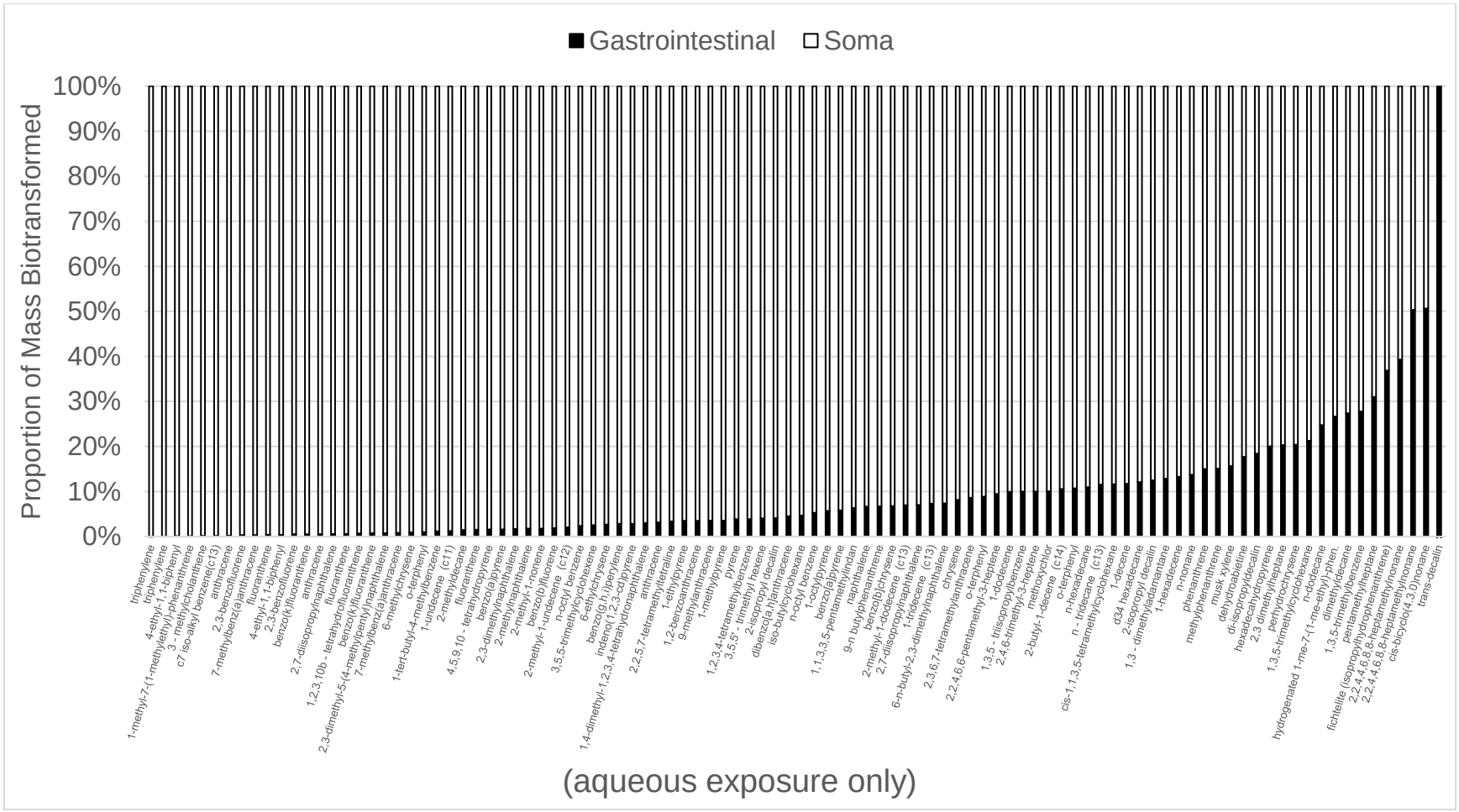


Figure 5.5 Contribution of somatic (white) and gastro-intestinal (black) biotransformation to the overall mass of chemical biotransformed in continuous dietary exposure environments (top) and in continuous water exposure environments (bottom).

Figure 5.5A illustrates the relative contribution of somatic and gastro-intestinal biotransformation for all the test chemicals when exposed only through the diet. It shows that for the majority of the ingested test chemicals, gastro-intestinal biotransformation contributes the majority of a substance's biotransformation. Figure 5.5B shows that test chemicals exposed via the respiratory route are in most cases primarily biotransformed in the fish body. In real world exposure scenarios, where exposure occurs via both respiratory and dietary routes, the relative contribution of somatic and intestinal biotransformation and hence the rate of biotransformation will depend on the relative concentrations of the chemical in the water and the diet. Figure C6 shows that for the chemicals tested there is not a general relationship between the somatic and gastro-intestinal biotransformation rate constants. This suggests that biotransformation pathways and associated metabolic stability of a chemical in the liver and the intestinal tract may differ substantially. These findings suggest that while biotransformation rate determinations in hepatocytes, liver tissues or liver homogenates such as S9 and liver microsomes are useful measures of somatic biotransformation rates, they do not fully characterize the ability of biotransformation processes in the fish to mitigate bioaccumulation of chemicals. Extra-hepatic biotransformation in the intestinal tract due to digestive and intestinal mucosal enzymes and resident bacteria is recognized for many food components and chemicals (*37*). The development of *in-vitro* bioassays for gastro-intestinal biotransformation may be a useful contribution to on-going hepatic *in-vitro* to *in-vivo* extrapolation methods for bioaccumulation assessments. Further research is needed to understand the role of fish enzymes and microflora on biotransformation in the intestinal tract and to characterize biotransformation pathways in the gut. Further analysis of dietary *in-vivo* bioaccumulation test data in relation to chemical structure may support the development of quantitative structure-activity relationships for both somatic and gastrointestinal biotransformation that are needed to advance *in-silico* methods for improving bioaccumulation model predictions.

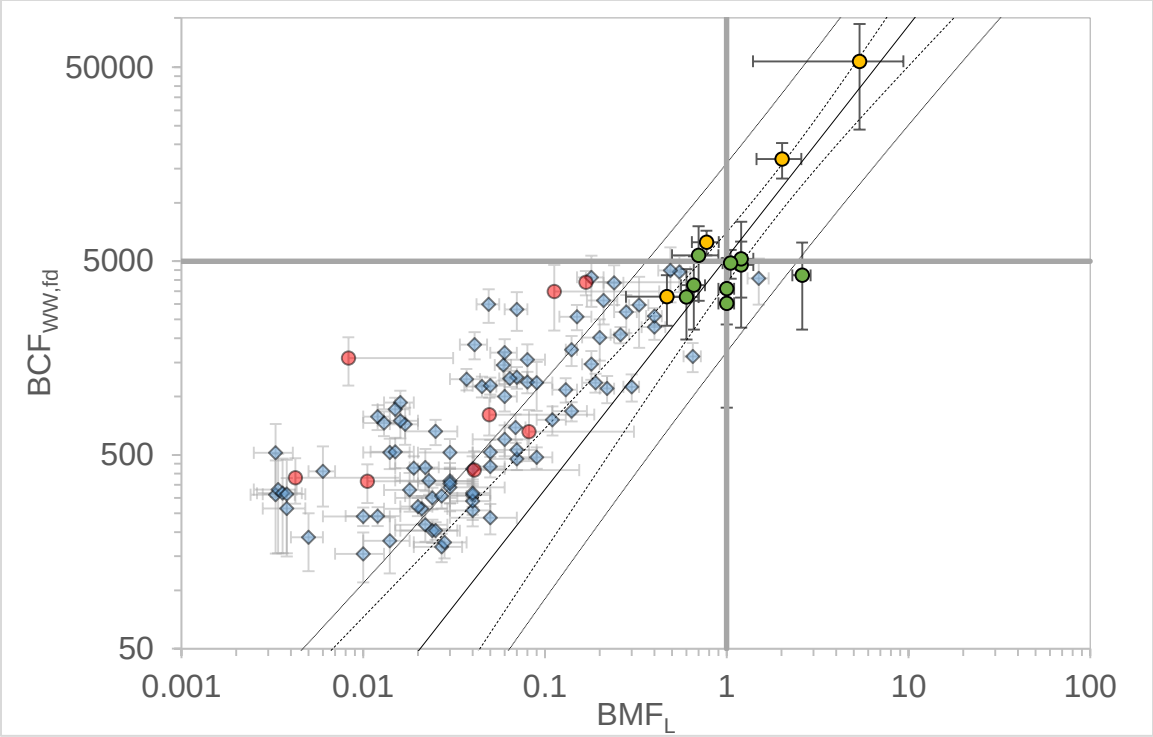


Figure 5.6 **$BCF_{ww,fd}$ vs BMF_L for test chemicals (blue and red circles) and reference chemicals (green and yellow circles) from present study (green, blue) and Lo et al. 2015 (yellow, red). The solid black line represents the predicted log BMF_L – log $BCF_{ww,fd}$ linear regression of the reference chemicals. Dotted lines represent the 95% prediction interval. The grey lines represent the regulatory criteria for BCF of 5000 and a BMF_L of 1 indicating chemical biomagnification.**

5.5.11. Relationship between BCF and BMF

Because the testing methodology explored in this study produces both BCF and BMF estimates, the relationship between the BCF and BMF can be explored. The BCF-BMF relationship is useful in interpreting data from dietary bioaccumulation tests in terms of the BCF required by regulations. Figure 5.6 illustrates that, within a single test, the freely dissolved wet weight BCF in rainbow trout, ($BCF_{ww,fd}$) and the lipid-normalized BMF (BMF_L) of non-biotransformable substances, represented by the reference chemicals, are closely related:

$$\log BCF_{ww,fd} = 1.20(SE\ 0.11)\cdot \log BMF_L + 3.72(SE\ 0.06),\ n=16,\ r^2=0.90,\ p < 0.001 \quad (5.14)$$

The close relationship between the $BCF_{WW,fd}$ and BMF_L is due to the fact that both the BCF and the BMF are a function of the same depuration rate constant (k_{BT}). The theoretical basis for the relationship between the BCF and BMF has been discussed in more detail in Mackay et al. (*25*). Equation 5.14 shows that if a non-biotransformable substance exhibits a $BCF_{WW,fd}$ equal to regulatory criterion of 5,000, then the mean predicted BMF_L can be expected to be approximately 1.0 with lower and upper 95% confidence intervals of 0.8 and 1.3 respectively. This confirms that the regulatory criterion for the BCF of 5,000 is a reasonable threshold for identifying non-biotransformable chemicals that have significant biomagnification potential. Non-biotransformable substances with $BCF_{WW,fd}$ less than the regulatory criterion of 5,000 do not show a significant biomagnification potential (Figure 5.6). Substances that are biotransformed exclusively in the body of the fish also adhere to the same $BCF_{WW,fd}$-BMF_L relationship applicable to non-biotransforming substances because somatic biotransformation contributes to the whole body depuration rate that controls both the biomagnification and bioconcentration factors. However, Figure 5.6 shows that a loss of the $BCF_{WW,fd}$ -BMF_L relationship occurs for substances that are biotransformed in the intestines of the fish. In all cases, intestinal biotransformation produces BMF_L that are less than expected from the $BCF_{WW,fd}$ -BMF_L relationship described by Equation 5.14. Gastro-intestinal biotransformation lowers the effective concentration of the chemical in the intestinal tract and reduces the chemical's dietary uptake efficiency. For substances subject to intestinal biotransformation, the BCF has a tendency to overestimate the biomagnification potential of substances. Substances that are significantly transformed in the intestinal tract do not have a biomagnification potential and may be of less concern for bioaccumulation than chemicals that biomagnify (i.e. BMF >1). Bioaccumulation tests using aqueous exposure can only identify the bioconcentration behavior of the test chemical because it is insensitive to the intestinal biotransformation rate. Dietary bioaccumulation tests, which are often less costly, time-involved and labor intensive than bioconcentration tests are more insightful than standard bioconcentration tests because of their ability to provide information on somatic and intestinal biotransformation rates as well as the BMF and BCF. Empirical correlations between the BCF and BMF (38), which are attractive because of their ability to express data from dietary bioaccumulation tests in terms of the regulatory required BCF pose considerable limitations since the correlation is highly sensitive to the inclusion of chemicals subject to high rates of intestinal biotransformation and low bioavailability in water. As this study

shows many hydrophobic organic chemicals are subject to intestinal biotransformation and exhibit a reduced bioavailability in water due to their high sorption potential to organic matter in the water phase. While this study demonstrates that intestinal biotransformation and bioavailability in water can modulate the relationship between the BCF and BMF, this study also supports a fairly simple rule of thumb that may be useful in the interpretation of dietary bioaccumulation tests, namely that chemicals with a BMF_L < 1 tend to exhibit BCFs based on either freely dissolved ($BCF_{ww,fd}$) or total concentration ($BCF_{ww,t}$) of the chemical in the water that are less than 5,000.

5.6. Acknowledgment

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Chapter 6.

In Vitro to In Vivo Extrapolation of Biotransforming Hydrophobic Chemicals in the Fish Body*

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6.1. Summary

In vitro and in vivo studies of four highly hydrophobic test chemicals in rainbow trout were conducted to test the ability of in vitro bioassays to assess the biotransformation capacity of chemicals in fish. In vitro studies with S9 liver extracts using solvent delivery and sorbent phase dosing methods were used to determine in vitro biotransformation rates as a function of the concentration of the test chemical in the in vitro assay and as a function of pre-exposure. In-vivo studies in rainbow trout were conducted to measure in vivo biotransformation rate constants. A method for the extrapolation of in vitro to in vivo biotransformation rate constants was developed and tested. In vitro assays showed good reproducibility of in vitro biotransformation rate constants among various methods with sorbent phase dosing experiments providing a cost effective alternative to multiple solvent delivery based tests required to elucidate the concentration dependence of the biotransformation rate constant. Somatic biotransformation rate constants of the test chemicals normalized to a 10 g fish derived from in vitro biotransformation rate constants through the in vitro to in vivo extrapolation (IVIVE) method described in this paper were in good agreement with in vivo measurements of somatic biotransformation rate constants for all test chemicals except 9-methylanthracene. This study points out the potential for IVIVE to support bioaccumulation assessment but also identifies the potential for “false negatives” where in vitro biotransformation assays indicate a low potential for biotransformation while in

vivo studies exhibit a higher potential for biotransformation. Fine-tuning of in vitro assays and additional efforts to test the IVIVE approach for bioaccumulation assessment are put forward as strategies to further improve bioaccumulation assessment through in vitro testing.

6.2. Introduction

A chemical's potential to bioaccumulate in fish is an important factor to consider when assessing its ability to cause harm to organisms. Bioaccumulation is a fundamental component of chemical classification schemes under international and national environmental programs (*1*-*5*). Under these regulatory programs, the laboratory-based bioconcentration factor (BCF; $C_{Organism}/C_{Water}$) is often a preferred metric to identify bioaccumulative substances. Canada also evaluates bioaccumulation using the bioaccumulation factor (BAF; $C_{Organism}/C_{Water}$) metric (*5*). In the European Union, the biomagnification factor (BMF; $C_{Organism}/C_{Diet}$) and the trophic magnification factor (TMF; antilog of the log-linear regression slope of $C_{Organism}$ vs trophic level) are also considered in a weight-of-evidence approach for bioaccumulation assessments (*6*-*7*). Although it is not preferred, the octanol-water partition coefficient ($K_{OW;}$ $C_{Octanol}/C_{Water}$) can be employed in the absence of organism-derived BCF and BAF data. Unfortunately, BCF and BAF data are often not available for many substances (*8*). As a result, most bioaccumulation assessments are based on K_{OW} or K_{OW}-based model estimates of the BCF (*9*-*10*). Recent bioaccumulation models have the capacity to include biotransformation by calculating in vivo biotransformation rate constants. Recently developed methods to derive in vivo biotransformation rate constants in the body of the fish from uptake and depuration studies in fish indicate that model estimated and empirical biotransformation rate constants are generally within the same order of magnitude, but that there may be considerable uncertainty in model estimated BCFs. Biotransformation plays a major role in chemical elimination, particularly for hydrophobic chemicals with a log K_{OW} larger than 5 which have the potential to bioaccumulate. For very high K_{OW} substances, the rates of chemical elimination in organisms are very low, causing biotransformation to be a major factor. While much progress has been made on estimating and measuring biotransformation rate constants, large data gaps remains and more efforts is needed.

An established method for including biotransformation in bioaccumulation assessments is through standardized OECD 305 in vivo aqueous bioconcentration tests

(*11*), where fish are exposed to a chemical in water. However, OECD 305 style in vivo tests are costly, time consuming, and require the use of many animals for each test. With thousands of commercial chemicals requiring classification and risk assessments, there are several initiatives to improve bioaccumulation assessments in a more cost and time effective manner, while also using less animals in testing.

One initiative to improve on the delivery of bioaccumulation assessments for commercial chemicals is through streamlined in vivo testing. The OECD 305 guideline was updated to include the option for dietary exposure tests, which are easier to conduct along with the option for multiple chemical testing for each feeding study to save costs (*11*). In addition to the BMF measurements, other valuable parameters such as empirical BCF measurements, the somatic biotransformation rate constant (k_{BM}), and the gastrointestinal biotransformation rate constant (k_{GM}) can also be measured in these dietary tests with some minor adjustments (*12-14*). While still requiring live animals in testing, these streamlined in vivo methods can more efficiently provide information for a weight-of-evidence approach to bioaccumulation assessments. Moreover, data gathered from these tests can act as benchmark values to further develop other initiatives for improving bioaccumulation assessments. A second initiative uses *in silico* methods to improve bioaccumulation assessments. This involves the back calculation of somatic biotransformation rates from BCF data using a bioaccumulation model such as AQUAWEB (*9*) and developing qualitative structure-activity relationships to somatic biotransformation rate constants (*15-17*). This approach is currently used in the US EPA EPI Suite program for estimating BCFs (*10*). A look at over 90 empirical in vivo somatic biotransformation rate constants normalized to the same size fish showed a general agreement between the empirical data and the EPI Suite QSAR estimates, but the 95% prediction interval spans approximately 2 orders of magnitude (*14*), consistent with the 1.5-1.7 orders of magnitude reported in Arnot et al. (*15*). While useful as a screening tool, the precision needs to be improved before bioaccumulation assessments can be based on in silico methods. A third initiative to improve bioaccumulation assessments is through in vitro testing. In this approach, chemical depletion bioassays using liver S9 extracts, microsomes, or hepatocytes are measured (*18-22*). This is followed by in vitro to in vivo extrapolation (IVIVE) models to make in vivo predictions for use in chemical assessments. Current IVIVE efforts for use in bioaccumulation assessments are based on physiologically based pharmacokinetic (PBPK) approaches (*23-24*). The IVIVE

approach estimates hepatic clearance in the whole animal, converts it to a first order rate constant, which is then used in a bioaccumulation model to estimate a BCF for use in chemical assessments. This approach has demonstrated its potential for improving BCF estimates of chemicals that have been shown to be metabolizable in in vitro bioassays (*18-20*). This PBPK approach is based on commonly used IVIVE methods from pharmaceutical research, in which hepatic drug clearance is a function of hepatic blood flow (*25-27*). This approach requires estimates for parameters such as cardiac output, fraction of blood flow through liver, and the volume of distribution (relative chemical concentration in the body to the blood at steady state) – parameters which may not be readily available. Potentially bioaccumulative substances, however, are very hydrophobic and their biotransformation capacity may not be limited by blood flow. Therefore, there is potential to simplify the IVIVE approach for potentially bioaccumulative substances. An alternative approach for the in vitro to in vivo extrapolation of bioaccumulation has been proposed for use in mammals (*28*).

The objective of the present study is to develop and test an IVIVE approach for fish to predict in vivo somatic biotransformation rate constants in fish. This study details the results of both in vitro (liver S9) and in vivo biotransformation experiments in rainbow trout. First order in vitro biotransformation rate constants of 4 poly aromatic hydrocarbons, measured using both thin-film sorbent dosing experiments and multiple solvent delivery-based experiments, are extrapolated and compared with first order in vivo biotransformation rate constants of the same test chemicals in the same species. Previous efforts in testing the IVIVE approach involved the comparison of extrapolated and empirical bioconcentration factors. In this study, the IVIVE approach is tested by comparing extrapolated and empirical biotransformation rate constants. This approach avoids the potential influence of confounding factors associated with the uptake of chemicals from water and the depuration of chemicals by depuration routes other than biotransformation. In the present study, *in vivo* biotransformation rates derived from *in vitro* studies using S9 rainbow trout liver extracts (*22*), which are then extrapolated to in vivo somatic biotransformation rates, which are then compared to in vivo somatic biotransformation rates in rainbow trout measured in dietary in vivo bioaccumulation experiments (*12, 14*). The direct comparison of predicted and observed in vivo biotransformation rate constants in rainbow trout allows for the assessment of the performance of the IVIVE method for bioaccumulation assessments. The overall goal of

the study is to make evaluations of bioaccumulation of commercial chemicals more precise and time efficient, less costly, while reducing animal use.

6.3. Theory

6.3.1. Modeling approach to IVIVE

In bioaccumulation assessment, one-compartment (*29-30*) and two-compartment (*12*) animal models are often used to describe the bioaccumulation of chemical contaminants. This modeling approach is consistent with the complexity of bioaccumulation tests, in which, in most cases, only concentrations of chemicals in the whole-body of the fish are measured (*11*). In pharmaceutical research, on the other hand, it is common practice to make measurements of the concentrations of drugs in multiple tissues (e.g. blood, muscle, organs, etc.) along with physiological parameters (e.g. cardiac output, renal excretion, etc.). Physiologically based pharmacokinetic (PBPK) animal models for pharmaceutical chemicals are therefore often more complex, using the information available from the associated tests. This approach is often necessary to investigate the specific requirements for potential drug candidates, i.e., favorable absorption, metabolic stability, tissue distribution, and elimination for assessing the optimal therapeutic dose at a target site in the body. The objectives in bioaccumulation assessments, on the other hand, are relatively straightforward i.e. to determine whether a chemical is bioaccumulative or not. Therefore, models used to describe the bioaccumulation behavior of contaminants often consider the concentration of the chemical in the whole body of the animal. In this paper, we describe an IVIVE method that is developed to use the available information from in vitro S9 fish liver extract (*22*) and the associated in vivo bioaccumulation (*12-13*) experiments. This method is designed for poorly water-soluble chemicals and also considers nonlinearities in elimination kinetics due to saturable in vitro metabolism (*22*).

In pharmacology, the elimination of chemicals (e.g. through hepatic biotransformation) is often expressed in terms of a clearance rate describing the volume of plasma from which a substance is completely removed per unit time (*27*). This is useful in pharmaceutical applications where drug responses are often related to the concentration of the drug in plasma. Uptake and elimination processes in animal bioaccumulation models, however, are described and measured in terms of first order

clearance rates constants (in units of L water.kg body weight^{-1}.day^{-1} for gill uptake and kg prey.kg body weight^{-1}.day^{-1}) and rate constants (in units of day^{-1}) (*9, 29*). It is possible to present an IVIVE approach that is able to estimate in vivo somatic biotransformation rates from in vitro first order depletion rate constant without interconversions to hepatic clearance (*28*). This approach is a simplification of the PBPK approach used in pharmacology but suited to the goals of the bioaccumulation assessments and recognizing the current limitations in bioaccumulation testing. This approach assumes, similar to the PBPK approach used in pharmacology, that the major site of biotransformation in the body of the fish, which excludes the gastrointestinal contents, is assumed to be the liver (*27*).

The proposed method for the in vitro to in vivo extrapolation of biotransformation rates in fish can be divided in the following steps:

Step 1 – Determining the maximum in vitro biotransformation rate constant ($k_{dep,C\rightarrow 0}$)

The first step is to measure the maximum first-order in vitro depletion rate constant of the chemical at an infinitesimally low substrate concentration, $k_{dep,C\rightarrow 0}$ (min^{-1}). A substrate depletion method is preferred over product formation methods because metabolic biotransformation pathways are often unknown for chemical contaminants and multiple metabolic products may be formed. The reason for extrapolating from high to low concentration of the chemical is that (i) in vitro assays often involve concentrations that are greater than those in fish in the environment; and (ii) biological reactions exhibit a concentration dependence. If the concentration of the substance in the incubation medium C_I (µM) is sufficiently below the Michaelis constant K_M (µM), then $k_{dep,C\rightarrow 0}$ can be estimated as the slope of the linear relationship between the natural logarithm of the remaining concentration of the substrate in the incubation medium ($C_{I,t}$) and incubation time (t):

$$k_{dep,C\rightarrow 0} \approx k_{dep} = \frac{\ln\left(\frac{C_{I,t=0}}{C_{I,t}}\right)}{t} \qquad (6.1)$$

where $C_{I,t=0}$ is the initial concentration of the substrate in the incubation medium. However, if the initial concentration of the substrate in the incubation medium is in the vicinity or greater than K_M, then $k_{dep,C\rightarrow 0}$ can be estimated as (*22, 31*):

$$k_{dep,C\rightarrow 0} = \frac{k_{dep}}{1-\frac{C_{I,t=0}}{C_{I,t=0} + K_M}} \quad (6.2)$$

This method requires that K_M is known. K_M can be determined by conducting multiple in vitro assays using an appropriate range in concentrations of the chemical in the incubation medium (*22*). Lo et al. (*22*) showed that a sorbent-phase dosing method can sometimes be used to determine a depletion rate constant $k_{dep,EVA}$ which approximates $k_{dep,C\rightarrow 0}$ without having to conduct multiple solvent-delivery dosing experiments. This is due to the initial substrate concentration in the incubation medium being zero and the slow-delivery of hydrophobic chemicals from the solvent phase into the aqueous incubation medium. If there is appreciable biotransformation, this should keep $C_I << K_M$ throughout the incubation. However, K_M cannot be verified through this sorbent-phase dosing approach.

Step 2 – Determining the maximum in vitro biotransformation rate constant of the unbound chemical ($k^*_{dep,C\rightarrow 0}$)

It is generally assumed that only the fraction of unbound chemical (f_u) is subjected to biotransformation. Since potentially bioaccumulative substances are generally very hydrophobic (log K_{OW} > 5), correction for the unbound fraction in incubations is a key component in the in vitro to in vivo extrapolation process.

Austin et al. (*32*) investigated the relationship between the ratio of bound to unbound fraction in the incubation medium to log K_{OW} for bases and log $D_{ph=7.4}$ for acids and neutral organics over a wide range of hydrophobicity. Han et al. (*19*) refined this log-linear relationship by including only hydrophobic chemicals that had empirical $f_{u,inc}$ measurements below 0.9. Although this relationship was developed with rat microsomal data, Escher et al. (*33*) found that biotransformation rates in microsomal rat liver extracts was similar to those fish in fish liver S9. Currently, empirical measurements of the unbound fraction of the chemical in liver extracts exists for liver extracts with protein concentrations of 0.25 mg/ml, 1 mg/ml, and 4 mg/ml by Austin et al. (*32*), 2 mg/ml by Escher et al. (*33*), 1 mg/ml by Nichols et al. (*34*), and 3.5 mg/ml by Lo et al. (*22*). Since binding studies to measure the unbound fraction involved different substances varying in K_{OW} and different incubation media varying in protein content, we used a multiple linear

regression model to derive an empirical relationship to estimate the fraction of unbound chemical in the incubation medium of S9 liver extracts:

$$\log\left(\frac{1 - f_{u,inc}}{f_{u,inc}}\right) = \alpha \cdot \log K_{OW} + \delta \cdot \log C_{S9} + \beta \quad (6.3)$$

where $f_{u,inc}$ is the fraction unbound in the incubation medium (and [1 – $f_{u,inc}$] is the bound fraction in the incubation medium); α and δ are regression coefficients relating to octanol-water partitioning (K_{OW}); C_{S9} (mg·ml^{-1}) is the concentration of S9 protein in the incubation medium, and β is the intercept. Equation 6.3 provides estimates of $f_{u,inc}$ as a function of the chemical's K_{OW} and the protein content of the incubation medium.

The maximum in vitro biotransformation rate constant, $k_{dep,C\rightarrow 0}$, can then be normalized to the fraction of unbound substrate in the incubation medium as:

$$k^{*}_{dep,C\rightarrow 0} = \frac{k_{dep,C\rightarrow 0}}{f_{u,inc}} \quad (6.4)$$

The normalized in vitro biotransformation rate constant, $k^{*}_{dep,C\rightarrow 0}$, represents the maximum in vitro biotransformation rate constant for the freely dissolved chemical in the incubation medium.

Step 3 – Estimating the in vivo hepatic biotransformation rate constant ($k_{met,H}$)

The hepatic biotransformation rate constant, $k_{met,H}$ can be estimated through a scaling factor (SF), the fraction unbound in the liver ($f_{u,H}$), the chemical concentration in the liver (C_H), and the in vivo hepatic Michaelis constant $K_{M,H}$ (μM) as:

$$k_{met,H} = k^{*}_{dep,C\rightarrow 0} \cdot SF \cdot f_{u,H} \cdot \left(1 - \frac{C_H}{C_H + K_{M,H}}\right) \quad (6.5)$$

where the term $\left(1 - \frac{C_H}{C_H + K_{M,H}}\right)$ describes the concentration dependence of the hepatic biotransformation rate constant as a result of possible enzyme saturation. The concentration dependence of the in vivo hepatic biotransformation $k_{met,H}$ and nonlinear pharmacokinetics can be observed if the chemical concentration at the liver (C_H) approaches $K_{M,H}$. However, in environmental situations where the concentration of the substrate can often expected to be much smaller than the concentration in bioassays,

the term $\left(1-\frac{C_{\mathrm{H}}}{C_{\mathrm{H}}+K_{\mathrm{M,H}}}\right)$is likely to approach 1. In many environmental exposures where the concentration of chemicals in the liver are likely far below $K_{M,H}$, equation 6.5 can be simplified as:

$$k_{\mathrm{met,H}} = k^{*}_{\mathrm{dep,C\rightarrow 0}} \cdot SF \cdot f_{\mathrm{u,H}} \tag{6.6}$$

For liver S9 preparations, the *SF* (scaling factor; unitless) is a factor that adjusts the hepatic biotransformation rate constant to the amount of liver present in the incubation medium according to the preparation of the liver S9. The *SF* is expressed as

$$SF = \frac{V_{\mathrm{inc}}}{V_{\mathrm{S9,inc}}} \cdot \frac{V_{\mathrm{S9}}}{V_{\mathrm{hom}}} \cdot \frac{V_{\mathrm{hom}}}{V_{\mathrm{H}}} = \frac{V_{\mathrm{inc}}}{V_{\mathrm{S9,inc}}} \cdot \frac{V_{\mathrm{S9}}}{W_{\mathrm{H}}} \cdot d_{\mathrm{H}} = \frac{V_{\mathrm{inc}}}{V_{\mathrm{S9,inc}}} \cdot \gamma_{\mathrm{S9}} \cdot d_{\mathrm{H}} \tag{6.7}$$

where V_{inc}, $V_{S9,inc}$, V_{hom}, and V_H are the volumes of the incubation mixture (mL), the S9 liver extract (mL) used in the incubation assay, the supernatant of the S9 fraction (mL) collected after the centrifugation of the liver extract and the liver (mL) used in the preparation of the liver S9. The V_H can be calculated by the wet weight of the liver (W_H; g) and the density of the liver (d_H = 1.05 g/mL). γ_{S9} is the yield of S9 fraction generated per gram of liver from the experiment (i.e. V_{S9} / W_H; mL/g liver).

In the absence of empirical data, the fraction unbound of the chemical in the liver of the fish ($f_{u,H}$; unitless) can be estimated from the partitioning behavior of the chemical between 3 phases in the liver tissue (i.e. lipids, proteins, and water):

$$f_{u,H} = \frac{f_{W,H}}{f_{L,H} \cdot K_{LW} + f_{P,H} \cdot K_{PW} + f_{W,H}} = \frac{f_{W,H}}{f_{L,H} \cdot K_{OW} + f_{P,H} \cdot \chi \cdot K_{OW} + f_{W,H}} \tag{6.8}$$

where $f_{W,H}$, $f_{L,H}$, and $f_{P,H}$ are the fractions of water, lipid, and protein of the liver (v/v; unitless), respectively. K_{LW} and K_{PW} are the lipid-water and protein-water partition coefficients, respectively. For neutral hydrophobic organic chemicals, K_{LW} can be approximated by K_{OW} (*35*), while K_{PW} can be approximated by K_{OW} multiplied by the sorptive capacity of proteins relative to that of lipids (χ) and can be assumed to be approximately 0.05 for animal protein (*36*). In this study, the liver lipid content, $f_{L,H}$, is 6% and the water content of the liver, $f_{W,H}$, is 73%. The liver protein content, $f_{P,H}$, is calculated as the remaining fraction of the liver content at 21%.

Step 4 – Estimating the somatic biotransformation rate constant (k_{BM})

The somatic biotransformation rate constant, k_{BM} (d^{-1}), can be approximated from the hepatic biotransformation rate constant, $k_{met,H}$ (d^{-1}), under two assumptions, i.e. (i) the liver is the main site of biotransformation in the fish body (soma); and (ii) the rate of distribution of the chemical between tissues is fast, and that the chemical in the liver is in near-equilibrium with the rest of the organism. Under these assumptions, the somatic biotransformation rate constant k_{BM} (d^{-1}) can be calculated as

$$k_{\mathrm{BM}} = k_{met,H} \cdot \frac{M_H}{M_B} \quad (6.9)$$

where M_H and M_B are the masses of the chemical in the liver and in the whole organism (including the liver), respectively. For neutral hydrophobic organic chemicals, the ratio of M_H / M_B (unitless) can be estimated through the partitioning behavior of the chemical between 3 phases (i.e. lipids, proteins, and water) in the liver tissue and in the body:

$$\frac{M_H}{M_B} = \phi_H \cdot \frac{f_{L,H} \cdot K_{OW} + f_{P,H} \cdot K_{PW} + f_{W,H}}{f_{L,B} \cdot K_{OW} + f_{P,B} \cdot K_{PW} + f_{W,B}} \quad (6.10)$$

where ϕ_H is the volumetric fraction of the liver in the organism (v/v; unitless), that is, V_H/V_B, where V_H is the volume of the liver and V_B is the volume of the organism ; and $f_{L,B}$, $f_{P,B}$, and $f_{W,B}$ are the fractions of lipid, protein, and water of the organism (v/v; unitless), respectively. When using equation 6.8 to characterize $f_{u,H}$ in the extrapolation process, the term $f_{L,H} \cdot K_{OW} + f_{P,H} \cdot K_{PW} + f_{W,H}$ which appears both in Equations 6.8 and 6.10 cancels out.

Step 5 – Estimating the somatic biotransformation rate constant normalized to 10 g fish ($k_{BM,10g}$)

The body weight of a fish is known to have a significant effect on bioaccumulation parameters such as uptake rate constants (*13, 29*), elimination rate constants (*9, 14, 38*) and biotransformation rate constants (*39*), and ultimately bioaccumulation metrics such as the BCF (*11*) and BAF (*9*). To improve comparisons between different studies, it is therefore important to normalize the data to the weight of

the fish appropriately. In an investigation of biotransformation rate constants from several laboratory studies of different sized fish, Arnot et al. (*39*) observed that smaller fish exhibited faster biotransformation rate constants than larger fish, and used exponent of -0.25 to normalize the biotransformation rate constant to 10 g fish ($k_{BM,10g}$):

$$k_{BM,10g} = k_{BM} (10/W_B)^{-0.25} \quad (6.11)$$

Where WB is the mean wet weight (g) of the fish in the test. This allometric scaling relationship has been applied in in vitro to in vivo extrapolation methods (24) and in QSAR approaches in EPI Suite (10).

6.4. Materials and Methods

6.4.1. Overview

To test the performance of the IVIVE model, both in vitro and in vivo biotransformation experiments in rainbow trout were conducted. Experimental details of in vitro (liver S9) experiments, conducted using both thin-film sorbent dosing experiments and multiple solvent delivery-based experiments, are summarized in Lo et al. (*22*). Experimental methods for conducting the dietary in vivo bioaccumulation experiments for measuring somatic biotransformation rate constants are detailed in Lo et al. (*12, 14*).

6.4.2. Fish

Rainbow trout (*Oncorhynchus mykiss*) was selected as the model organism in the in vitro S9 (in Lo et al. (*22*) and in the present study) and in vivo (*12, 14*) bioaccumulation experiments. Parameters for the test fish in the in vitro and in vivo experiments are presented in Table D1. To achieve the necessary liver yields for conducting S9 incubation bioassays, large fish were selected for the in vitro studies (*22*). Small fish were selected in the in vivo dietary bioaccumulation experiments to increase sample size. In all in vitro and in vivo experiments, fish were acclimatized for at least 2 weeks under similar conditions.

6.4.3. Test Chemicals

9-methylanthracene (log K_{OW} = 5.07) (*40*), pyrene (log K_{OW} = 5.18) (*41*), chrysene (log K_{OW} = 5.81) (*42*), and benzo[*a*]pyrene (log K_{OW} = 6.13) (*42*) were selected as the test chemicals in the present study. All 4 polyaromatic hydrocarbons exhibited biotransformation in in vitro bioassays (Lo et al. (*22*) and present study) and dietary in vivo (*12, 14*) studies with Rainbow trout. The test chemicals also exhibit log K_{OW} in excess of 5.

6.4.4. Rainbow trout in vitro liver S9 bioassays

Maximum first-order biotransformation rate constants ($k_{dep,C\rightarrow 0}$) were measured for 9-methylanthracene, chrysene, and benzo[*a*]pyrene, and pyrene using both thin-film sorbent phase dosing and multiple solvent delivery-based in vitro S9 experiments (*22*). Additional in vitro bioassays using multiple solvent delivery-based experiments varying in initial substrate concentrations were conducted in the present study for the 4 test chemicals using fish that had been pre-exposed for the same 14 day exposure period with the same dietary formulation containing the same concentrations of the test chemicals as those used in the in vivo dietary bioaccumulation study conducted in Lo et al. (*12*). Experiments with pre-exposed fish liver were conducted to account for any potential upregulation (induction) or downregulation of biotransforming enzymes in the fish body. With the exception of the pre-exposure to the contaminated diet prior to the preparation of the liver S9, all other procedures and conditions remained the same as those in Lo et al. (*22*). Experimental details for liver S9 preparation, bioassays using the sorbent-phase dosing approach (*21-22*) and multiple solvent delivery-based experiments approach (*22, 31*), incubation conditions, chemical extraction and GC/MS analysis can be found in Lo et al. (*22*). Experimental parameters and results from the in vitro liver S9 bioassays can be found in Table D2.

6.4.5. Rainbow trout in vivo dietary bioaccumulation bioassays.

Somatic biotransformation rate constants (k_{BM}) were measured for 9-methylanthracene, chrysene, and benzo[*a*]pyrene for an in vivo study conducted at SFU (*12-13***)**, and for all four test chemicals for in vivo studies conducted at ExxonMobil

Biomedical Sciences Inc. (*14*). The k_{BM} for benzo[*a*]pyrene was measured in two in vivo studies conducted at ExxonMobil Biomedical Sciences Inc. (EMBSI).

In vivo bioaccumulation tests were performed according to the OECD 305 Guideline dietary bioaccumulation tests (*11*), with some modifications in the studies conducted at Simon Fraser University (SFU), i.e., the inclusion of biotransformation-resistant reference chemicals with a wide range of log K_{OW} (*12*). Uptake and elimination data from these reference chemicals in comparison with test chemicals helped determine somatic and gastrointestinal biotransformation rate constants through non-linear (*12*) and linear regression (*13*). Experiments conducted at Exxon Mobil Biomedical Sciences Inc. (EMBSI) included at least 1 biotransformation-resistant reference chemical and was complimented by the SFU study to derive the in vivo biotransformation rate constants for a range of biotransformable substances (*14*). Specific laboratory procedures and quantitative methods for measuring in vivo k_{BM} can be found in Lo et al. (*12*), Gobas and Lo (*13*), and Lo et al. (*14*). Experimental parameters and results from the in vivo studies can be found in Table D3.

6.4.6. Unbound Fraction

To derive a method for estimating the fraction of unbound chemical in the in vitro incubation medium (fu,inc), reported measurements of fu,inc in fish liver S9 were compiled from 4 independent studies that used different chemical substances (22, 32-34), and S9 fractions that varied in protein concentrations from 0.25 mg/ml and 4 mg/ml.

A multiple linear regression model was then used to investigate the relationship between $\log\left(\frac{1 - f_{u,inc}}{f_{u,inc}}\right)$ and the logarithms of K_{OW} and the concentrations of S9 in the incubation medium (CS9; mg·ml-1). Organic chemicals varying in log K_{OW} between 3.1 and 7.6 were used in the analysis.

6.4.7. In vitro to in vivo extrapolation

Maximum in vitro biotransformation rate constants ($k_{dep,C\rightarrow 0}$; min^{-1}) for 9-methylanthracene, pyrene, chrysene, and benzo[*a*]pyrene measured from Lo et al. (*22*) and the present study (for fish pre-exposed to a contaminated diet prior to the preparation of the liver S9) extrapolated to somatic biotransformation rate constants

normalized to 10 g fish ($k_{BM,IVIVE,10g}$; d^{-1}) following the 5 step IVIVE process described in the present study. In vitro to in vivo extrapolated ($k_{BM,10g}$) data are compared to in vivo ($k_{BM,in\text{-}vivo,10g}$) data from studies conducted at SFU (*12*) and EMBSI (*14*). Table D2 shows the in vitro experimental parameters used in each study to calculate the scaling factor (SF) for estimating the hepatic biotransformation rate constant, $k_{met,H}$. Lipid ($f_{L,B}$), protein ($f_{P,B}$), and water ($f_{W,B}$) compositions in the fish body were 0.06, 0.19, and 0.75.

Given the allometric relationship between biotransformation rate constants and body weight (*39*) and ultimately the BCF and BAF (*9, 11*), both IVIVE-derived and in vivo-derived biotransformation rate constants were normalized to 10 g fish according to Equation 6.11.

6.5. Results and Discussion

6.5.1. In vitro biotransformation

Maximum in vitro biotransformation rate constants ($k_{dep,C\rightarrow 0}$) in S9 liver extracts of fish that were pre-exposed to the test chemicals were not significantly different from those of fish that were not pre-exposed (Figure 6.1). This suggests that there was not a significant induction or inhibition of biotransforming enzymes during the 14 days of pre-exposure during which fish were exposed to the 4 test chemicals through the diet. This implies that it is justified to compare in vitro biotransformation rates derived from S9 liver extracts of fish that were not pre-exposed to the test chemicals to in vivo biotransformation rates derived from fish that were pre-exposed during the uptake phase of the in vivo experiment. In vitro S9 bioassays typically are conducted using fish livers that have not been pre-exposed to test chemicals (*19-20, 22*). In-vivo depuration rate constants measured in bioaccumulation tests to derive the BCF or biotransformation rate constant are determined in fish that have been pre-exposed during the uptake phase.

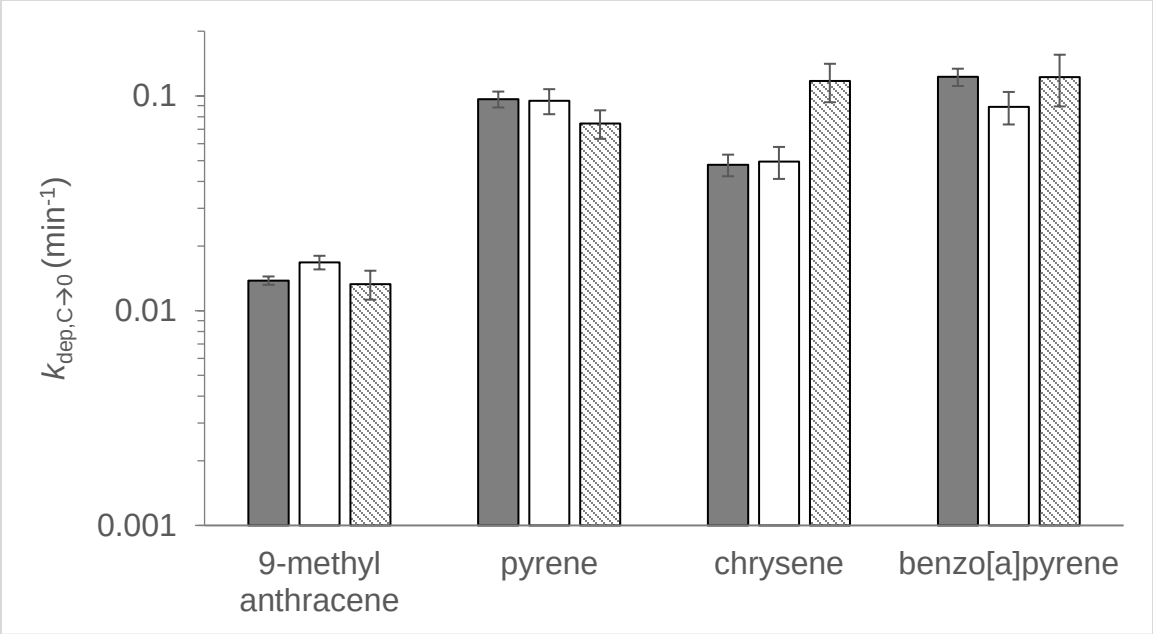

Figure 6.1 Maximum in vitro biotransformation rate constants ($k_{dep,C\rightarrow0}$; min^{-1}) measured using the multiple dosing concentration method (grey and white bars) and the sorbent phase-dosing method (downward diagonal bars) using livers from fish that were not pre-exposed to test chemicals. In vitro S9 experiments using fish that were pre-exposed to test chemicals are in grey. Error bars indicate standard errors of the mean estimates.

Maximum in vitro biotransformation rate constants ($k_{dep,C\rightarrow0}$) in S9 fish liver extracts derived from multiple solvent delivery based experiments conducted at varying initial concentrations of the test chemical in the incubation medium, were not statistically different from in vitro biotransformation rate constants derived in single experiments using the sorbent phase dosing method for three of the four test chemicals (Figure 6.1). Only for chrysene, the sorbent phase dosing method provided an in vitro biotransformation rate constant that was greater than that found using the multiple concentration solvent delivery approach. The inability to derive a complete record of the concentration dependence of the depletion rate constant in the original study (*22*) due to the lack of detection of the depletion rate constant at the lowest concentrations may explain this exception. This suggests that the sorbent phase delivery method may be an adequate and resource efficient method for measuring the maximum in vitro biotransformation rate constants ($k_{dep,C\rightarrow0}$) in fish needed for the IVIVE approach.

9-methylanthracene appeared to be biotransformed in vitro at the slowest rate (i.e., $k_{dep,C\rightarrow0}$ = 0.013 [SE 0.002] and 0.017 [SE 0.001] min^{-1} using the sorbent phase

dosing method and multiple concentration solvent delivery approach respectively with non pre-exposed S9, and $k_{dep,C\rightarrow 0}$ = 0.014 [SE 0.001] min^{-1} using the multiple concentration solvent delivery approach with pre-exposed S9) while benzo[*a*]pyrene was biotransformed at the highest rate (i.e., $k_{dep,C\rightarrow 0}$ = 0.12 [SE 0.03] and 0.09 [SE 0.02] min^{-1} using the sorbent phase dosing method and multiple concentration solvent delivery approach respectively with non pre-exposed S9, and $k_{dep,C\rightarrow 0}$ = 0.12 [SE 0.01] min^{-1} using the multiple concentration solvent delivery approach with pre-exposed S9) (Figure 6.1).

6.5.2. Unbound fraction

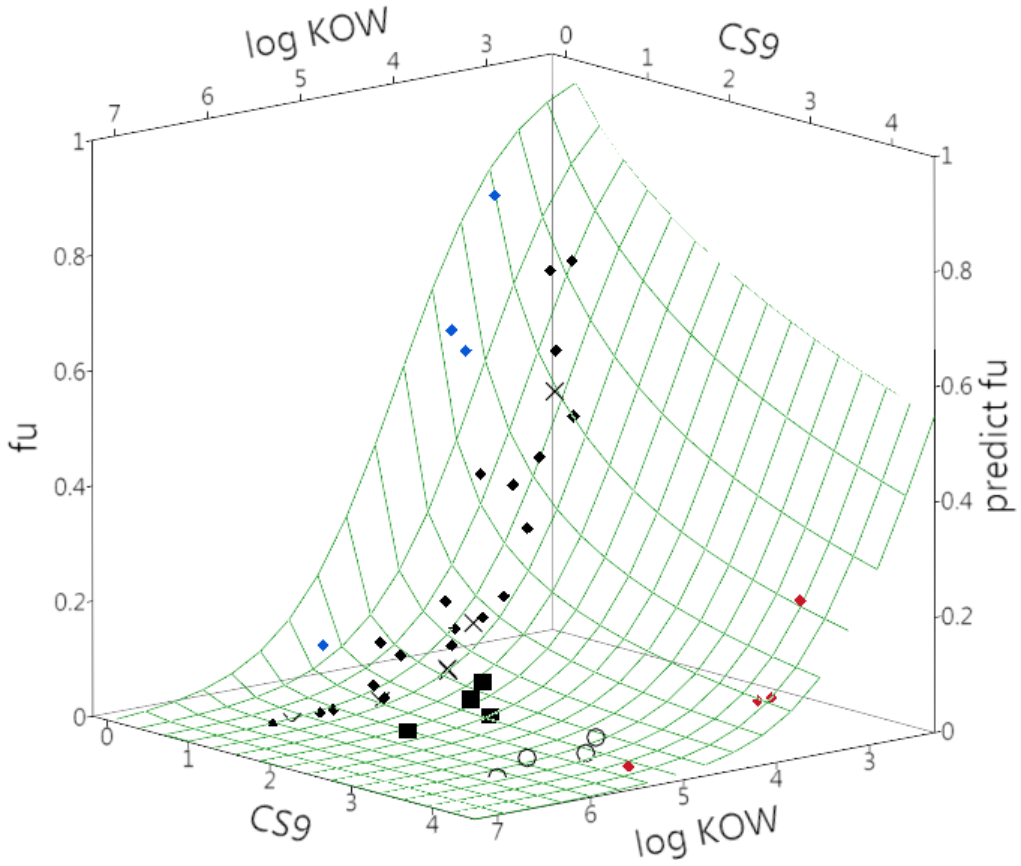

Figure 6.2 **Relationship between $f_{u,inc}$ reported in 4 studies, K_{OW}, and the concentration of S9 (CS9) in the incubation medium in units of mg·ml^{-1}. Data are from Austin et al. (*32*) (n = 31; blue, red, and black diamonds), Escher et al. (*33*) (n = 4; black squares), Nichols et al. (*34*) (n = 6; black crosses), and Lo et al. (*22*) (n = 4; black circles).**

The fraction of unbound chemical in the in vitro incubation medium ($f_{u,inc}$), was found to follow significant relationships with the concentration of S9 in the incubation medium (C_{S9}; mg·ml^{-1}), and the chemical's octanol-water partition coefficient (K_{OW}) (Figure 6.2). The multiple linear regression (Figure D1) indicated that a single model provided a reasonable fit of the available data with a root mean square error (RMSE) of 0.38:

$$\log\left(\frac{1 - f_{u,inc}}{f_{u,inc}}\right) = 0.73(\text{SE } 0.05)\cdot\log K_{OW} + 0.83\ (\text{SE } 0.17)\cdot\log C_{S9} + -2.30\ (\text{SE } 0.24)$$
$$\text{DFE} = 41;\ \text{RMSE} = 0.38 \qquad (6.12)$$

This equation provides a method for estimating the fraction of unbound chemical in the incubation medium ($f_{u,inc}$) as a function of the chemical's K_{OW} and the protein content of the incubation medium. The normalized $k^{*}_{dep,C\rightarrow 0}$ can then be calculated from $k_{dep,C\rightarrow 0}$ following Equation 6.4 with $f_{u,inc}$ estimated from Equation 6.12. Table D2 tabulates the concentration of S9 (C_{S9}) in each in vitro test and the test chemical's K_{OW} used to calculate the $f_{u,inc}$ for each study. The fraction of unbound chemical in the incubation medium ($f_{u,inc}$) of non-pre-exposed S9 (C_{S9} was 3.5 mg·mL^{-1}) was estimated at 0.014 for 9-methylanthracene, and dropped with increasing log K_{OW} of the test chemical to 0.002 for benzo[*a*]pyrene (Figure 6.3). In experiments with pre-exposed S9 (C_{S9} of 2.9 mg·mL^{-1}), $f_{u,inc}$ for 9-methylanthracene was estimated at 0.017 and 0.003 for benzo[*a*]pyrene (Figure 6.3).

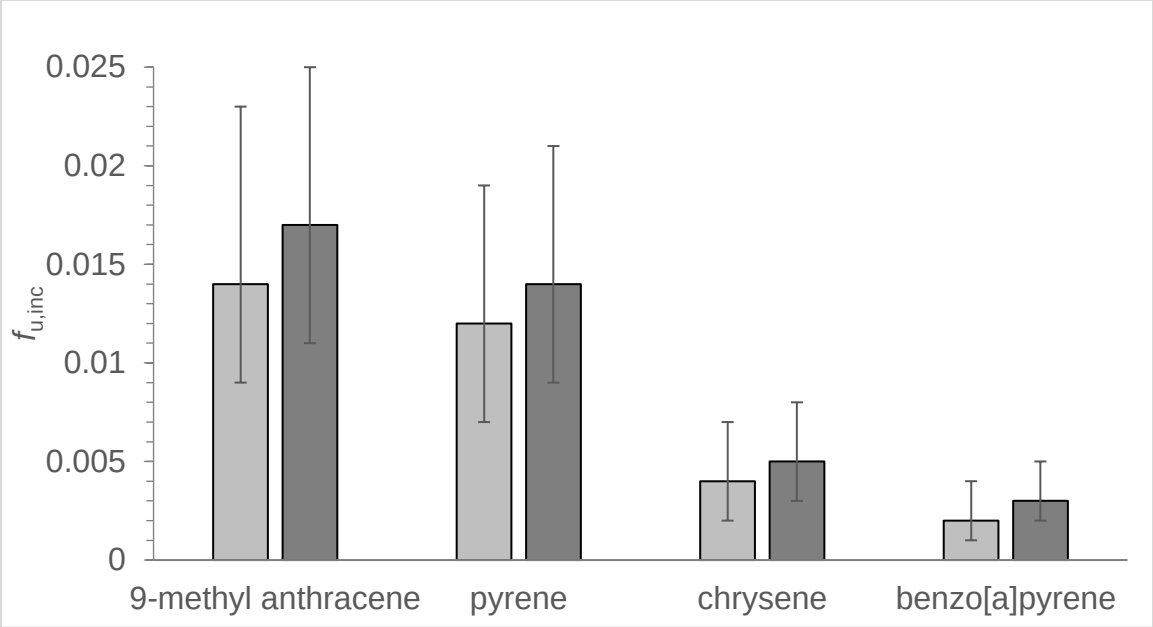

Figure 6.3 The fraction of unbound chemical in the incubation medium $f_{u,inc}$ derived from Equation 12 in experiments with non-pre-exposed (light grey) and pre-exposed (dark grey) livers.

6.5.3. In vitro to in vivo extrapolation

Somatic biotransformation rate constants of the test chemicals ($k_{BM,10}$) estimated from in vitro measurements of $k_{dep,C\rightarrow 0}$ and normalized to a 10 g fish show good reproducibility among the different assays. As a result, the mean of the $k_{BM,10}$ derived from $k_{dep,C\rightarrow 0}$ determined in various assays exhibits a relatively small standard error (Figure 6.4). This suggests that in vitro liver S9 biotransformation assays can be conducted with a reasonable level of reproducibility within a single lab. Recent studies by Embry et al. (*43*) indicate that inter-laboratory variability in measurements of liver S9 extract depletion rate constants is also sufficiently small (i.e., a coefficient of variation of 10 to 40%, n=6) to have confidence in the results of in vitro biotransformation assays. Attention to the effect of substrate concentrations and S9 concentrations in the assays may further improve inter-laboratory reproducibility of in vitro biotransformation rate measurements.

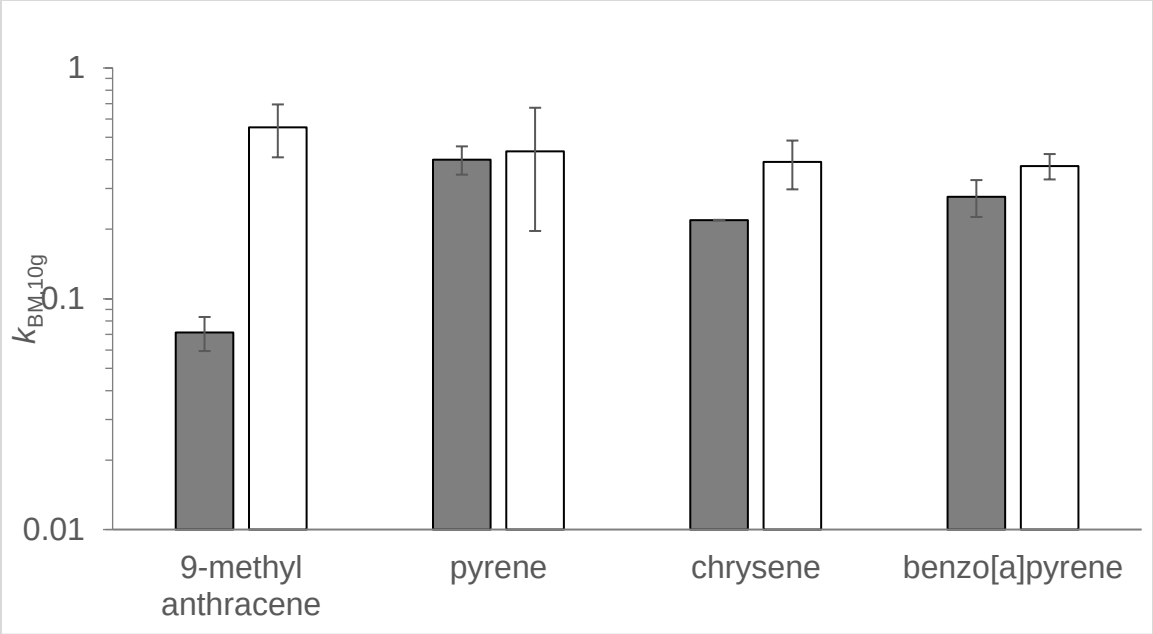

Figure 6.4 Mean (n = 3) in vitro to in vivo extrapolated somatic biotransformation rate constants normalized to 10 g fish (grey bars) compared with the mean (between 1 – 3 *in vivo* experiments) of in vivo somatic biotransformation rate constants normalized to 10 g fish (white bars). Error bars represent standard errors of the means.

In vivo measurements of somatic biotransformation rate constants $k_{BM,10}$ obtained from OECD 305 dietary bioaccumulation tests also indicate a reasonable level of accuracy (Figure 6.4). This suggests that the in vivo biotransformation rate data depicted in Figure 6.4, may provide a reasonable data set for a preliminary test of in vitro to in vivo extrapolation methods of the biotransformation rate constant. Figure 6.4 shows that with the exception of 9-methylanthracene, in vitro estimates of biotransformation rate constant in a 10g rainbow trout are in good agreement with in vivo measurements of somatic biotransformation rate constants. This clearly indicates the potential of fish liver S9 extracts to assess in vivo somatic biotransformation potential. However, for 9-methylanthracene, the in vitro derived mean $k_{BM,10g}$ was found to be approximately 7.7 times lower than the corresponding in vivo measurements of $k_{BM,10g}$. This suggests that the subcellular S9 fraction obtained from the test fish in the in vitro procedure may not contain all of the metabolic capacity to biotransform 9-methylanthracene in the whole fish. S9 in vitro systems prepared in this study contain cytochrome P450 enzymes and co-factors necessary for Phase I metabolism. However, the in vitro systems do not include all enzymes and co-factors necessary for Phase II metabolism. It is possible that the in vitro enzyme systems do not contain the necessary co-factors for metabolizing 9-

methylanthracene, resulting in an underestimate of the in vivo somatic biotransformation rate constant. A second explanation for the lack of the in vitro depletion rate constants to accurately estimate the in vivo somatic biotransformation rate constant is the possible role of extrahepatic biotransformation. One of the main assumptions of the IVIVE approach is that the liver is the main site of biotransformation. This assumption may not hold for all chemicals.

This study points out the potential for IVIVE to support bioaccumulation assessment but also points out the potential for "false negatives" where in vitro tests indicate a low potential for biotransformation while in vivo studies identify a higher potential for biotransformation. Earlier studies showed that high concentrations of the test chemicals in in vitro tests can cause in vitro studies to underestimate the in vivo biotransformation potential (*14*). This study identifies the possible role of extrahepatic metabolism as a cause for the underestimation of in-vivo biotransformation potential. Fine-tuning of in vitro assays and additional efforts to test the IVIVE approach for bioaccumulation assessment are put forward as strategies for further improving bioaccumulation assessment through in vitro testing.

6.6. Acknowledgment

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Chapter 7.

Conclusions

The assessment of the bioaccumulation potential of substances plays a large role in the effective management of commercial chemicals in the environment. To properly identify a bioaccumulative substance, the current solution under regulatory frame works is to conduct the laboratory-derived BCF test in accordance with the OECD 305 Test Guideline (OECD 2012). This solution is costly in time, money and in animal use for testing. With thousands of commercial chemicals requiring chemical evaluations for risk assessments, there is a need for streamlined approaches to bioaccumulation testing that are cost and time effective and use minimal animals in testing compared to the OECD 305 Test Guideline.

The overall objective of this research was to develop new methods for measuring and estimating the bioaccumulation capacity of neutral organic chemicals in fish in order to identify and manage chemicals that are harmful to environmental and human health. To meet this objective, in vitro and in vivo protocols were developed for measuring biotransformation rates and the bioaccumulation potential of hydrophobic substances in fish. These methods are based on scientifically-sound principles that are suitable for risk assessment and chemical management purposes, while also requiring less cost, time, and animal use for testing. The overall conclusions of this research is as follows:

7.1. In vitro testing protocols

The results of the in vitro research suggest that in vitro biotransformation rates are highly dependent on the chemical concentration in the test system. The current convention of a 1 µM initial substrate concentration, for example, may underestimate the in vitro biotransformation rate constant and, ultimately, the bioconcentration factors to be overestimated following in vitro-to-in vivo extrapolations.As a result, it is recommended that in vitro methods for bioaccumulation assessments be based on conducting (1) solvent delivery based tests conducted at multiple initial substrate concentrations or (2) sorbent phase delivery base tests.

Solvent delivery based tests conducted at multiple initial substrate concentrations provides the most accurate method for determining the in vitro biotransformation rate constant. This method allows for the estimation of the maximum first order biotransformation rate constant and the Michaelis-Menten Constant. As an alternative method, a single sorbent phase delivery based test method test appears to provide a resasonable estimate of the maximum first order depletion rate constant for the test chemicals in this research. Although the full Michaelis-Menten relationship cannot be described in the sorbent phase delivery based test, it does not require multiple dosing experiments and therefore may be a more efficient method in terms of cost, time and animal use in testing. Additionally, sorbent phase delivery based tests provide measurements of the fraction unbound of the chemical in the in vitro system (a valuable extrapolation parameter) and requires no solvents, and prevents incomplete dissolution of the test chemical in the incubation medium (Lo et al. 2015b).

7.2. Dietary in vivo testing protocols

Protocols for the measurement of biotransformation rates and bioaccumulation potential were developed using dietary bioaccumulation tests that involved the addition of non-metabolizable reference chemicals that make it possible to determine in vivo biotransformation rate constants and the BCF in OECD 305-style dietary fish bioaccumulation tests. Results show that dietary bioaccumulation studies of gastro-intestinal biotransformation contribution to the overall biotransformation in fish is significant for the majority of test chemicals in the SFU in vivo study. Gastro-intestinal biotransformation can only be measured through dietary experiments, suggesting that aqueous bioconcentration tests described in the OECD 305 Test Guideline may not be able to account for the full degree of biotransformation of the test chemical in natural situations where uptake is predominantly via the diet (Lo et al. 2015). Based on the overall results of the in vivo research, it is recommended that (1) reference chemicals be included and (2) dietary exposure methods be given preference over aqueous exposure methods in bioaccumulation testing.

Dietary bioaccumulation tests can also determine bioconcentration factors. In this approach, the measurement of chemical concentrations in the water is not required. Bioconcentration factors measured in the dietary studies using this approach showed a good agreement with empirical BCF values for the same chemicals from other literature

sources (Gobas and Lo 2016). Dietary in vivo testing methods can provide cost savings over conventional bioconcentration tests, by reducing test completion times and animal use in testing (Gobas and Lo 2016).

Applying these analytical approaches to a larger set of dietary OECD 305 fish tests revealed details on the biotransformation and bioaccumulation behavior of structurally diverse chemicals, and the general relationship between the bioconcentration factor and the biomagnification factor for substances subject to somatic and gastrointestinal biotransformation. The results from Lo et al. 2016 show that there is a large contribution of gastro-intestinal biotransformation for chemicals in fish if the chemical uptake in fish is via the diet. The results lend additional support that, for the majority of test chemicals, the relative contribution of gastro-intestinal biotransformation exceeds somatic biotransformation when fish are exposed to chemicals through the diet. However, modeling results show that test chemicals that would be exposed solely through the respiratory route will be primarily be biotransformed in the fish body. In real world exposure scenarios, the relative contribution of gastro-intestinal and somatic biotransformation will depend on the relative degree of exposure through the dietary and aqueous exposure routes (Lo et al. 2016).

The relationship of the freely dissolved wet-weight BCF ($BCF_{WW,fd}$) and the lipid normalized BMF (BMF_L) shows that, for non-biotransformable substances, $BCF_{WW,fd}$ of 5,000 corresponds well to a mean BMF_L of 1. This confirms that the commonly used regulatory criterion for the BCF of 5,000 is a reasonable threshold for identifying potentially bioaccumulative substances. For substances that don't biotransform, the relationship between the BCF and BMF is strong because both metrics are a function of a common depuration rate constant. For substances that biotransform in the fish body but not in the gastro-intestinal tract, the strong correlation remains because the somatic biotransformation rate constant is also shared for the BCF and BMF. For substances that are biotransformed in the gastro-intestinal tract, however, there is a loss of relationship between the BMF_L and the $BCF_{WW,fd}$. In these instances, results show that the BCF has a tendency to overestimate the biomagnification potential of chemicals. For substances that are significantly biotransformed in the gastro-intestinal tract, there may be a lower bioaccumulation concern (i.e. BMF < 1) than what the BCF (i.e. > 5000) would suggest because the chemical is unable to biomagnify.

7.3. In vitro to in vivo extrapolation protocols

The results of the in vitro to in vivo extrapolation research show a good agreement between in vitro-derived and in vivo measurements of the somatic biotransformation rate constants for the majority of the chemicals tested. Among in vitro-derived measurements of the somatic biotransformation rate constants, there is also a good agreement between biotransformation rates derived by multiple solvent delivery based depletion method and sorbent dosing method. Likewise, there is a reasonable agreement among in vivo measurements of the somatic biotransformation rate constants for chemicals that were tested. Overall, this research demonstrates potential for fish liver S9 extracts to assess in vivo biotransformation potential in the fish body.

However, for 9-methylanthracene, the mean in vitro derived somatic biotransformation rate constant was found to be lower than the corresponding mean in vivo measurements by a factor of 7.7. This suggest that the S9 fraction and S9 in vitro system may not contain all of the enzymes and co-factors to biotransform the chemical compared to the whole fish. It is possible, therefore, that subcellular in vitro systems may underestimate the in vivo somatic biotransformation rate constant. Another explanation is the possibility of significant extrahepatic biotransformation that is not captured by liver-based in vitro systems. One of the main assumptions of the in vitro (followed by IVIVE) approach for chemical assessments is that the liver is the main site of biotransformation. However, as discovered in the dietary in vivo research, that may not always be the case for many neutral organic chemicals.

7.4. Future work

With the addition of non-metabolizable reference chemicals, dietary bioaccumulation tests have the potential to empirically measure the bioconcentration factor (BCF) along with additional bioaccumulation metrics such as the biomagnification factor (BMF), the somatic biotransformation rate constant (k_{BM}), and the gastro-intestinal biotransformation rate constant (k_{GM}). With additional analyses from future dietary bioaccumulation tests, a database of empirical BCF, BMF, k_{BM}, and k_{GM} values can be developed to help supplement regulatory chemical evaluations as provisions are developed for considering these additional bioaccumulation metrics and as bioaccumulation assessments move towards a weight of evidence approach (ECHA

2014). The development a database of somatic biotransformation rate constants (k_{BM}) and gastro-intestinal biotransformation rate constants (k_{GM}) can also help improve and support the development in silico methods for improving bioaccumulation predictions through quantitative structure-activity relationships.

Results from this research suggest that gastro-intestinal biotransformation generally contributes more to the overall biotransformation in fish than somatic biotransformation for most chemicals that are exposed through the diet. In combination with hepatic in vitro bioassays for estimating somatic biotransformation, the development of in vitro bioassays for estimating gastro-intestinal biotransformation may be a useful contribution to in vitro initiatives for improving bioaccumulation assessments. Further research is needed to understand and identify the potential contributions of enzymes from the fish and microflora in the intestinal tract.

7.5. References

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Appendix A.

Supplemental Information for Chapter 2

Supplemental information for “Concentration Dependence of Biotransformation in Fish Liver S9: Optimizing Substrate Concentrations to Estimate Hepatic Clearance for Bioaccumulation Assessment”

Table A1 Value of KM reported in the literature for benzo[a]pyrene oxidation, and the range of substrate concentrations used in their determination.

Reaction Monitored (species and liver preparation)	Apparent K_M (µM)	Substrate concentration range examined (µM)	Reference
Depletion of parent compound (rainbow trout liver S9)	0.18 (0.08 SE)	0.056 – 10	*the present study*
Formation of fluorescent phenolic metabolites (bluegill liver microsomes)	0.125 - 0.32	0.08 – 19.8	Karr et al., 1985
Formation of 3-OH metabolite (scup liver microsomes)	0.4	0.08 – 4	Stegeman et al., 1979
Formation of 3-OH metabolite (scup liver microsomes)	38	2 – 20	Stegeman et al., 1979
Formation of water soluble ^{14}C metabolites (rainbow trout liver microsomes)	33-125	5 – 60	Carpenter et al., 1990
Formation of water soluble ^{14}C metabolites (Atlantic salmon liver microsomes)	7.77 – 10.4	5 – 360	Vignier et al., 1996
Formation of OH-metabolites (rainbow trout liver microsomes)	1.63	1 – 50	Hermens et al., 1990
Formation of water soluble ^{3}H metabolites (English sole hepatocytes)	29 (10 SE)	2 – 40	Nashimoto et al., 1992
Formation of 3-OH metabolite (European sea bass liver microsomes)	9.93 (1.52 SE)	2.5 – 12.5	Lemaire et al., 1992

Table A2 **Depletion rate constants (k_{dep}) from single depletion curve incubations at different initial substrate concentrations (left). These rate constants were used to estimate the maximum depletion rate constants ($k_{dep,C\rightarrow 0}$) and Michaelis constants (K_M) using weighted nonlinear regression.**

	Single Depletion Experiments				Multiple Depletion Experiments	
	Log $C_{l,t=0}$ (µM)	k_{dep} ± SE (min^{-1})	n	Coefficient of Variation (R^2)	$k_{dep,C\rightarrow 0}$ ± SE (min^{-1})	K_M ± SE (µM)
pyrene	-1.25	0.10 ± 0.02	4	0.91	0.09 ± 0.01	0.31 ± 0.08
	-0.75	0.06 ± 0.01	5	0.92		
	-0.51	0.059 ± 0.006	5	0.97		
	-0.25	0.046 ± 0.007	5	0.93		
	0.00	0.029 ± 0.002	5	0.99		
	0.25	0.011 ± 0.002	5	0.94		
	0.50	0.008 ± 0.001	4	0.95		
	0.75	0.0021 ± 0.0003	4	0.95		
	1.00	0.0010 ± 0.0009	5	0.28		
	-1.00	0.06 ± 0.03	5	0.52		
	-0.75	0.09 ± 0.01	4	0.98		
	-0.51	0.046 ± 0.007	5	0.94		
	-0.25	0.038 ± 0.005	5	0.96		
	0.00	0.021 ± 0.007	5	0.75		
	0.25	0.012 ± 0.004	5	0.76		
	0.50	0.003 ± 0.002	5	0.43		
	0.75	-0.0003 ± 0.001	5	0.02		
	1.00	0.002 ± 0.004	5	0.09		
	-1.25	0.08 ± 0.01	3	0.98		
	-1.00	0.06 ± 0.01	5	0.84		
	-0.75	0.037 ± 0.003	5	0.98		
	-0.51	0.05 ± 0.02	5	0.76		
	-0.25	0.045 ± 0.006	5	0.94		
	0.25	0.020 ± 0.003	5	0.93		
	0.50	0.0085 ± 0.0008	5	0.97		
	0.75	0.001 ± 0.001	5	0.17		
	1.00	-0.002 ± 0.002	5	0.28		
9-methylantracene	-1.25	0.015 ± 0.001	5	0.97	0.017 ± 0.001	1.6 ± 0.4
	-1.00	0.010 ± 0.002	5	0.86		
	-0.77	0.0162 ± 0.0007	5	0.99		
	-0.50	0.015 ± 0.001	5	0.98		
	-0.25	0.0138 ± 0.0009	5	0.99		
	0.00	0.011 ± 0.002	5	0.87		
	0.23	0.0075 ± 0.0006	5	0.98		
	0.50	0.005 ± 0.002	5	0.73		
	0.75	0.003 ± 0.001	5	0.70		
	1.25	0.002 ± 0.002	5	0.26		

	Single Depletion Experiments					Multiple Depletion Experiments	
	Log $C_{l,t=0}$ (µM)	k_{dep} ± SE (min^{-1})		n	Coefficient of Variation (R^2)	$k_{dep,C\rightarrow 0}$ ± SE (min^{-1})	K_M ± SE (µM)
chrysene	-1.00	0.032	± 0.004	5	0.96	0.049 ± 0.08	0.14 ± 0.05
	-0.75	0.019	± 0.003	5	0.93		
	-0.50	0.016	± 0.002	5	0.95		
	0.00	0.004	± 0.001	5	0.78		
	0.25	-0.001	± 0.003	5	0.03		
	0.75	-0.005	± 0.006	5	0.18		
	-1.25	0.031	± 0.003	5	0.98		
	-1.00	0.034	± 0.004	5	0.97		
	-0.75	0.024	± 0.004	5	0.92		
	-0.50	0.015	± 0.004	5	0.81		
	-0.25	0.012	± 0.006	5	0.59		
benzo[a]pyrene	-1.25	0.063	± 0.007	4	0.98	0.09 ± 0.02	0.18 ± 0.08
	-1.00	0.057	± 0.002	5	0.99		
	-0.75	0.053	± 0.004	5	0.98		
	-0.51	0.04	± 0.01	5	0.85		
	-0.25	0.018	± 0.003	5	0.92		
	0.00	0.016	± 0.006	5	0.69		
	0.25	0.000	± 0.005	5	0.00		
	0.50	0.001	± 0.002	5	0.90		
	0.75	-0.010	± 0.006	5	0.52		
	1.00	-0.004	± 0.003	5	0.35		

Table A3 Chromatography peak areas of test chemicals and internal standard (chrysene d^{12}) in extraction tests using various extraction medium (S9 incubation medium at 0, 30, and 60 minutes, EVA medium, and HPLC-grade n-hexane blank

	Extraction medium	Chromatography		
		Analyte (peak area)	Internal Standard (peak area)	Analyte / IS ratio
Pyrene	0 min (incubation)	474113	304999	0.6433044
	0 min (incubation)	350817	257269	0.7333425
	0 min (incubation)	439015	299455	0.6821065
	30 min (incubation)	367382	276435	0.7524457
	30 min (incubation)	492665	333977	0.6778988
	30 min (incubation)	531832	333992	0.6280028
	60 min (incubation)	421246	304619	0.723138
	60 min (incubation)	411788	276584	0.671666
	60 min (incubation)	460692	282602	0.6134294
	EVA	438018	276915	0.6322
	EVA	536419	304478	0.5676123
	EVA	387380	273409	0.7057902
	hexane	479621	326456	0.6806541
	hexane	352805	250432	0.7098312
	hexane	365660	213297	0.5833206
9-methylanthracene	0 min (incubation)	443175	235753	0.5319637
	0 min (incubation)	500718	241935	0.4831762
	0 min (incubation)	437598	204704	0.4677901
	30 min (incubation)	427245	193313	0.452464
	30 min (incubation)	396752	196940	0.4963806
	30 min (incubation)	498926	220429	0.441807
	60 min (incubation)	520178	238071	0.4576722
	60 min (incubation)	425526	222666	0.5232724
	60 min (incubation)	458476	203898	0.4447299
	EVA	417716	208386	0.49887
	EVA	408183	185112	0.4535025
	EVA	463613	206711	0.4458697
	hexane	509118	229886	0.4515378
	hexane	403786	185152	0.4585399
	hexane	411667	212685	0.5166433
chrysene	0 min (incubation)	361019	585036	1.620513
	0 min (incubation)	409764	595041	1.4521554
	0 min (incubation)	431496	639747	1.4826256
	30 min (incubation)	452838	675737	1.4922268
	30 min (incubation)	380132	587445	1.5453711
	30 min (incubation)	461652	659314	1.4281623

	Extraction medium	Chromatography		
		Analyte (peak area)	Internal Standard (peak area)	Analyte / IS ratio
	60 min (incubation)	380998	588974	1.5458716
	60 min (incubation)	497588	671170	1.3488468
	60 min (incubation)	413946	587020	1.4181077
	EVA	424944	697021	1.6402655
	EVA	392300	556079	1.4174841
	EVA	359523	506512	1.4088445
	hexane	425354	595862	1.4008614
	hexane	498234	709431	1.4238912
	hexane	361939	570082	1.5750776
benzo[a]pyrene	0 min (incubation)	385597	1898051	4.9223697
	0 min (incubation)	442957	2094490	4.7284274
	0 min (incubation)	406863	1866971	4.5886969
	30 min (incubation)	376003	1862550	4.9535509
	30 min (incubation)	381364	1916583	5.0256002
	30 min (incubation)	454317	2081639	4.5819087
	60 min (incubation)	444346	2082355	4.6863368
	60 min (incubation)	373357	2010317	5.3844363
	60 min (incubation)	407527	1954263	4.7954197
	EVA	367251	1881266	5.122562
	EVA	405310	1942463	4.7925366
	EVA	378794	1832151	4.8368005
	hexane	403708	2002826	4.9610758
	hexane	420516	2141844	5.093371
	hexane	447744	1946378	4.3470778

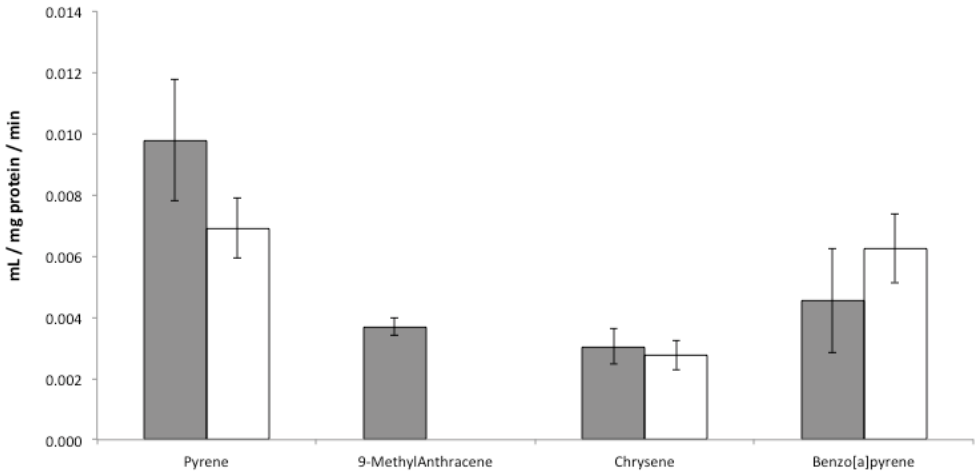
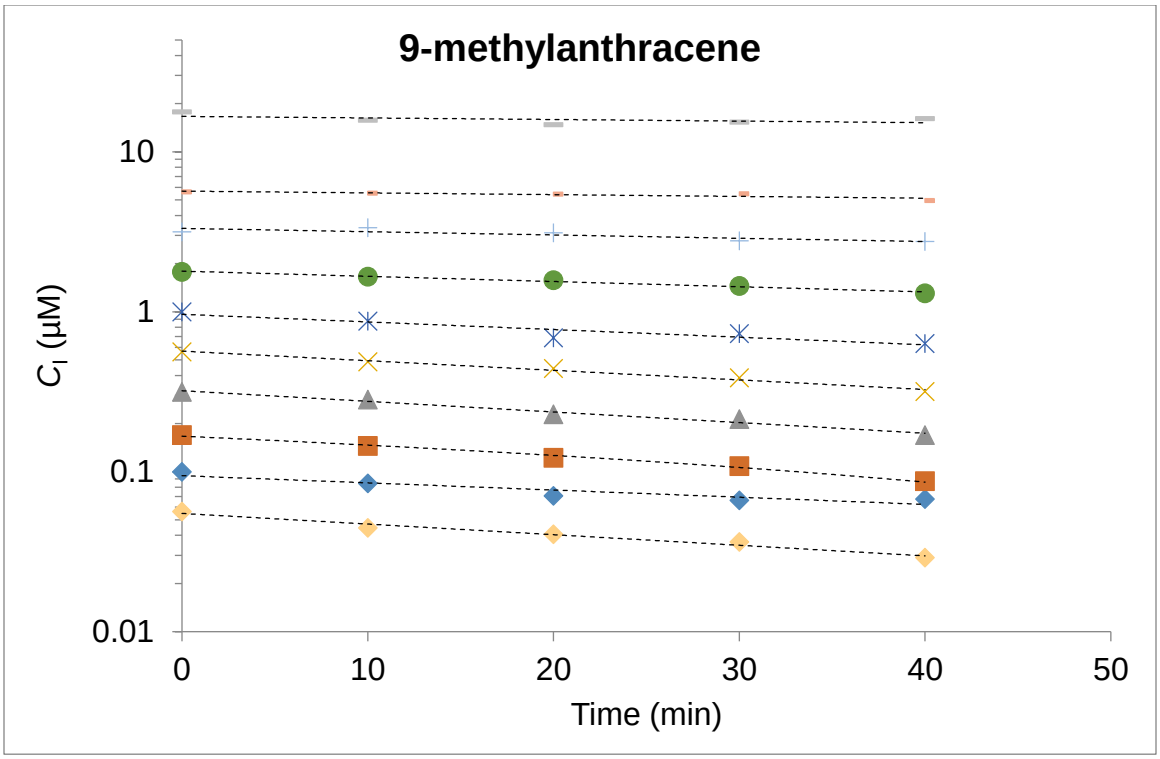
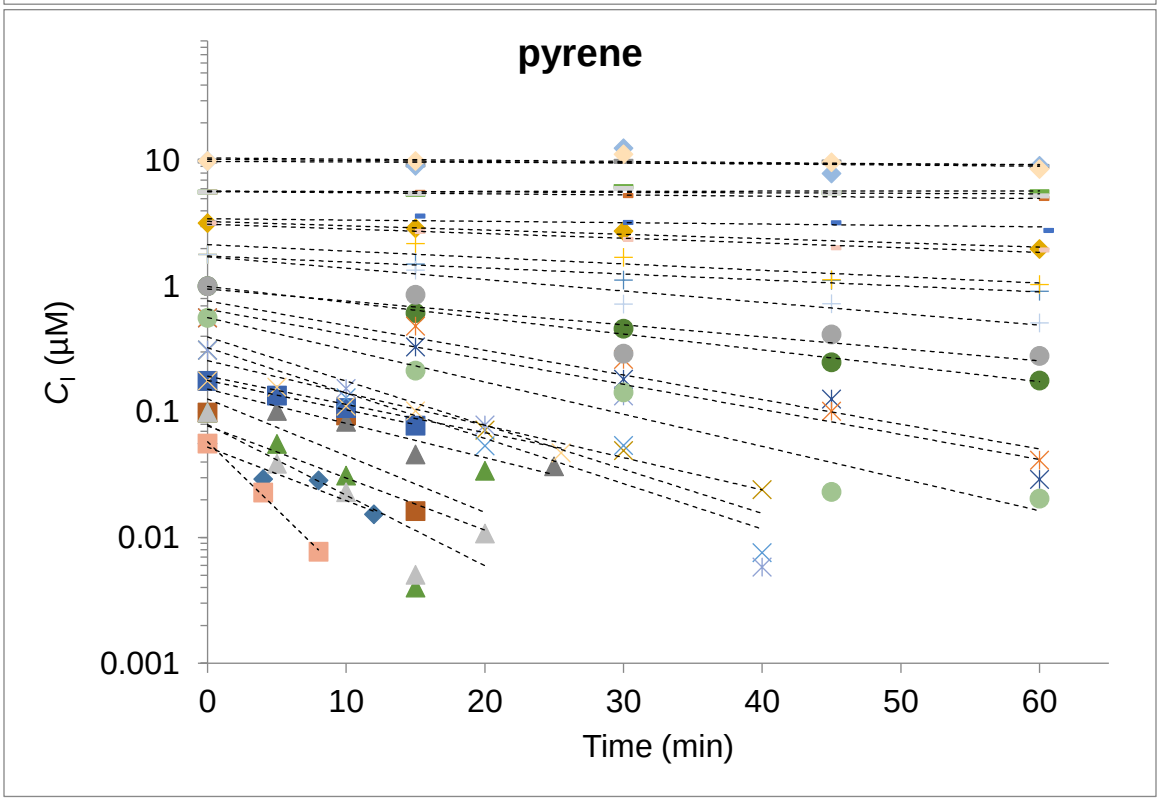
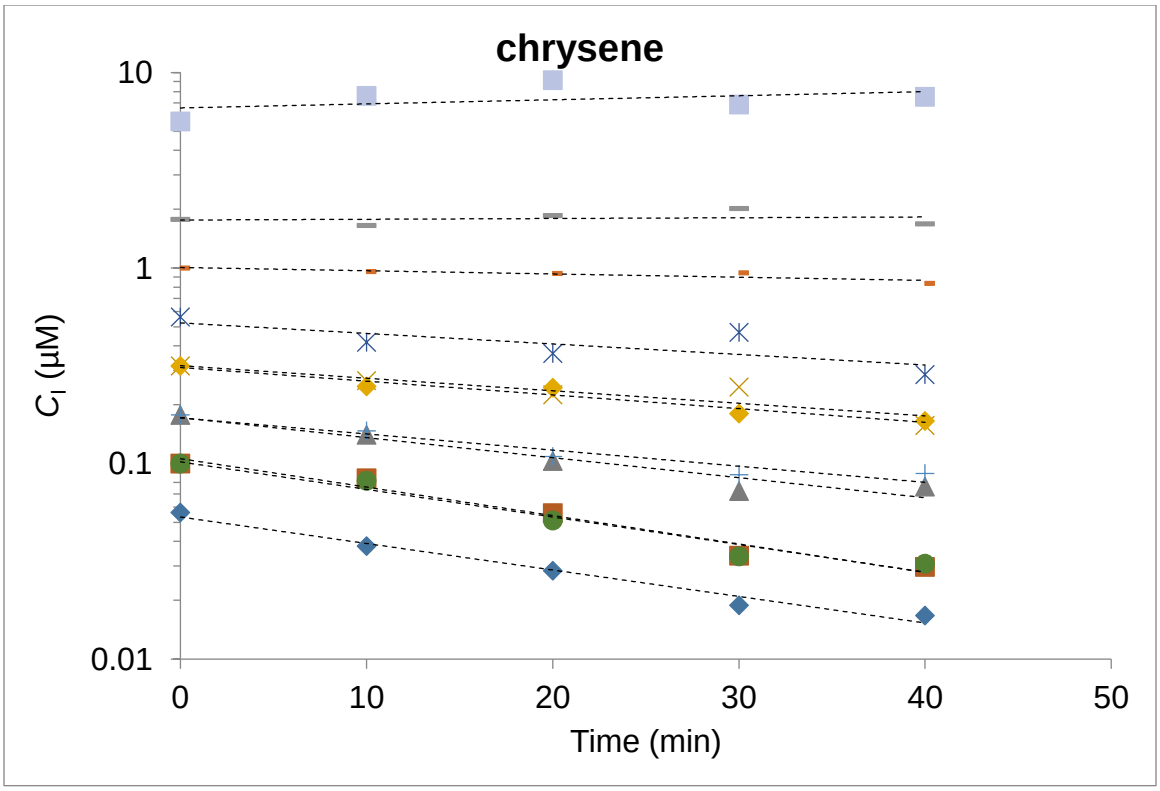


Figure A1 **Measured depletion rate constants (ml.mg protein^{-1}.min^{-1}) of pyrene, 9-methyl-anthracene, chrysene and benzo[a]pyrene in rainbow trout liver S9 measured in (i) incubations where liver S9 homogenates were added to the test chemical solution in the incubation medium (grey bars) and (ii) incubations where the test chemical solution was added to the liver S9 homogenates in the incubation medium (white bars). Error bars represent standard deviations.**


9-methylanthracene
10
1
0.1
0.01
C_l (µM)
0
10
20
30
40
50
Time (min)


pyrene
10
1
0.1
0.01
0.001
C_l (µM)
0
10
20
30
40
50
60
Time (min)


chrysene
10
1
0.1
0.01
C_l (µM)
0
10
20
30
40
50
Time (min)

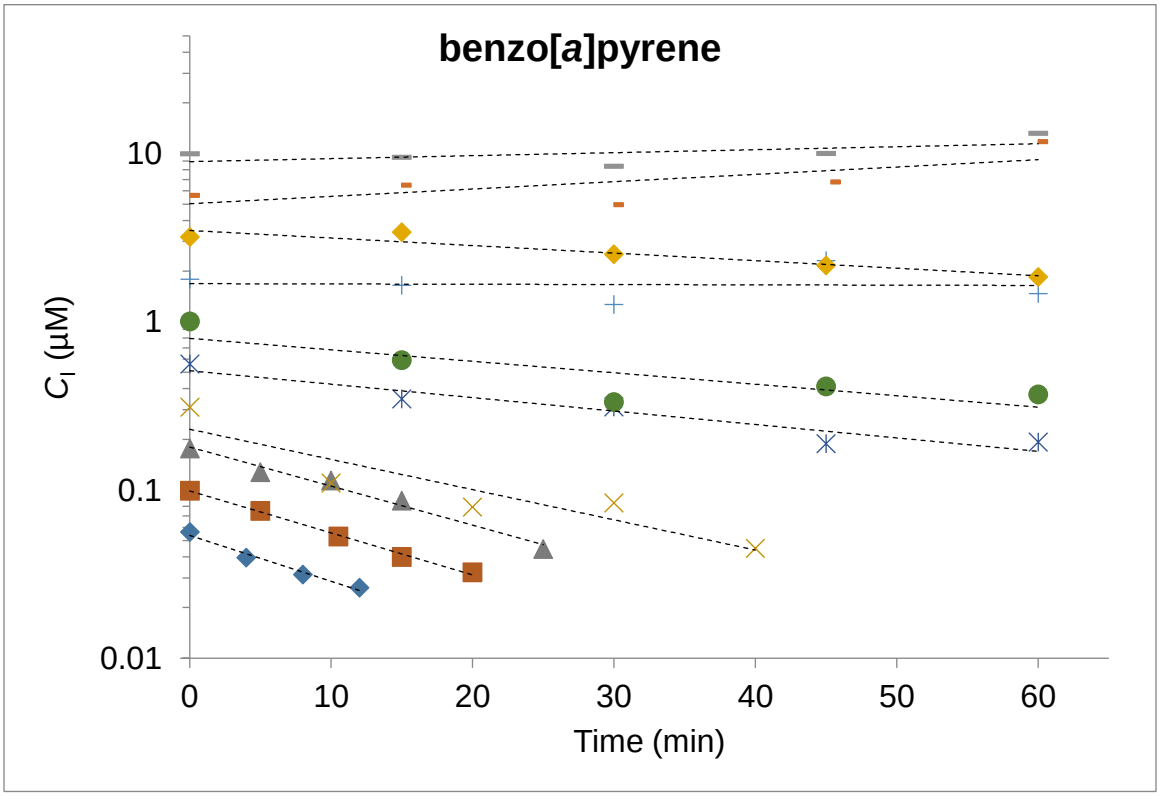

benzo[a]pyrene
10
1
0.1
0.01
C_l (µM)
0
10
20
30
40
50
60
Time (min)

Figure A2 **Decline of the concentrations of 9-methylanthracene, pyrene, chrysene and benzo[*a*]pyrene in the incubation medium for different initial substrate concentrations in the incubation medium (C_I) in solvent-delivery dosing experiments.**

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Appendix B.

Supplemental Information for Chapter 3

Supplemental information for “Somatic and Gastro-intestinal In vivo Biotransformation Rates of Hydrophobic Chemicals in Fish”

The role of water absorption with food

The purpose of this section is to demonstrate that for very hydrophobic neutral organic substances with a very high K_{OW} (log K_{OW} > 4) absorption of water with the food by fish does not have a significant effect on the determination of intestinal biotransformation rates and that calculations based on a dry weight basis will produce estimates of biotransformation rates that are not significantly different from those conducted on a wet weight basis. The main reason for the insignificant role of water on the dietary uptake dynamics of very hydrophobic substances in fish is that water has a negligible capacity to solubilize very hydrophobic chemical compared to lipids, proteins and other organic materials. As a result, the mass balance equations for the uptake of very hydrophobic chemicals in fish can be described on a wet or dry weight basis without introducing a significant error. This is advantageous in dietary bioaccumulation experiments because often, as is the case in this study, the chemical is administered in the form of a dried food that is applied to water. Because the water content of the actual diet and digesta of the fish are in most cases not characterized in dietary bioaccumulation experiments, wet weight based calculations involving the feeding and fecal egestion rates are difficult to perform.

To demonstrate the insignificant effect of water absorption with food, we present the calculations of k_{GM}^{*} using both wet and dry weight calculations. k_{GM}^{*} and $E_{D,M}^{*}$are the main bioaccumulation metrics explored in this study. In this illustration, we will assume that the diet is administered in the form of dry food (0% water) and that it is absorbed by fish with an equal amount of water (50% water). We will present the calculation of k_{GM}^{*} using calculations on a dry weight calculations, which ignore the absorption of water with the diet by the fish, and calculations on a wet weight basis, which include water

absorption with the food. We will further assume the same characteristics of the food as described in the accompanying paper.

Food Composition

We will assume that the dry food is made up of 18.6 g lipid, 46.6 g protein and 32.4 g of non-digestibles. Hence, on a dry weight basis, the lipid, protein and indigestible matter contents are:

Lipid content = 18.6 / (18.6 + 46.6 + 32.4) = 0.19 kg lipid.kg food dry weight^{-1}

Protein content = 46.6/(18.6 + 46.6 + 32.4) = 0.48 kg protein.kg food dry weight^{-1}

Non-digestible content = 32.4/ (18.6 + 46.6 + 32.4) = 0.33 kg non-digestibles.kg food dry weight^{-1}

If the same food is absorbed with an equal amount (i.e. 18.6 + 46.6 + 32.4 = 97.6 g) of water, then

Lipid content = 18.6 / ((18.6 + 46.6 + 32.4) × 2) = 0.095 kg lipid.kg food wet weight^{-1}

Protein content = 46.6 / ((18.6 + 46.6 + 32.4) × 2) = 0.24 kg protein.kg food wet weight^{-1}

Indigestible content = 32.4/ ((18.6 + 46.6 + 32.4) × 2) = 0.165 kg non-dig.kg food wet weight^{-1}

Water content = 97.6/ ((18.6 + 46.6 + 32.4) x 2) = 0.50 kg water.kg food wet weight^{-1}

Digesta Composition

Using assimilation efficiencies of 92% for lipids, 75% for proteins, 0% for non-digestibles and 50% for water, we can calculate the composition of the digesta according to equations 3.23, 3.24, 3.25 and 3.26 on a dry weight basis as:

ϕ_{GL} = (1-0.92).0.19 / {(1-0.92).0.19 + (1-0.75).0.48 + (1-0).0.33 + (1-0.5).0} = 0.03 kg lipid.kg digesta dry weight^{-1}

ϕ_{GP} = (1-0.75).0.48 / {(1-0.92).0.19 + (1-0.75).0.48 + (1-0).0.33 + (1-0.5).0} = 0.26 kg protein.kg digesta dry weight^{-1}

ϕ_{GN} = (1-0).0.33 / {(1-0.92).0.19 + (1-0.75).0.48 + (1-0).0.33 + (1-0.5).0} = 0.71 kg non-digestibles.kg digesta dry weight^{-1}

ϕ_{GW} = (1-0.5).0 / {(1-0.92).0.19 + (1-0.75).0.48 + (1-0).0.33 + (1-0.5).0} = 0 kg water.kg digesta dry weight^{-1}

On a wet weight basis, taking into account water absorption, the composition of the digesta can be calculated as:

ϕ_{GL} = (1-0.92).0.095 / {(1-0.92).0.0.095 + (1-0.75).0.24 + (1-0).0.165 + (1-0.5).0.5} = 0.016 kg lipid.kg digesta wet weight^{-1}

ϕ_{GP} = (1-0.75).0.24 / {(1-0.92).0.0.095 + (1-0.75).0.24 + (1-0).0.165 + (1-0.5).0.5} = 0.124 kg protein.kg digesta wet weight^{-1}

ϕ_{GN} = (1-0).0.165 / {(1-0.92).0.0.095 + (1-0.75).0.24 + (1-0).0.165 + (1-0.5).0.5} = 0.343 kg non-digestibles.kg digesta wet weight^{-1}

ϕ_{GW} = (1-0.5).0.5 / {(1-0.92).0.0.095 + (1-0.75).0.24 + (1-0).0.165 + (1-0.5).0.5} = 0.517 kg water.kg digesta wet weight^{-1}

Feeding Rate

In our experiment, the feeding rate on a dry weight basis was 0.43 g dry weight food per fish, where the fish had an average wet weight of 37 g. If we assume that fish absorb their food with an equal amount of water, then the feeding rate on a wet weight basis is 0.86 g wet weight food per fish.

Food Assimilation efficiency

Using assimilation efficiencies of 92% for lipids, 75% for proteins, 0% for non-digestibles and 50% for water, we can calculate assimilation efficiencies on dry weight basis as (0.92 × 0.19 + 0.75 × 0.48 + 0 × 0.33) or 0.53. On a wet weight basis, the assimilation efficiency is (0.92 × 0.095 + 0.75 × 0.24 + 0 × 0.165 + 0.5 × 0.5) or 0.52.

Diet-digesta partition coefficient

On a dry weight basis, the diet-digesta partition coefficient (K_{DG}) for a substance with a log Kow of 7 is:

$K_{DG} = (0.19 . 10^7 + 0.48 . 0.05 . 10^7 + 0.33 . 0.05 . 10^7 + 0) / (0.03.K_{OW} + 0.26 . 0.05 . 10^7 + 0.71 . 0.05 . 10^7 + 0) = 2.85$

On a wet weight basis, the diet-digesta partition coefficient (K_{DG}) for a substance with a log K_{OW} of 7 is:

$K_{DG} = (0.095.10^7 + 0.24. 0.05.10^7 + 0.165.0.05.10^7 + 0.5) / (0.016.10^7 + 0.124. 0.05.10^7 + 0.343.0.05.10^7 + 0.517) = 2.95$

Calculation of k^*_{GM}

The gastro-intestinal biotransformation rate k^*_{GM} (d^{-1}) that prevents gastro-intestinal magnification can be derived on a dry weight basis as:

$k^*_{GM} = (0.43 / 0.20) (2.85 – (1-0.53)) = 5.11$

and on a wet basis k^*_{GM} (d^{-1}) is:

$k^*_{GM} = (0.86 / 0.41) (2.95 – (1-0.52)) = 5.18$

The small difference in the dry and wet weight based calculations of k^*_{GM} is largely due to rounding errors in the calculations. However, the dry and wet weight based calculations of k^*_{GM} are not identical. Ignoring water absorption with the food

produces an error. For substances with a high K_{OW} this error is very small and is insignificant compared with other errors in typical bioaccumulation experiments.

Gastro-Intestinal Fugacity Ratio

The purpose of this section is to present the derivation of k^*_{GM} and $E^*_{D,M}$ which are respectively the gastro-intestinal biotransformation rate constant and the dietary uptake efficiency of a biotransformable substance that are expected to produce a gastro-intestinal magnification factor of 1, i.e. equal chemical fugacities in the digesta and the diet of the fish. A gastro-intestinal magnification factor of 1 is expected to produce a biomagnification factor of l or less than 1, i.e. equal chemical fugacities in the body of the fish that is equal or less than that in the diet of the fish.

Following the fugacity based model of the dietary bioaccumulation of hydrophobic chemicals in fish [B.1], we can write the mass balance equations for the body of the fish and the gastro-intestinal tract in a typical dietary bioaccumulation test where the aqueous concentration C_W is held at a 0 concentration as:

$$V_B.Z_B.df_B/dt = D_{GB}.f_G - (D_{BG} + D_{B2} + D_{BM} + D_{GD}).f_B \quad (B.1)$$

and

$$V_G.Z_G.df_G/dt = D_I.f_D + D_{BG}.f_B – (D_{GB} + D_{GE} + D_{GM}).f_G \quad (B.2)$$

where

f_B :fugacity in the body of the fish (Pa)

f_D :fugacity in the diet (Pa)

f_G :fugacity in the digesta (Pa)

V_B : volume of the body of the fish (m^3)

V_G : volume of the digesta (m^3)

Z_B : fugacity capacity of the body of the fish ($mol.m^{-3}.Pa^{-1}$)

Z_G : fugacity capacity of the digesta (mol.m^{-3}.Pa^{-1})

D_I :transport parameter for food ingestion (mol.Pa^{-1}.day^{-1})

D_{GE} :transport parameter for the egestion of digesta from the gastro-intestinal tract (mol.Pa^{-1}.day^{-1})

D_{GM} :transport parameter for gastro-intestinal biotransformation (mol.Pa^{-1}.day^{-1})

D_{BG} :transport parameter for chemical transfer from the body of the fish to the digesta (mol.Pa^{-1}.day^{-1})

D_{GB} :transport parameter for chemical transfer from the digesta to the body of the fish (mol.Pa^{-1}.day^{-1})

D_{B2} :transport parameter for chemical transfer from the body of the fish to the water via the respiratory surface (e.g. gills) and skin (mol.Pa^{-1}.day^{-1})

D_{BM} :transport parameter for somatic biotransformation (mol.Pa^{-1}.day^{-1})

D_{GD} :transport parameter for growth dilution (mol.Pa^{-1}.day^{-1})

Assuming a steady-state mass balance of the chemical in the gastro-intestinal tract (i.e. $V_G.Z_G.df_G/dt = 0$) and the body of the fish (i.e. $V_B.Z_B.df_B/dt = 0$) and substituting equation B.1 into B.2, it follows that gastro-intestinal magnification factor, i.e. the steady-state ratio of the fugacities of the chemical in the gastro-intestinal tract f_G and the diet f_D (i.e. F_{GD}) is:

$$F_{GD} = \frac{f_G}{f_D} = \frac{D_I}{D_{GE} + D_{GM} + D_{GB}(1-(\frac{D_{BG}}{(D_{BG} + D_{B2} + D_{BM} + D_{GD})}))} \qquad \text{(B.3)}$$

Equation B.3 shows that if chemical elimination via the gills is insignificant (i.e. $D_{B2} = 0$), the chemical is not biotransformed in the body of the fish or in the gastro-intestinal tract (i.e. $D_{BM} = D_{GM} = 0$) and growth dilution is insignificant (i.e. $D_{GD} = 0$) then F_{GD} reaches its maximum value, i.e.

$$F_{GD} = \frac{f_G}{f_D} = \frac{D_I}{D_{GE}} = \frac{G_I.Z_D}{G_{GE}.Z_G} \quad \text{(B.4)}$$

Where

$$D_I = G_I.Z_D \quad \text{(B.5)}$$

$$D_{GE} = G_{GE}.Z_G \quad \text{(B.6)}$$

G_I : dietary ingestion rate ($m^3.day^{-1}$)

G_{GE} : fecal egestion from the gastro-intestinal tract ($m^3.day^{-1}$)

Z_D :fugacity capacity of the chemical in the diet ($mol.Pa^{-1}.m^{-3}$)

Z_G : fugacity capacity of the chemical in the digesta ($mol.Pa^{-1}.m^{-3}$)

Equation B.4 illustrates that the maximum gastro-intestinal magnification factor is controlled by the degree to which the diet is assimilated, expressed as the ratio of the feeding and fecal egestion rates, and the degree to which the dietary matrix is digested, expressed as the ratio of the fugacity capacities of the diet and the digesta, i.e. Z_D/Z_G, which equals the dimensionless diet to digesta partition coefficient K_{DG}.

Equation B.3 shows that under the same simplifying conditions (i.e. $D_{B2} = 0$, $D_{BM} = 0$ and $D_{GD} = 0$), a substance that is biotransformed in the gastro-intestinal tract (i.e. $D_{GM} \neq 0$) achieves a gastro-intestinal magnification factor F_{GD} below its theoretical maximum limit expressed in equation B.4, i.e.

$$F_{GD} = \frac{f_G}{f_D} = \frac{D_I}{D_{GE} + D_{GM}} = \frac{G_I.Z_D}{G_{GE}.Z_G + V_G.Z_G.k_{GM}} \quad \text{(B.7)}$$

where

$$D_{GM} = V_G.k_{GM}.Z_G \quad \text{(B.8)}$$

k_{GM} : rate constant for gastro-intestinal biotransformation (day^{-1})

V_G : volume of the digesta (m^3)

To find the k_{GM} for which $F_{GD} = 1$ (i.e. no gastro-intestinal magnification), we can substitute $F_{GD} = 1$ into equation B.7 to give

$$k_{GM}^{*} = \frac{G_I}{V_G} \cdot \frac{Z_D}{Z_G} - \frac{G_{GE}}{V_G} \quad \text{(B.9)}$$

If we further recognize that

$$G_{GE} = \gamma_{GI} G_I \quad \text{(B.10)}$$

where γ_{GI} is the unitless ratio of the fecal egestion and dietary ingestion rates, representing the assimilation of food by the fish body; and that the chemical partition coefficient between the diet and the digesta K_{DG} (unitless) can be defined as

$$K_{DG} = \frac{Z_D}{Z_G} \quad \text{(B.11)}$$

It follows that substituting equations B.10 and B.11 into equation B.9:

$$k_{GM}^{*} = \frac{G_I}{V_G}(K_{DG} - \gamma_{GI}) \quad \text{(B.12)}$$

Eqaution B.11 is equivalent to equation 3.27 in the manuscript. However, in equation B.11, G_I, V_G, K_{DG} and γ_{GI} are expressed in units of $m^3.d^{-1}$, m^3, unitless, and unitless respectively, while in equation 3.27 G_I, V_G, K_{DG} and γ_{GI} are expressed in units of kg food.d^{-1}, kg digesta, kg digesta.kg food^{-1} and kg digesta.kg food^{-1} respectively.

To find the chemical's maximum dietary uptake efficiency $E_{D,M}^{*}$ for gastro-intestinal magnification cannot occur, i.e. $F_{GD} \leq 1$, we can substitute equation B.11 into equation 3.14 in the manuscript, here reproduced as

$$(E_{D,M}^{*\,-1} - E_{D,N}^{-1}) = k_{GM} / k_{GB} \quad \text{(B.13)}$$

where k_{GB} is given by equation 3.15, here reproduced

$$k_{GB} = (E_{D,N} / (1-E_{D,N})).(G_{GE} / V_G) \quad \text{(B.14)}$$

Hence, combining equations B.11, B.13 and B.14 gives a relatively simple expression to determine the threshold dietary uptake efficiency $E^{*}_{D,M}$, which if not exceeded, indicates that the substance is not subject to gastro-intestinal magnification and hence is not expected to biomagnify in fish:

$$\frac{1}{E^{*}_{D,M}} = \frac{K_{DG}}{\gamma_{GI}} \cdot \frac{(1 - E_{D,N})}{E_{D,N}} + \frac{1}{E_{D,N}} \qquad \text{(B.15)}$$

This equation is similar to equation 3.29 in the manuscript with the only difference that K_{DG} and γ_{GI} are unitless in equation B.15, while in equation 3.29 K_{DG} and γ_{GI} are expressed in units of kg digesta.kg food^{-1}.

Illustration of Experimental Derivation of $E^{*}_{D,M}$

In our present study, a non-biotransformed reference substance with a log K_{OW} of 7 exhibits an $E_{D,N}$ (following equation 3.31 in the manuscript) of approximately:

$$E_{D,N} = (5.6.10^{-9} \,.\, 10^{7} + 1.9)^{-1} = 0.51 \qquad \text{(B.16)}$$

The diet of the fish consisted of dried fish food containing 18.6% lipids (ϕ_{DL} = 0.186), 46.6% protein (ϕ_{DP} = 0.466), 32.4% non-digestible organic matter (ϕ_{DN} = 0.324) and 2.4% water (ϕ_{DW} = 0.466). For very hydrophobic chemicals, one can ignore the water content of the diet as it contains a negligible amount of chemical. This assumption simplifies the derivation of several bioaccumulation metrics in a dietary bioaccumulation study as it ignores the absorption of water with the intake of food, which is poorly known and usually not characterized in dietary bioaccumulation experiments. Hence, if the water content is ignored and the derivation of bioaccumulation metrics is conducted on a dry weight basis, the composition of the food on a dry weight basis is 19.1% lipids (ϕ_{DL} = 0.191), 47.7% protein (ϕ_{DP} = 0.477), 33.2% non-digestible organic matter (ϕ_{DN} = 0.332) and 0% water (ϕ_{DW} = 0).

To find K_{DG} it is important to characterize the composition of the digesta. This can be done by using equations 3.22 to 3.25 in the manuscript applying a dietary lipid assimilation efficiency in rainbow trout of 92% (ε_{L}=0.92), a protein assimilation efficiency

of 75% (ε_P=0.75) and an assimilation efficiency for non-digestible organic matter of 0%. In our experiment this produces the following

$$\phi_{GL} = (1\text{-}0.92).0.191 / \{(1\text{-}0.92).0.191 + (1\text{-}0.75).0.477 + (1\text{-}0).0.332\} = 0.033 \quad \text{(B.17)}$$

$$\phi_{GP} = (1\text{-}0.75).0.477 / \{(1\text{-}0.92).0.191 + (1\text{-}0.75).0.477 + (1\text{-}0).0.332\} = 0.256 \quad \text{(B.18)}$$

$$\phi_{GN} = (1\text{-}0).0.332 / \{(1\text{-}0.92).0.191 + (1\text{-}0.75).0.477 + (1\text{-}0).0.332\} = 0.711 \quad \text{(B.19)}$$

On a dry weight basis, the diet-digesta partition coefficient (K_{DG}) for a substance with a log Kow of 7 is:

$K_{DG} = (0.191 \,.\, 10^7 + 0.477 \,.\, 0.05 \,.\, 10^7 + 0.332 \,.\, 0.05 \,.\, 10^7 + 0) / (0.033.K_{OW} + 0.256 \,.\, 0.05 \,.\, 10^7 + 0.711 \,.\, 0.05 \,.\, 10^7 + 0) = 2.85$

and:

$$\gamma_{GI} = (1\text{-}0.92).0.191 + (1\text{-}0.75).0.477 + (1\text{-}0).0.332 = 0.47$$

hence,

$$\frac{1}{E^*_{D,M}} = \frac{2.85}{0.47} \cdot \frac{1\text{-}0.51}{0.51} + \frac{1}{0.51} = 7.79$$

and:

$$E^*_{D,M} = \frac{1}{7.79} = 0.13$$

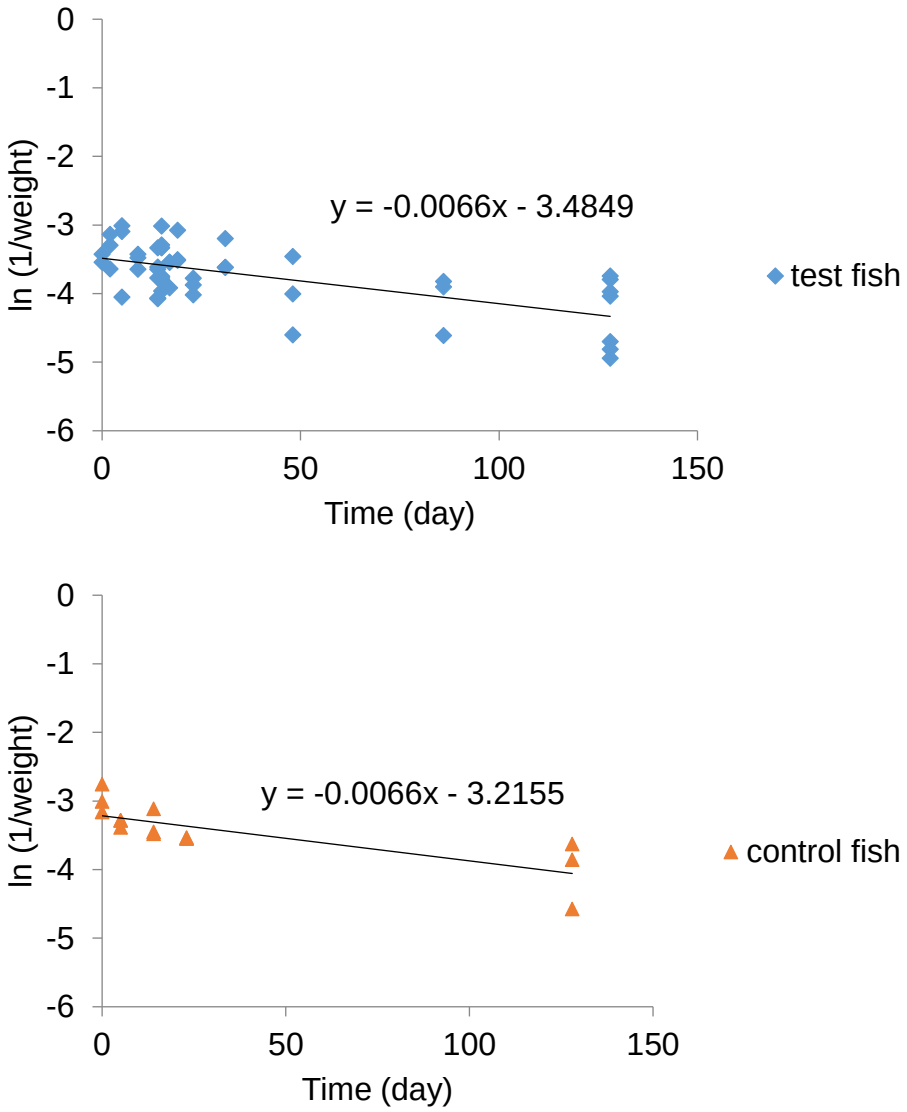
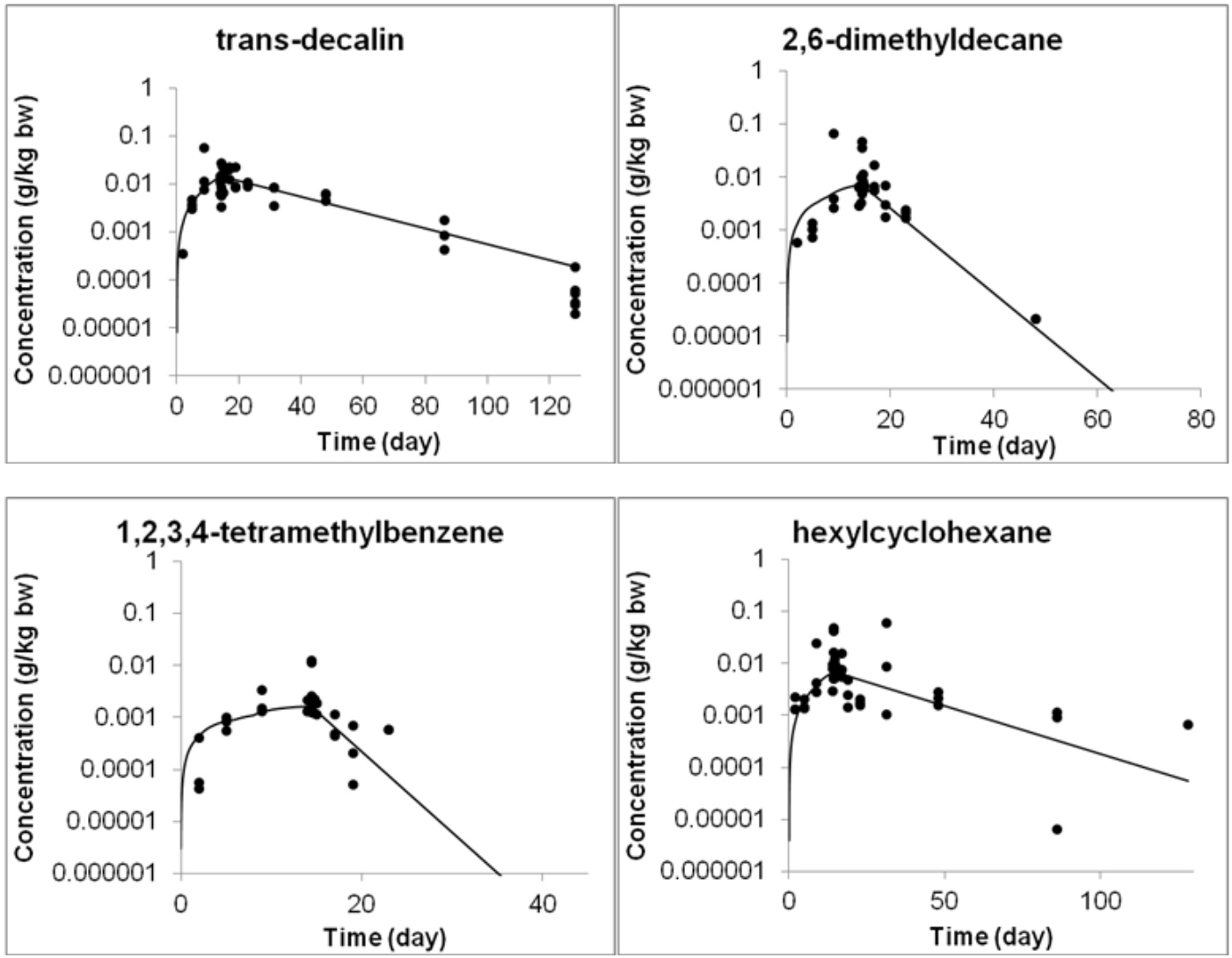
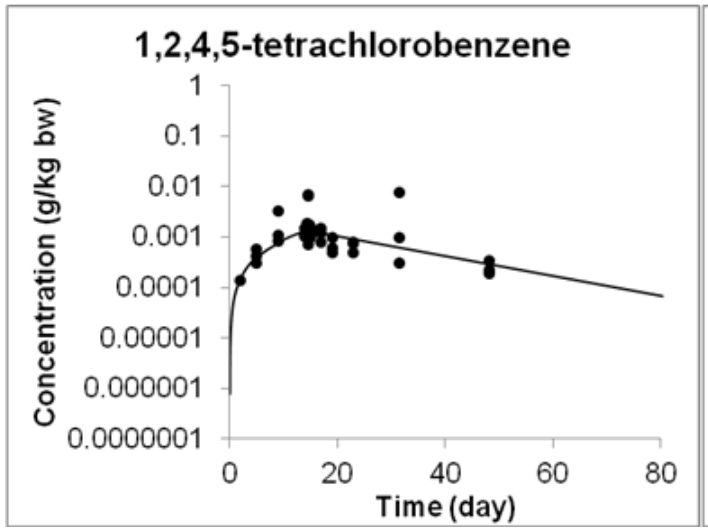
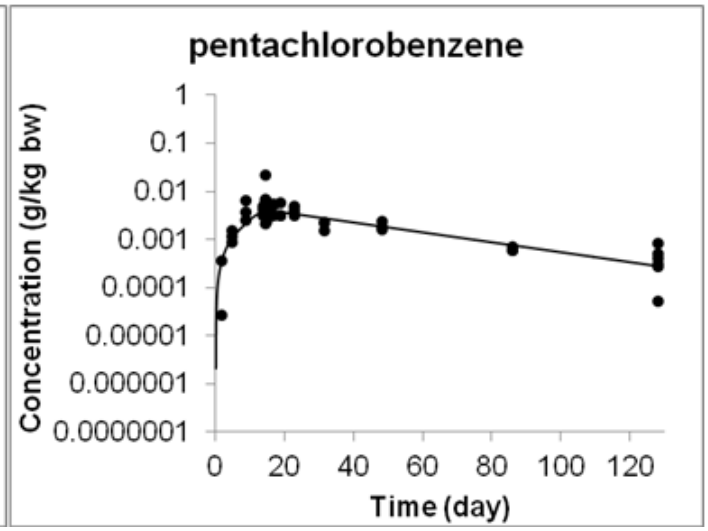
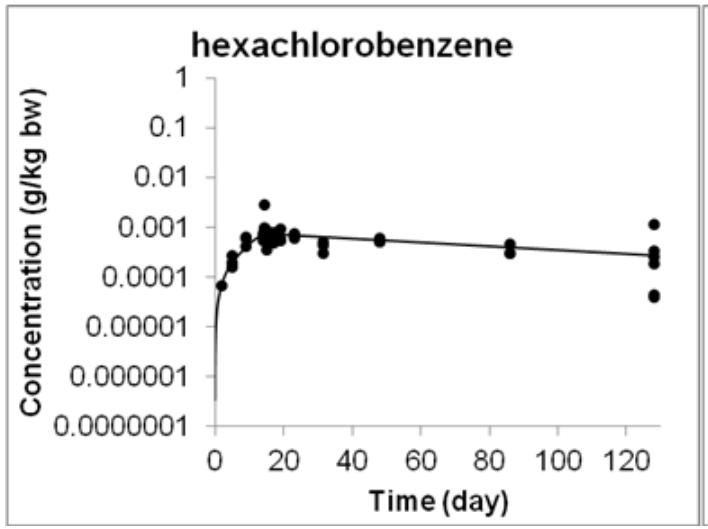


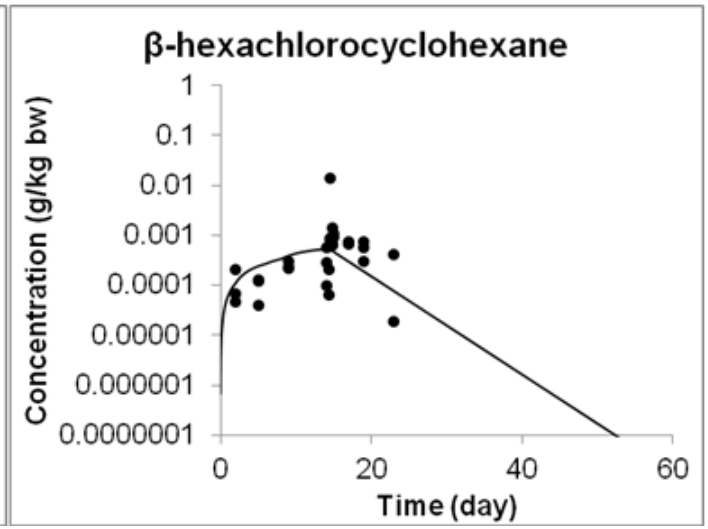
Figure B1 **Growth rate constants calculated as the slope of the natural logarithm of 1/weight (g) vs. time (day) for test and control fish.**

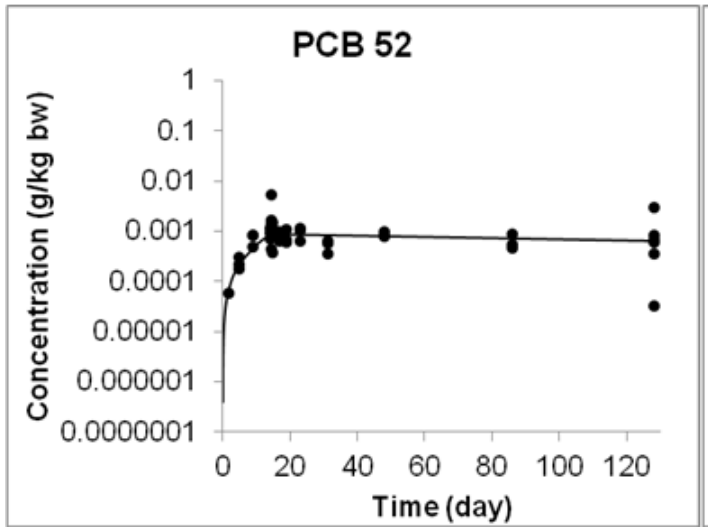

trans-decalin
Concentration (g/kg bw)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0
20
40
60
80
100
120
Time (day)
2,6-dimethyldecane
Concentration (g/kg bw)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0
20
40
60
80
Time (day)
1,2,3,4-tetramethylbenzene
Concentration (g/kg bw)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0
20
40
Time (day)
hexylcyclohexane
Concentration (g/kg bw)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0
50
100
Time (day)

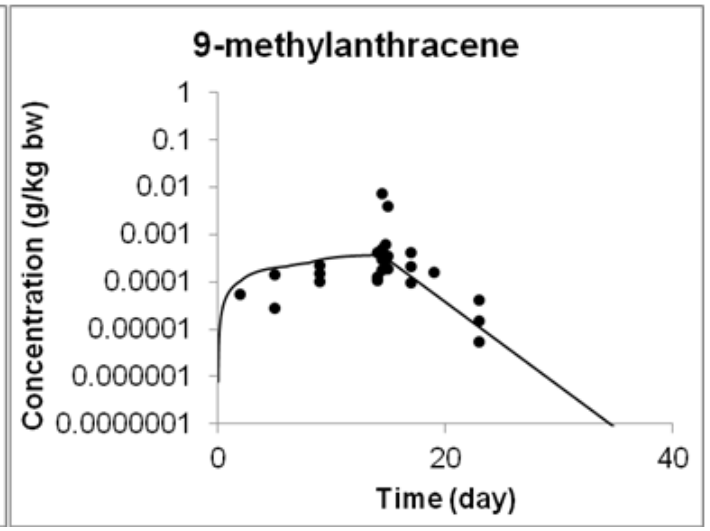

1,2,4,5-tetrachlorobenzene
Concentration (g/kg bw)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0.0000001
0
20
40
60
80
Time (day)

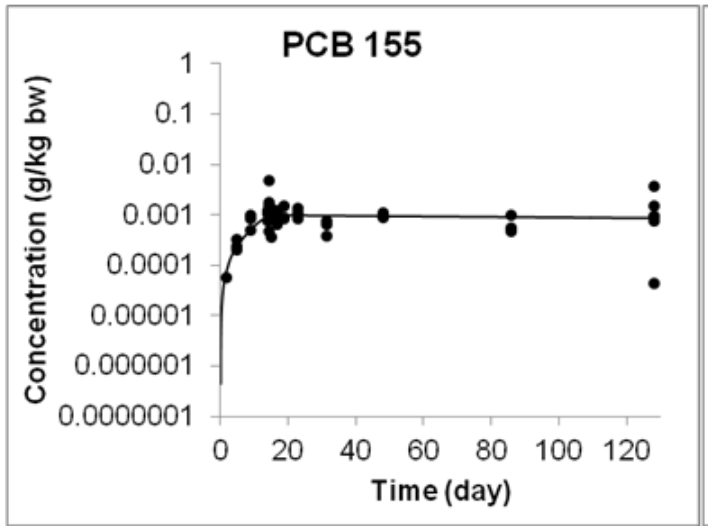

pentachlorobenzene
Concentration (g/kg bw)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0.0000001
0
20
40
60
80
100
120
Time (day)

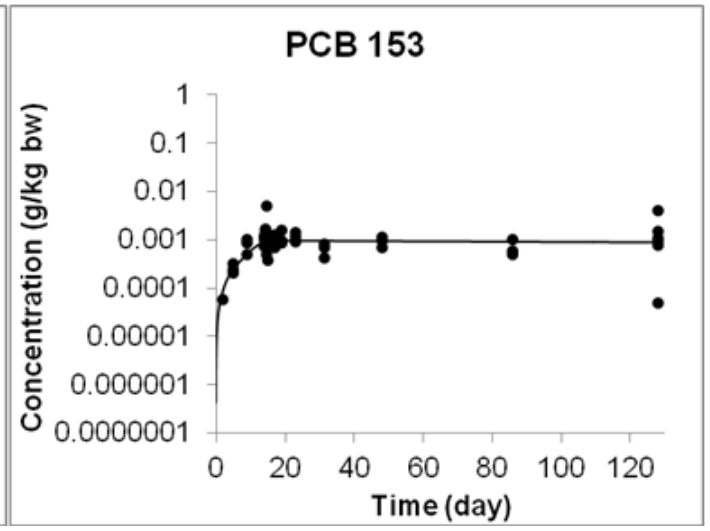

hexachlorobenzene
Concentration (g/kg bw)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0.0000001
0
20
40
60
80
100
120
Time (day)

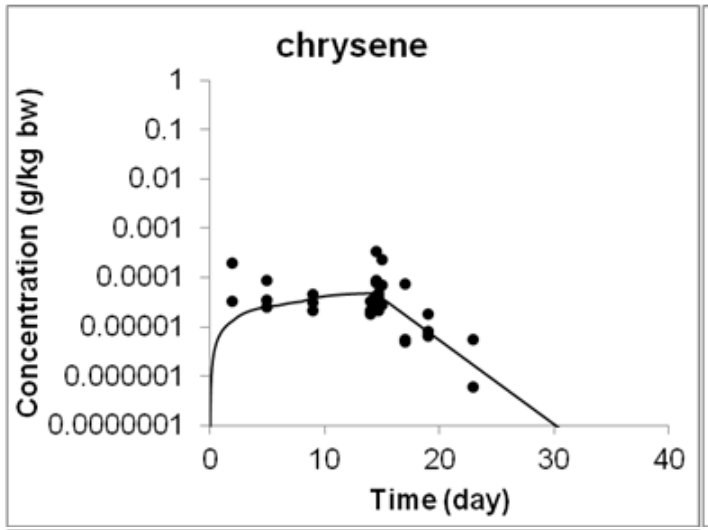

β-hexachlorocyclohexane
Concentration (g/kg bw)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0.0000001
0
20
40
60
Time (day)

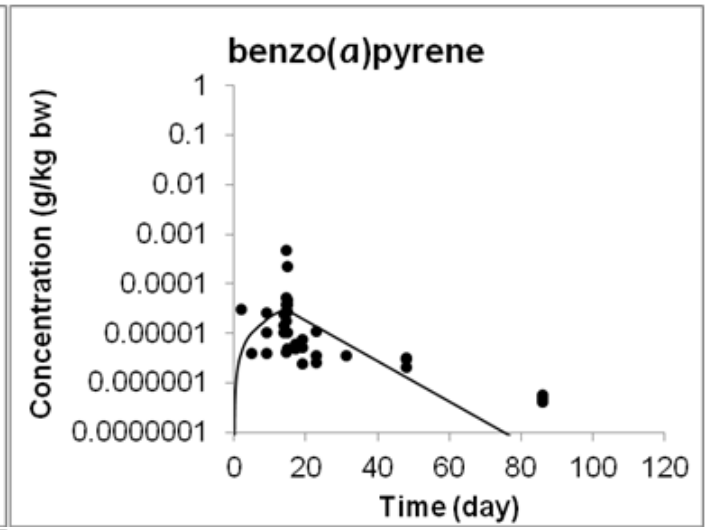

PCB 52
Concentration (g/kg bw)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0.0000001
0
20
40
60
80
100
120
Time (day)


9-methylanthracene
Concentration (g/kg bw)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0.0000001
0
20
40
Time (day)


PCB 155
Concentration (g/kg bw)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0.0000001
0
20
40
60
80
100
120
Time (day)


PCB 153
Concentration (g/kg bw)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0.0000001
0
20
40
60
80
100
120
Time (day)


chrysene
Concentration (g/kg bw)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0.0000001
0
10
20
30
40
Time (day)


benzo(a)pyrene
Concentration (g/kg bw)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0.0000001
0
20
40
60
80
100
120
Time (day)

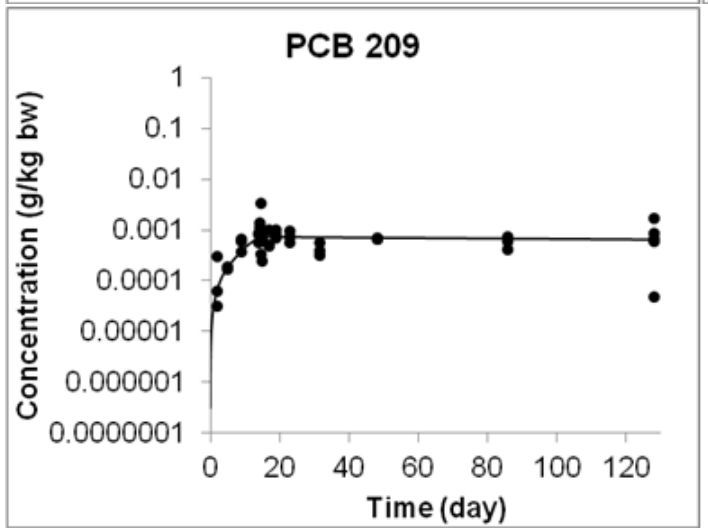

PCB 209
Concentration (g/kg bw)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0.0000001
0
20
40
60
80
100
120
Time (day)

Figure B2 **Concentrations of the reference and test chemicals in the fish throughout the uptake and depuration phase of the dietary bioaccumulation experiment.**

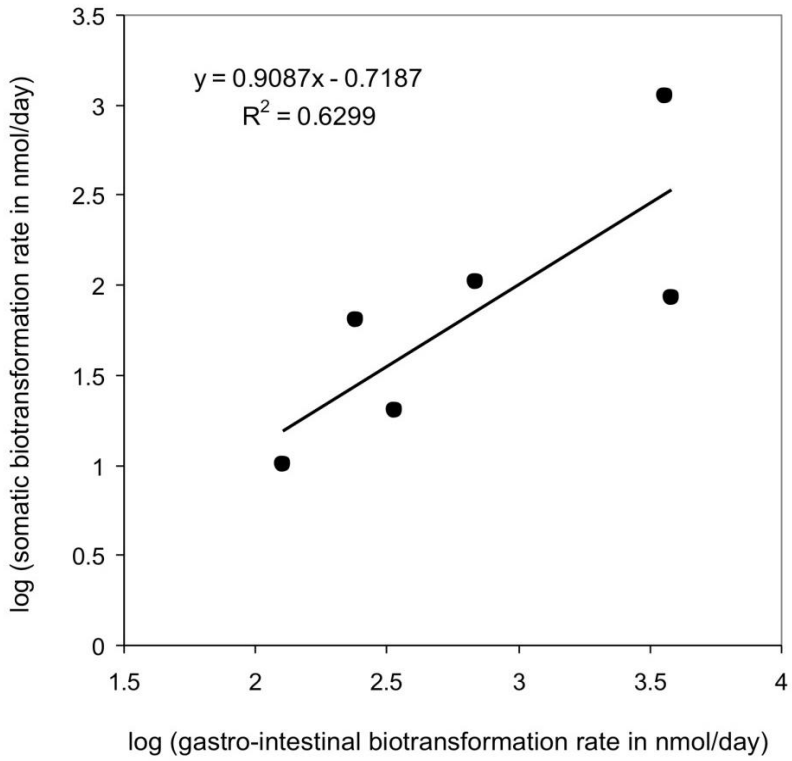


Figure B3 **Observed relationship between the logarithms of the gastro-intestinal and somatic biotransformation rates expressed in units of nmol.day^{-1} for 6 of the 8 test chemicals that were biotransformed in both the body and the gastro-intestinal tract of the fish.**

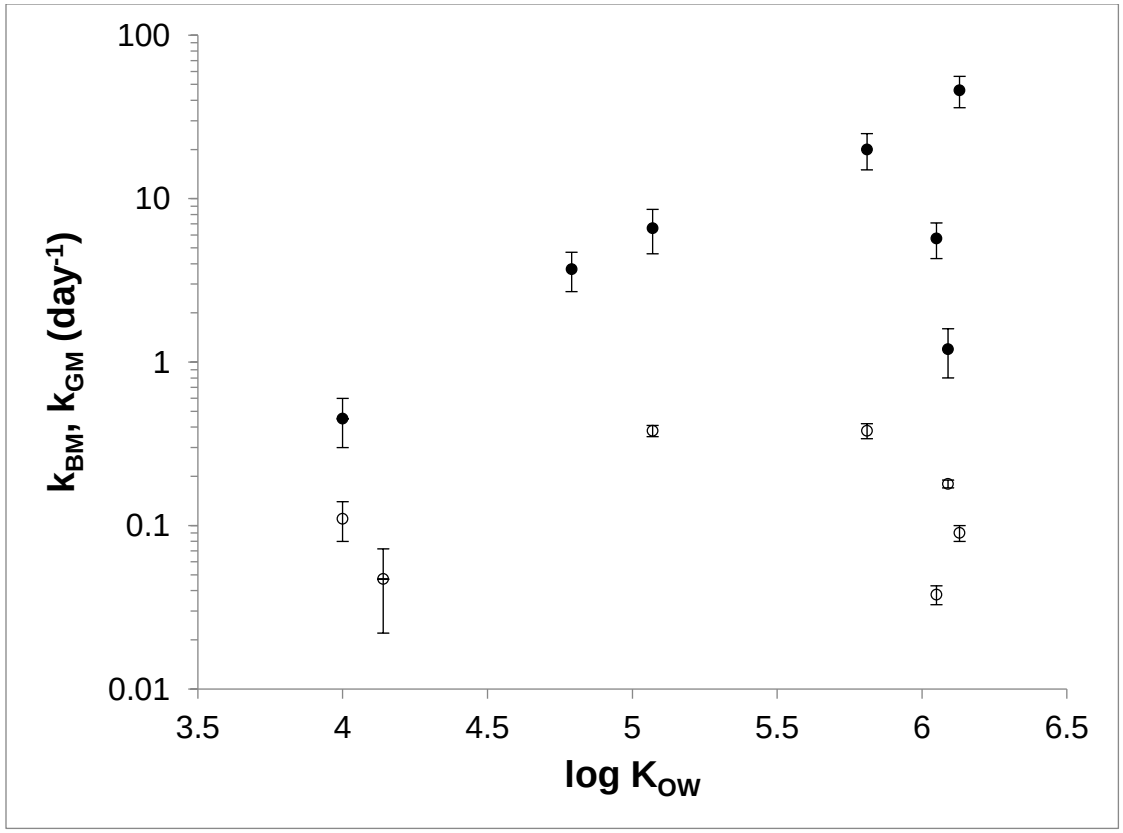


Figure B4 **The somatic (open symbols, k_{BM}) and gastro-intestinal (closed symbols, k_{GM}) biotransformation rate constant of the test chemicals as a function of log K_{OW}. Error bars are the standard error of the mean estimates.**

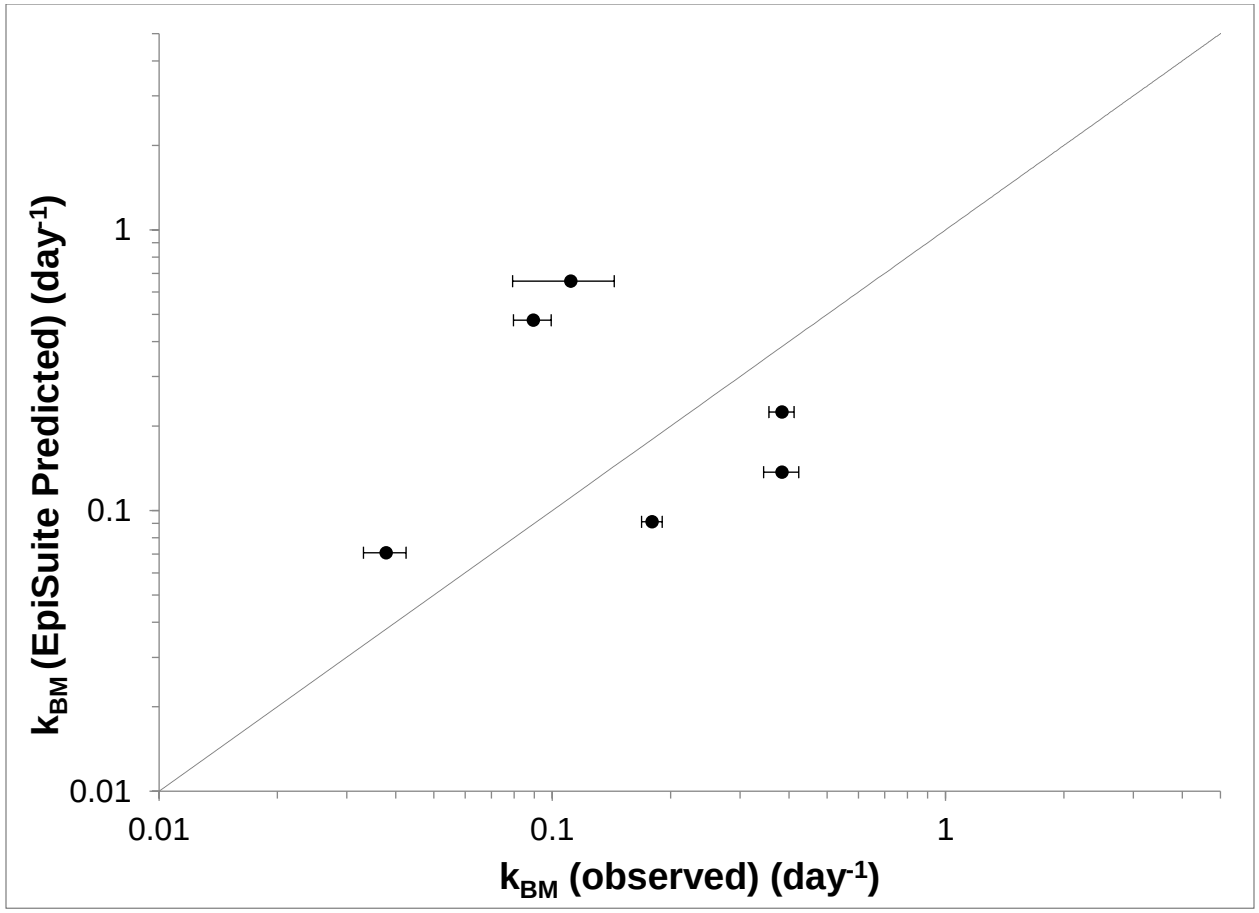


Figure B5 **EpiSuite (BCFBAF) predicted biotransformation rate constant as a function the measured somatic biotransformation rate constants (day^{-1}). From left to right: hexylcyclohexane, benzo[*a*]pyrene, 1,2,3,4-tetramethylbenzene, 2,6-dimethyldecane, 9-methylanthracene (top), chrysene (bottom). Data are listed in Table 2. Trans-decalin is not included.**

Table B1 Model parameters used to calibrate the Arnot and Gobas (2004) bioaccumulation model for fish to observed depuration rate constants of the reference chemicals.

Symbol	Parameter	Value	Source
T	Water temperature	12.6 °C	Measured
W_B	Whole body weight	0.062 kg	Measured
χ	sorptive capacity of protein relative to octanol	0.05	[deBryun and Gobas 2004]
S	Water dissolved oxygen saturation	90%	Measured
G_I	Feeding rate	0.44 g/day	Measured
ϕ_{FL}	Lipid content of organism	6.7%	Model Estimate
ϕ_{FP}, ϕ_{FW}	Protein, and water content of organism	18%, 73.7%	[Arnot and Gobas 2004]
ϕ_{DL}, ϕ_{DP}, ϕ_{DN}, ϕ_{DW}	Lipid, protein, non-digestable, and water content of diet	18.6%, 46.6%, 32.4% 0.025%	Measured (ϕ_{DN}, ϕ_{DW})
ε_L, ε_N, ε_W	Lipid, protein, and water assimilation efficiencies	92%, 75%, 50%	[Gobas et al. 1999 for ε_L]
k_G	Growth rate constant	0.0005 (se 0.002) /day	Model Estimate

References

1. Gobas FAPC, Wilcockson JWB, Russell RW, Haffner GD. 1999. Mechanism of biomagnification in fish under laboratory and field conditions. *Environmental Science and Technology* 33:133-141.
2. Arnot JA, Gobas FAPC. 2004. A food web bioaccumulation model for organic chemicals in aquatic ecosystems. *Environmental Toxicology and Chemistry* 23:2343-2355.
3. deBruyn AMH, Gobas FAPC. 2007. The soprtive capcity of animal protein. *Environmental Toxicology and Chemistry* 9:1803-1808.
4. Gobas FAPC, Wilcockson JWB, Russell RW, Haffner GD. 1999. Mechanism of biomagnification in fish under laboratory and field conditions. *Environmental Science and Technology* 33:133-141.

Appendix C

Supplemental Information for Chapter 5

Supplemental information for “In Vivo Biotransformation Rates of Organic Chemicals in Fish: Relationship with Bioconcentration and Biomagnification Factors”

Theory

Deriving k_{BG}

The chemical transfer from the body to the GI content rate constant, k_{BG}, can be determined as (C1):

$$k_{BG} = K_{GB} \cdot k_{GB} \cdot W_G / W_B \quad \text{(C1)}$$

where K_{GB} is the chemical partitioning between the gastro-intestinal contents and the body; W_G (kg) is the steady state amount of digesta in the gastrointestinal tract; and W_B is the weight of the body of the fish. K_{BG} is determined as:

$$K_{GB} = (\phi_{GL} \cdot K_{OW} + \phi_{GP} \cdot \chi \cdot K_{OW} + \phi_{GN} \cdot \theta \cdot K_{OW} + \phi_{GW}) / (\phi_{BL} \cdot K_{OW} + \phi_{BP} \cdot \chi \cdot K_{OW} + \phi_{BN} \cdot \theta \cdot K_{OW} + \phi_{BW}) \quad \text{(C2)}$$

where ϕ_{GL}, ϕ_{GP}, ϕ_{GN}, ϕ_{GW}, ϕ_{BL}, ϕ_{BP}, ϕ_{BN}, ϕ_{BW} are the proportions of lipid, protein, non-digestible organic matter, and water in the gastro-intestinal contents and the body, respectively. χ and θ, the sorptive capacities of protein and non-digestible organic matter relative to octanol, are estimated to be 0.05. The composition of the digesta ϕ_{GL}, ϕ_{GP}, ϕ_{GN}, ϕ_{GW} are often difficult to measure, but can be estimated as:

$$\Phi_{GL} = (1-\varepsilon_L)\,\Phi_{DL} \,/\, \{(1-\varepsilon_L)\,\Phi_{DL} + (1-\varepsilon_P)\,\Phi_{DP} + (1-\varepsilon_N)\,\Phi_{DN} + (1-\varepsilon_W)\,\Phi_{DW}\} \quad \text{(C3)}$$

$$\Phi_{GP} = (1-\varepsilon_P)\,\Phi_{DP} \,/\, \{(1-\varepsilon_L)\,\Phi_{DL} + (1-\varepsilon_P)\,\Phi_{DP} + (1-\varepsilon_N)\,\Phi_{DN} + (1-\varepsilon_W)\,\Phi_{DW}\} \quad \text{(C4)}$$

$$\Phi_{GN} = (1-\varepsilon_N)\,\Phi_{DN} \,/\, \{(1-\varepsilon_L)\,\Phi_{DL} + (1-\varepsilon_P)\,\Phi_{DP} + (1-\varepsilon_N)\,\Phi_{DN} + (1-\varepsilon_W)\,\Phi_{DW}\} \quad \text{(C5)}$$

$$\Phi_{GW} = (1-\varepsilon_W)\,\Phi_{DW} \,/\, \{(1-\varepsilon_L)\,\Phi_{DL} + (1-\varepsilon_P)\,\Phi_{DP} + (1-\varepsilon_N)\,\Phi_{DN} + (1-\varepsilon_W)\,\Phi_{DW}\} \quad \text{(C6)}$$

where ε_L, ε_P, ε_N, ε_W are the dietary assimilation efficiencies of the lipids, protein, non-digestible organic matter, and water and estimated to be 0.92 (*2*), 0.75, 0, 0.5. Φ_{DL}, Φ_{DP},, Φ_{DN} and Φ_{DW} are the fractions of lipid (kg lipid.kg food^{-1}), protein (kg protein.kg food^{-1}), non-digestible organic matter (kg non-digestible organic matter.kg food^{-1}) and water (kg water.kg food^{-1}) of the fish's diet.

Derivation of the digesta evacuation rate constant (d^{-1})

The intestinal evacuation can be described as:

$$dM/dt = -\delta.M \qquad (C7)$$

where M is the mass of intestinal content (g); δ is the digesta evacuation rate constant (d^{-1}); and t is time (d). Rearranging equation C7 gives:

$$dM/M = -\delta.dt \qquad (C8)$$

Integration from t=0 to t then produces:

$$\ln M = -\delta.t + \ln M_{t=0} \qquad (C9)$$

where $M_{t=0}$ is the mass of digesta at t=0 when the evacuation initiated. Then, after rearranging:

$$\ln (M_{t=0}/M) = \delta.t \qquad (C10)$$

The 95% digesta evacuation time ($t_{E,95}$) can be defined as the time at which $M_{t=0}/M = 20$ or $M/M_{t=0} = 0.05$. The ln(20) is approximately 3. Hence δ can be approximated as $3/t_{E,95}$ where the 95% digesta evacuation time ($t_{E,95}$) is assumed to be similar to the 100% digesta evacuation time reported.

Materials and Methods

Chemical analysis

One-gram aliquots of diet contaminated with the spiked test compounds were extracted with 10 mL or 25 mL of a 1:1 solvent mixture of dichloromethane and acetone

using a combination of sonication and mechanical agitation. One-milliliter portions of the extracts were spiked with a semi-volatile internal standard solution consisting of a series of deuterated aromatic hydrocarbons (1,4-dichlorobenzene-d4, naphthalene-d8, acenaphthene-d10, phenanthrene-d10, chrysene-d12, perylene-d12). Depending on the test chemical mixture composition, alternative internal standards were used to provide structurally similar chemical analogs. Extracts were analyzed by gas chromatography-mass spectrometry operated in the selective ion monitoring mode (GC-MS (SIM)). Triplicate diet aliquots were extracted and analyzed at both the initiation and conclusion of the uptake phase to demonstrate stability of the test compound in diet and to calculate the mean dietary concentration to which fish were exposed.

Individual whole fish were homogenized with a metal spatula in glass beakers with three grams of solvent-extracted Hydromatrix® and permitted to dry from four hours to overnight, depending on the relative volatility of compounds under investigation. The dried fish-desiccant samples were extracted by accelerated solvent extraction (ASE) at 1500 psi and 120^{o}C using a 1:1 mixture of dichloromethane and acetone. Collected extract volumes were adjusted to final volumes of either 15 or 20 mL with dichloromethane. Portions of each extract were diluted in hexane and subject to normal-phase solid phase extraction (SPE) clean-up using disposable silica or alumina cartridges containing 1-2 grams of sorbent. The cleaned–up extracts were spiked with the semi-volatile internals standard solution or alterantive internal standard mixture and evaporated under nitrogen to a final volume of 0.5 or 1.0 mL in methylene chloride. The fish extracts were analyzed by GC-MS (SIM) using the same instrument conditions for analysis of the corresponding diet extracts except that large volume injections (LVI) of 10 to 40 µL were made in order to achieve the necessary analytical sensitivity for tissue analysis. Recoveries of test compounds from spiked control fish homogenates indicated quantitative recovery (80%-120%) of nearly all test compounds.

Lipid content analysis

Diet and fish lipid content was determined gravimetrically based on the method described in Dionex Application Note 337. Briefly, diet or one or more whole fish with a total mass of between one and four grams were homogenized with three grams of Hydromatrix and permitted to dry overnight. The dried mixtures were extracted by ASE at 1500 psi and 125^{o}C using hexane. The collected solvent was evaporated under

nitrogen. The extracts were then dried in an oven at 105°C for one hour before being weighed to determine the exact lipid mass.

Total tissue concentrations

Experiment 1 involved the highest total dietary test concentrations and resulting tissue concentrations at the end of the exposure period than all other tests. Chronic toxic units ($\Sigma TU_{chronic}$) associated with this experiment were calculated by dividing the lipid normalized tissues for each test substance at the end of exposures periods by the corresponding chronic critical target lipid model estimated for rainbow trout using the target lipid model and then summing these ratios (*3*). Calculations, detailed in Table C6, show that the $\sum TU_{chronic} = 0.3$, consistent with the lack of growth effects observed in this study.

Kinetic analysis

First, a single segmented regression was performed to determine the dietary uptake efficiency (E_D); the proportional bodyweight feeding rate (F_D) and the total body elimination rate constant (k_{BT}) from the concentrations of the chemical in the fish during the dietary exposure and depuration phases of the test, using:

$$C_B = (E_D \cdot F_D \cdot C_D / k_{BT}) [1 - \exp(k_{BT} \cdot t)] \quad \text{(C11)}$$

during the uptake phase and

$$C_B = C_{B,t0} \cdot \exp[-k_{BT} \cdot (t - t_0)] \quad \text{(C12)}$$

during the depuration phase. Here, t_0 (d) is the time at which the contaminated diet was replaced with the uncontaminated diet, and $C_{B,t0}$ is the concentration in the body of the fish at t_0. To use most of the experimental information available, a single regression combining Equation C11 for days < t_0 and Equation C12 for days > t_0 (Figure C1) was used to solve for ($E_D \cdot F_D \cdot C_D$) and k_{BT}.

Secondly, a weighted multiple linear regression (weights equal to the reciprocal of the standard error) was used to fit the observed depuration rate constant ($k_{BT,R}$) for the

reference chemicals (i.e., hexachlorobenzene in 9 tests and transdecalin in 1 test) from the present study (n = 10) and from Lo et al. (*1*) (n = 8) to Equation 5.7 to find α and b.

Third, k_{BM} of the test chemicals was calculated by subtracting the total body elimination rate constants k_{BT} of each test and the corresponding $k_{BT,R}$, derived from the linear regression using the K_{OW} of the test chemical (Equation 5.5). The $BCF_{ww,fd}$ was converted into the $BCF_{WW,t}$ following Equation 5.9 using a χ_{OC} of 2 mg/L i.e., equal to the maximum recommended total organic carbon concentration in a OECD 305 guideline (*4*) and a dissolved organic carbon/water partition coefficient (K_{OC}) calculated from log K_{OW} according to (*5*):

$$\log K_{OC} = 0.97.\log K_{OW} - 1.27 \qquad \text{(C13)}$$

Fourth, to determine the dietary uptake efficiency of the test chemicals $E_{D,M}$ given uncertainty in the actual feeding rate of the fish, the dietary uptake efficiency $E_{D,N}$ of the reference chemical hexachlorobenzene was set to a mean value of 52.7%, determined in (*1*). Since C_D of hexachlorobenzene was measured and $E_{D,N} \cdot F_D \cdot C_D$ determined via linear regression of the concentration data for hexachlorobenzene, the feeding rate (F_D) was estimated and then used to determine the dietary uptake efficiency ($E_{D,M}$) of the test chemicals. This calculation is not necessary if actual feeding rates are known and has only marginal effects on the derivation of the intestinal biotransformation rate constants of the test chemicals (Figure C2).

Fifth, *g*astro-intestinal biotransformation rate constants (k_{GM}) of the test chemicals were derived according to Equation 5.10 from the dietary uptake efficiencies of the test chemical ($E_{D,M}$) and the corresponding dietary uptake efficiencies of the non-biotransformed reference chemical ($E_{D,N}$), derived from Equation C13 using the K_{OW} of the test chemical. A γ_{GI} for rainbow trout of 0.48 kg digesta dry weight·kg food dry weight^{-1} (*2*) and a $t_{E,95}$ in rainbow trout of 1.45 d (*6*) were used in the calculations.

Table C1 **General parameters of the bioaccumulation studies, including test number, uptake time (t_{uptake}), proportion bodyweight feeding rates (F_D), average weights of test fish ($W_{B,test}$), fish body lipid content (ϕ_{BL}), dietary lipid content (ϕ_{DL}) and growth dilution rate constants of test ($k_{GD,\ test}$) and control fish ($k_{GD,\ control}$).**

Test	t_{uptake} d	F_D d^{-1}	$W_{B,test}$ g	ϕ_{BL}	ϕ_{DL}	$k_{GD,\ test}$ d^{-1}	$k_{GD,\ control}$ d^{-1}
1	10	0.07 (SE 0.01)	1.0 (SE 0.1)	2.80%	15.60%	0.047 (SE 0.006)	0.044 (SE 0.006)
2	13	0.04 (SE 0.01)	2.3 (SE 0.3)	4.77%	15.60%	0.032 (SE 0.003)	0.0033 (SE 0.003)
3	10	0.032 (SE 0.008)	0.88 (SE 0.05)	2.39%	15.60%	0.039 (SE 0.005)	0.039 (SE 0.004)
4	14	0.03 (SE 0.01)	1.12 (SE 0.04)	3.22%	15.60%	0.041 (SE 0.002)	0.040 (SE 0.002)
5	13	0.03 (SE 0.01)	1.37 (SE 0.05)	3.46%	15.60%	0.036 (SE 0.002)	0.034 (SE 0.003)
6	13	0.021 (SE 0.006)	1.9 (SE 0.2)	3.27%	15.60%	0.034 (SE 0.003)	0.037 (SE 0.003)
7	11	0.024 (SE 0.009)	1.9 (SE 0.2)	5.58%	15.60%	0.027 (SE 0.004)	0.024 (SE 0.003)
8	11	0.024 (SE 0.008)	1.41 (SE 0.07)	3.38%	15.60%	0.041 (SE 0.002)	0.043 (SE 0.002)
9	13	0.041 (SE 0.007)	1.6 (SE 0.2)	5.11%	15.00%	0.042 (SE 0.002)	0.040 (SE 0.002)

Table C2 **The log KOW, mean concentrations of test chemicals in the diet of the fish CD with standard deviation, number of observations in regression analysis n, total elimination rate constant from the fish body kBT with its standard error, total elimination rate constant from the fish body for non-metabolizable chemical kBT,R with standard error, and somatic biotransformation rate kBM with standard error.**

Test	Chemical	log K_{OW}	C_D ± SD (µg.g^{-1})	n	k_{BT} ± SE (d^{-1})	$k_{BT,R}$ ± SE (d^{-1})	k_{BM} ± SE (d^{-1})
1	hexachlorobenzene	5.73	100.0 ± 4.8	57	0.08 ± 0.02	0.067 ± 0.006	0.01 ± 0.02
	2,6-dimethyldecane	6.09	96.3 ± 0.8	60	0.22 ± 0.02	0.056 ± 0.006	0.17 ± 0.02
	2,3 dimethylheptane	4.61	78.9 ± 13.3	60	0.29 ± 0.02	0.303 ± 0.006	-0.01 ± 0.02
	1-methylphenanthrene	5.08	130.0 ± 4.9	39	0.45 ± 0.07	0.134 ± 0.006	0.32 ± 0.07
	n-dodecane	6.1	100.0 ± 0.4	60	0.22 ± 0.02	0.055 ± 0.006	0.17 ± 0.02
	n-nonane	4.76	88.1 ± 13.4	50	0.43 ± 0.04	0.228 ± 0.006	0.2 ± 0.04
	phenanthrene	4.46	105.0 ±2.8	50	0.47 ± 0.04	0.408 ± 0.006	0.06 ± 0.04
	2,2,4,6,6 -pentamethylheptane	5.94	93.7 ± 8.6	60	0.19 ± 0.02	0.059 ± 0.006	0.13 ± 0.02
	trans-decalin	4.2	100.0 ±0.9	60	0.2 ± 0.02	0.216 ± 0.006	-0.02 ± 0.02
	1,3,5-trimethylbenzene	3.66	101.0 ± 8.8	30	0.8 ± 0.1	2.327 ± 0.006	-1.6 ± 0.1
	1,3,5-trimethylcyclohexane	4.42	80.1 ± 10.8	60	0.29 ± 0.02	0.443 ± 0.006	-0.15 ± 0.02
2	hexachlorobenzene	5.73	26.5 ± 1.3	30	0.06 ± 0.02	0.042 ± 0.003	0.02 ± 0.02
	3,5,5-trimethylcyclohexene	4.25	85.8 ± 1.3	20	0.58 ± 0.06	0.326 ± 0.003	0.25 ± 0.06
	2,4,6-trimethyl-3-heptene	4.95	78.4 ± 0.1	25	0.23 ± 0.02	0.091 ± 0.003	0.14 ± 0.03
	cis-1,1,3,5-tetramethylcyclohexane	4.88	86.6 ± 0.4	30	0.12 ± 0.02	0.101 ± 0.003	0.02 ± 0.02
	1-decene	5.12	91.2 ± 1.0	25	0.22 ± 0.02	0.072 ± 0.003	0.15 ± 0.03
	iso-butylcyclohexane	4.99	90.8 ± 0.6	30	0.23 ± 0.02	0.086 ± 0.003	0.15 ± 0.02
	cis-bicyclo(4.3.0)nonane	3.71	95.5 ± 1.4	20	0.52 ± 0.06	1.049 ± 0.003	-0.53 ± 0.06
	2,2,4,6,6-pentamethyl-3-heptene	5.85	106.0 ± 1.4	25	0.25 ± 0.02	0.04 ± 0.003	0.21 ± 0.03
	1-tert-butyl-4-methylbenzene	5.17	102.0 ± 0.7	20	0.57 ± 0.06	0.067 ± 0.003	0.5 ± 0.06
	1,2,3,4-tetramethylbenzene	4	98.1 ± 4.2	20	0.64 ± 0.06	0.554 ± 0.003	0.08 ± 0.06
	1-dodecene	6.1	88.2 ± 4.2	25	0.22 ± 0.02	0.036 ± 0.003	0.18 ± 0.03
	1,4-dimethyl-1,2,3,4-tetrahydronaphthalene	4.79	106.0 ± 4.2	25	0.33 ± 0.02	0.117 ± 0.003	0.21 ± 0.03
	1-hexadecene	8.06	96.2 ± 0.8	30	0.06 ± 0.02	0.032 ± 0.003	0.03 ± 0.02
3	hexachlorobenzene	5.73	8.0 ± 0.2	56	0.11 ± 0.03	0.063 ± 0.005	0.05 ± 0.04
	naphthalene	3.3	114.0 ± 7.8	23	1.8 ± 0.4	6.296 ± 0.005	-4.5 ± 0.4

Test	Chemical	log K_{OW}	C_D ± SD (µg.g^{-1})	n	k_{BT} ± SE (d^{-1})			$k_{BT,R}$ ± SE (d^{-1})			k_{BM} ± SE (d^{-1})		
	2-methylnaphthalene	3.86	97.5 ±1.6	23	2.1	±	0.4	1.762	±	0.005	0.4	±	0.4
	2,3-dimethylnaphthalene	4.4	96.8 ± 0.8	23	1.8	±	0.4	0.536	±	0.005	1.3	±	0.4
	deuterated hexadecane	8.2	115.0 ± 3.5	56	0.11	±	0.03	0.039	±	0.005	0.07	±	0.04
	anthracene	4.45	109.0 ± 1.4	23	1.3	±	0.4	0.482	±	0.005	0.8	±	0.4
	9-methylanthracene	5.07	104.0 ± 1.4	23	1.1	±	0.4	0.146	±	0.005	0.9	±	0.4
	pyrene	4.88	98.6 ± 1.3	23	1	±	0.4	0.204	±	0.005	0.8	±	0.4
	1-methylpyrene	5.48	105.0 ± 1.4	23	1	±	0.4	0.081	±	0.005	1	±	0.4
	1,2-benzoanthracene	5.76	101.0 ± 0.7	23	1.1	±	0.4	0.061	±	0.005	1	±	0.4
	benzo[a]pyrene	6.13	94.5 ± 0.2	31	0.6	±	0.2	0.049	±	0.005	0.6	±	0.2
	dibenzo[a,h]anthracene	6.54	88.7 ± 1.0	31	0.8	±	0.2	0.043	±	0.005	0.8	±	0.2
	hexachlorobenzene	5.73	24.3 ± 3.1	30	0.09	±	0.02	0.058	±	0.002	0.03	±	0.02
	1,1,3,3,5-pentamethylindan	6.3	48.2 ± 3.1	29	0.29	±	0.02	0.046	±	0.002	0.24	±	0.02
	2,2,5,7-tetramethyltetraline	5.92	84.3 ± 1.4	20	0.61	±	0.06	0.052	±	0.002	0.55	±	0.06
	6-n-butyl-2,3-dimethylnaphthalene	6.28	79.9 ± 6.6	25	0.4	±	0.03	0.046	±	0.002	0.35	±	0.03
	2,3-dimethyl-5-(4-methylpentyl)naphthalene	7.19	93.8 ± 8.1	15	1.2	±	0.1	0.042	±	0.002	1.2	±	0.1
	fichtelite (isopropylhydrophenanthrene)	6.54	43.7 ± 5.1	30	0.08	±	0.02	0.044	±	0.002	0.04	±	0.02
4	dehydroabietine	6.43	86.7 ± 10.7	30	0.17	±	0.02	0.045	±	0.002	0.12	±	0.02
	9-n butylphenanthrene	6.37	91.6 ± 17.6	25	0.43	±	0.03	0.045	±	0.002	0.38	±	0.03
	1-ethylpyrene	5.97	46.2 ± 5.9	20	0.73	±	0.06	0.051	±	0.002	0.68	±	0.06
	2,3,6,7-tetramethylanthracene	6.53	46.8 ± 5.2	25	0.34	±	0.03	0.044	±	0.002	0.29	±	0.03
	chrysene	5.81	83.4 ± 11.7	25	0.36	±	0.03	0.055	±	0.002	0.31	±	0.03
	1-octylpyrene	8.92	42.7 ± 1.1	30	0.1	±	0.02	0.041	±	0.002	0.06	±	0.02
	benzo[b]chrysene	6.54	28.8 ± 1.8	19	0.42	±	0.06	0.044	±	0.002	0.37	±	0.06
	hexachlorobenzene	5.73	28.6 ± 1.7	29	0.06	±	0.02	0.051	±	0.002	0.01	±	0.02
	3,5,5' - trimethyl hexene	4.44	88.9 ± 1.8	20	0.5	±	0.08	0.323	±	0.002	0.18	±	0.08
	2-methyl-1-nonene	5.27	100.0 ± 0.4	13	0.9	±	0.2	0.079	±	0.002	0.8	±	0.2
5	2-methyldecane	5.67	95.1 ± 0.9	15	1	±	0.2	0.053	±	0.002	0.9	±	0.2
	c7 iso-alkyl benzene	5.38	51.9 ± 0.1	15	1.3	±	0.2	0.069	±	0.002	1.2	±	0.2
	1-undecene	5.61	110.0 ± 0.9	15	1.1	±	0.2	0.056	±	0.002	1	±	0.2
	2-methyl-1-undecene	6.15	55.8 ± 3.0	14	0.8	±	0.2	0.042	±	0.002	0.8	±	0.2
	2-methyl-1-dodecene	6.65	58.5 ± 2.8	25	0.3	±	0.04	0.038	±	0.002	0.27	±	0.04

Test	Chemical	log K_{OW}	C_D ± SD (µg.g^{-1})	n	k_{BT} ± SE (d^{-1})	$k_{BT,R}$ ± SE (d^{-1})	k_{BM} ± SE (d^{-1})
	1-tridecene	6.59	61.0 ± 3.3	20	0.26 ± 0.08	0.038 ± 0.002	0.22 ± 0.08
	n - tridecane	6.73	50.7 ± 0.3	25	0.15 ± 0.04	0.038 ± 0.002	0.11 ± 0.04
	2-butyl-1-decene	7.14	65.9 ± 4.8	25	0.2 ± 0.04	0.037 ± 0.002	0.16 ± 0.04
6	hexachlorobenzene	5.73	22.3 ± 0.6	36	0.08 ± 0.02	0.049 ± 0.003	0.03 ± 0.02
	1,3 - dimethyladamantane	4.84	99.6 ± 0.8	36	0.17 ± 0.02	0.151 ± 0.003	0.02 ± 0.02
	1,3,5 - triisopropylbenzene	6.36	89.2 ± 0.5	36	0.11 ± 0.02	0.038 ± 0.003	0.07 ± 0.02
	2,6-di-isopropyldecalin	6.85	92.5 ± 1.0	36	0.1 ± 0.02	0.035 ± 0.003	0.07 ± 0.02
	hexadecahydropyrene	5.94	118.0 ± 0.2	36	0.07 ± 0.02	0.043 ± 0.003	0.02 ± 0.02
	hydrogenated 1-me-7-(1-me-ethyl)-phen.	6.96	108.0 ± 0.7	36	0.07 ± 0.02	0.035 ± 0.003	0.04 ± 0.02
	1,2,3,10b - tetrahydrofluoranthene	4.39	84.4 ± 0.1	18	1.3 ± 0.2	0.363 ± 0.003	1 ± 0.2
	4,5,9,10 - tetrahydropyrene	5.04	36.7 ± 0.4	18	0.9 ± 0.2	0.108 ± 0.003	0.8 ± 0.2
	1-methyl-7-(1-methylethyl)-phenanthrene	6.35	58.9 ± 0.9	18	1.7 ± 0.2	0.038 ± 0.003	1.7 ± 0.2
	3 - methylcholanthene	6.42	53.0 ± 0.6	18	1.7 ± 0.2	0.037 ± 0.003	1.6 ± 0.2
	indeno[1,2,3-cd]pyrene	6.7	35.9 ± 1.5	24	0.57 ± 0.07	0.036 ± 0.003	0.53 ± 0.07
	benzo(g,h,i)perylene	6.63	28.9 ± 2.0	24	0.56 ± 0.07	0.036 ± 0.003	0.53 ± 0.07
7	hexachlorobenzene	5.73	26.9 ± 0.1	30	0.05 ± 0.02	0.036 ± 0.004	0.02 ± 0.02
	2,2,4,4,6,8,8-heptamethylnonane	7.79	73.1 ± 1.6	30	0.04 ± 0.02	0.027 ± 0.004	0.01 ± 0.02
	2-isopropyl decalin	5.52	42.9 ± 0.9	30	0.25 ± 0.02	0.041 ± 0.004	0.21 ± 0.02
	n-octyl benzene	6.3	90.6 ± 1.4	18	0.43 ± 0.09	0.03 ± 0.004	0.4 ± 0.09
	4-ethyl-1,1-biphenyl	4.8	86.4 ± 1.6	11	1.4 ± 0.3	0.101 ± 0.004	1.3 ± 0.3
	2,7-diisopropylnaphthalene	6.08	52.3 ± 0.3	11	0.8 ± 0.3	0.031 ± 0.004	0.8 ± 0.3
	Anthracene	4.45	94.6 ± 0.4	11	1.1 ± 0.3	0.191 ± 0.004	0.9 ± 0.3
	o-terphenyl	5.52	73.2 ± 0.6	30	0.11 ± 0.02	0.041 ± 0.004	0.07 ± 0.02
	Fluoranthene	5.18	74.6 ± 0.9	14	1 ± 0.2	0.058 ± 0.004	0.9 ± 0.2
	2,3-benzofluorene	5.77	45.2 ± 0.6	13	0.9 ± 0.2	0.035 ± 0.004	0.9 ± 0.2
	Triphenylene	5.49	86.1 ± 1.5	15	1.4 ± 0.2	0.042 ± 0.004	1.4 ± 0.2
	7-methylbenz[a]anthracene	6.07	47.8 ± 0.5	15	1.2 ± 0.2	0.031 ± 0.004	1.2 ± 0.2
	benzo[k]fluoranthene	6.11	45.0 ± 0.9	15	1.2 ± 0.2	0.031 ± 0.004	1.2 ± 0.2
8	hexachlorobenzene	5.73	24.6 ± 1.2	30	0.09 ± 0.02	0.056 ± 0.002	0.04 ± 0.02
	perhydrochrysene	6.2	136.0 ± 2.1	30	0.1 ± 0.02	0.046 ± 0.002	0.05 ± 0.02
	Fluoranthene	5.18	87.0 ± 0.9	17	0.72 ± 0.08	0.094 ± 0.002	0.62 ± 0.08

Test	Chemical	log K_{OW}	C_D ± SD (µg.g^{-1})	n	k_{BT} ± SE (d^{-1})	$k_{BT,R}$ ± SE (d^{-1})	k_{BM} ± SE (d^{-1})
	benzo[b]fluorene	5.77	42.4 ± 3.6	18	0.82 ± 0.08	0.055 ± 0.002	0.77 ± 0.08
	6-methylchrysene	6.07	49.0 ± 6.6	18	0.96 ± 0.08	0.048 ± 0.002	0.91 ± 0.08
	6-ethylchrysene	6.56	73.8 ± 2.8	18	0.59 ± 0.08	0.043 ± 0.002	0.55 ± 0.08
	hexachlorobenzene	5.73	22.1 ± 0.4	33	0.06 ± 0.008	0.051 ± 0.002	0.008 ± 0.009
	musk xylene	4.9	54.7 ± 0.3	33	0.112 ± 0.008	0.106 ± 0.002	0.006 ± 0.009
9	o-terphenyl	5.52	45.5 ± 0.8	33	0.14 ± 0.009	0.057 ± 0.002	0.083 ± 0.009
	Methoxychlor	5.08	96.8 ± 1.8	26	0.24 ± 0.01	0.084 ± 0.002	0.15 ± 0.01
	benzo[a]pyrene	6.13	151.0 ± 2.0	10	1.1 ± 0.1	0.046 ± 0.002	1 ± 0.1

Table C3 **Respiratory uptake rate constant k_{B1} with standard error, respiratory elimination rate constant k_{B2} with standard error, freely dissolved wet weight bioconcentration factor $BCF_{WW,fd}$ with standard error, bioavailability-corrected bioconcentration factor normalized to 5% lipid $BCF_{5\%,t}$ with standard error. Bioavailability correction was made to a concentration of organic carbon in the water of 2 mg/L.**

Test	Chemical	k_{B1} ± SE (d^{-1})			k_{B2} ± SE (d^{-1})			$BCF_{WW,fd}$ ± SE			$BCF_{5\%,t}$ ± SE		
1	hexachlorobenzene	324.2	±	36.9	0.019	±	0.002	4225	±	1126	7263	±	1935
	2,6-dimethyldecane	324.2	±	36.9	0.0085	±	0.001	1456	±	203	2392	±	333
	2,3 dimethylheptane	324.2	±	36.9	0.26	±	0.03	1100	±	142	1958	±	253
	1-methylphenanthrene	324.2	±	36.9	0.087	±	0.01	720	±	140	1274	±	248
	n-dodecane	324.2	±	36.9	0.0083	±	0.0009	1469	±	205	2409	±	337
	n-nonane	324.2	±	36.9	0.18	±	0.02	760	±	109	1351	±	195
	Phenanthrene	324.2	±	36.9	0.36	±	0.04	693	±	97	1235	±	172
	2,2,4,6,6-pentamethylheptane	324.2	±	36.9	0.012	±	0.001	1746	±	260	2935	±	438
	trans-decalin	324.2	±	36.9	0.17	±	0.02	1613	±	233	2866	±	414
	1,3,5-trimethylbenzene	324.2	±	36.9	2.3	±	0.3	431	±	98	769	±	174
	1,3,5-trimethylcyclohexane	324.2	±	36.9	0.4	±	0.05	1122	±	145	1999	±	259
2	hexachlorobenzene	276.5	±	27	0.0097	±	0.0009	4780	±	1466	4824	±	1479
	3,5,5-trimethylcyclohexene	276.5	±	27	0.29	±	0.03	477	±	68	499	±	71
	2,4,6-trimethyl-3-heptene	276.5	±	27	0.059	±	0.006	1190	±	173	1239	±	180
	cis-1,1,3,5-tetramethylcyclohexane	276.5	±	27	0.069	±	0.007	2282	±	387	2379	±	404
	1-decene	276.5	±	27	0.04	±	0.004	1232	±	182	1279	±	189
	iso-butylcyclohexane	276.5	±	27	0.053	±	0.005	1181	±	143	1229	±	149
	cis-bicyclo(4.3.0)nonane	276.5	±	27	1.02	±	0.1	530	±	80	555	±	84
	2,2,4,6,6-pentamethyl-3-heptene	276.5	±	27	0.0074	±	0.0007	1127	±	159	1124	±	158
	1-tert-butyl-4-methylbenzene	276.5	±	27	0.035	±	0.003	485	±	70	503	±	73
	1,2,3,4-tetramethylbenzene	276.5	±	27	0.52	±	0.05	435	±	59	456	±	62
	1-dodecene	276.5	±	27	0.0041	±	0.0004	1260	±	189	1213	±	182
	1,4-dimethyl-1,2,3,4-tetrahydronaphthalene	276.5	±	27	0.085	±	0.008	841	±	104	877	±	108
	1-hexadecene	276.5	±	27	5E-05	±	4E-06	4388	±	1247	570	±	162
3	hexachlorobenzene	331.5	±	38.9	0.023	±	0.003	3029	±	1029	6100	±	2073
	Naphthalene	331.5	±	38.9	6.3	±	0.7	188	±	52	393	±	108
	2-methylnaphthalene	331.5	±	38.9	1.7	±	0.2	154	±	36	323	±	76

Test	Chemical	k_{B1} ± SE (d^{-1})	k_{B2} ± SE (d^{-1})	$BCF_{WW,fd}$ ± SE	$BCF_{5\%,t}$ ± SE
	2,3-dimethylnaphthalene	331.5 ± 38.9	0.5 ± 0.06	180 ± 48	377 ± 100
	deuterated hexadecane	331.5 ± 38.9	8E-05 ± 9E-06	2967 ± 990	582 ± 194
	Anthracene	331.5 ± 38.9	0.44 ± 0.05	265 ± 98	553 ± 204
	9-methylanthracene	331.5 ± 38.9	0.11 ± 0.01	312 ± 133	646 ± 276
	Pyrene	331.5 ± 38.9	0.16 ± 0.02	331 ± 149	688 ± 311
	1-methylpyrene	331.5 ± 38.9	0.041 ± 0.005	317 ± 138	649 ± 282
	1,2-benzoanthracene	331.5 ± 38.9	0.022 ± 0.003	314 ± 135	630 ± 271
	benzo[a]pyrene	331.5 ± 38.9	0.009 ± 0.001	513 ± 176	980 ± 336
	dibenzo[a,h]anthracene	331.5 ± 38.9	0.0036 ± 0.0004	412 ± 117	697 ± 198
	hexachlorobenzene	312.6 ± 34.1	0.016 ± 0.002	3603 ± 800	5385 ± 1196
	1,1,3,3,5-pentamethylindan	312.6 ± 34.1	0.0044 ± 0.0005	1086 ± 136	1481 ± 185
	2,2,5,7-tetramethyltetraline	312.6 ± 34.1	0.011 ± 0.001	516 ± 75	756 ± 110
	6-n-butyl-2,3-dimethylnaphthalene	312.6 ± 34.1	0.0046 ± 0.0005	788 ± 102	1080 ± 139
	2,3-dimethyl-5-(4-methylpentyl)naphthalene	312.6 ± 34.1	0.0006 ± 6E-05	258 ± 42	199 ± 32
	fichtelite (isopropylhydrophenanthrene)	312.6 ± 34.1	0.0025 ± 0.0003	3867 ± 907	4855 ± 1139
4	Dehydroabietine	312.6 ± 34.1	0.0032 ± 0.0004	1853 ± 273	2427 ± 358
	9-n butylphenanthrene	312.6 ± 34.1	0.0037 ± 0.0004	732 ± 92	978 ± 123
	1-ethylpyrene	312.6 ± 34.1	0.009 ± 0.001	427 ± 57	621 ± 84
	2,3,6,7-tetramethylanthracene	312.6 ± 34.1	0.0026 ± 0.0003	931 ± 127	1174 ± 160
	Chrysene	312.6 ± 34.1	0.014 ± 0.001	860 ± 114	1276 ± 169
	1-octylpyrene	312.6 ± 34.1	1E-05 ± 1E-06	2994 ± 582	94 ± 18
	benzo[b]chrysene	312.6 ± 34.1	0.0025 ± 0.0003	751 ± 139	943 ± 175
	hexachlorobenzene	303.7 ± 32	0.015 ± 0.002	5133 ± 1983	7140 ± 2759
	3,5,5' - trimethyl hexene	303.7 ± 32	0.29 ± 0.03	601 ± 110	867 ± 158
	2-methyl-1-nonene	303.7 ± 32	0.042 ± 0.004	340 ± 91	484 ± 129
	2-methyldecane	303.7 ± 32	0.017 ± 0.002	310 ± 67	433 ± 94
5	c7 iso-alkyl ben	303.7 ± 32	0.033 ± 0.003	237 ± 43	336 ± 60
	1-undece	303.7 ± 32	0.019 ± 0.002	289 ± 60	405 ± 84
	2-methyl-1-undecene	303.7 ± 32	0.0056 ± 0.0006	368 ± 92	483 ± 121
	2-methyl-1-dodecene	303.7 ± 32	0.0018 ± 0.0002	1000 ± 157	1109 ± 174
	1-tridecene	303.7 ± 32	0.002 ± 0.0002	1179 ± 365	1346 ± 417

Test	Chemical	k_{B1} ± SE (d^{-1})			k_{B2} ± SE (d^{-1})			$BCF_{WW,fd}$ ± SE			$BCF_{5\%,t}$ ± SE		
	n - tridecane	303.7	±	32	0.0015	±	0.0002	2019	±	519	2141	±	551
	2-butyl-1-decene	303.7	±	32	0.0006	±	6E-05	1551	±	324	1176	±	246
6	hexachlorobenzene	292.8	±	29.8	0.015	±	0.002	3755	±	1008	5529	±	1484
	1,3 - dimethyladamantane	292.8	±	29.8	0.12	±	0.01	1684	±	254	2562	±	386
	1,3,5 - triisopropylbenzene	292.8	±	29.8	0.0035	±	0.0004	2735	±	568	3611	±	749
	2,6-di-isopropyldecalin	292.8	±	29.8	0.0011	±	0.0001	2821	±	599	2928	±	622
	hexadecahydropyrene	292.8	±	29.8	0.0093	±	0.0009	4473	±	1399	6443	±	2016
	hydrogenated 1-me-7-(1-me-ethyl)-phen.	292.8	±	29.8	0.0009	±	9E-05	4125	±	1201	3930	±	1145
	1,2,3,10b - tetrahydrofluoranthene	292.8	±	29.8	0.33	±	0.03	218	±	35	333	±	53
	4,5,9,10 - tetrahydropyrene	292.8	±	29.8	0.074	±	0.007	329	±	70	500	±	106
	1-methyl-7-(1-methylethyl)-phenanthrene	292.8	±	29.8	0.0036	±	0.0004	168	±	23	222	±	31
	3 - methylcholanthene	292.8	±	29.8	0.0031	±	0.0003	177	±	26	229	±	34
	indeno[1,2,3-cd]pyrene	292.8	±	29.8	0.0016	±	0.0002	515	±	80	588	±	92
	benzo[g,h,i]perylene	292.8	±	29.8	0.0019	±	0.0002	519	±	81	616	±	96
7	hexachlorobenzene	286.7	±	28.6	0.0086	±	0.0009	5351	±	2480	4618	±	2141
	2,2,4,4,6,8,8-heptamethylnonane	286.7	±	28.6	8E-05	±	7E-06	6839	±	4016	1260	±	740
	2-isopropyl decalin	286.7	±	28.6	0.014	±	0.001	1139	±	158	997	±	138
	n-octyl benzene	286.7	±	28.6	0.0023	±	0.0002	661	±	156	520	±	123
	4-ethyl-1,1-biphenyl	286.7	±	28.6	0.073	±	0.007	204	±	44	182	±	39
	2,7-diisopropylnaphthalene	286.7	±	28.6	0.0038	±	0.0004	356	±	124	294	±	102
	Anthracene	286.7	±	28.6	0.16	±	0.02	264	±	70	236	±	63
	o-terphenyl	286.7	±	28.6	0.014	±	0.001	2584	±	621	2262	±	544
	Fluoranthene	286.7	±	28.6	0.031	±	0.003	300	±	59	266	±	52
	2,3-benzofluorene	286.7	±	28.6	0.0079	±	0.0008	307	±	66	264	±	57
	Triphenylene	286.7	±	28.6	0.015	±	0.001	203	±	30	178	±	26
	7-methylbenz[a]anthracene	286.7	±	28.6	0.0039	±	0.0004	241	±	39	200	±	32
	benzo[k]fluoranthene	286.7	±	28.6	0.0036	±	0.0004	242	±	39	199	±	32
8	hexachlorobenzene	303.7	±	32	0.015	±	0.002	3262	±	872	4645	±	1242
	Perhydrochrysene	303.7	±	32	0.0047	±	0.0005	3132	±	809	4132	±	1068
	Fluoranthene	303.7	±	32	0.053	±	0.006	424	±	64	620	±	94
	benzo[b]fluorene	303.7	±	32	0.014	±	0.001	369	±	52	523	±	74

Test	Chemical	k_{B1} ± SE (d^{-1})	k_{B2} ± SE (d^{-1})	$BCF_{WW,fd}$ ± SE	$BCF_{5\%,t}$ ± SE
	6-methylchrysene	303.7 ± 32	0.0069 ± 0.0007	318 ± 42	434 ± 58
	6-ethylchrysene	303.7 ± 32	0.0022 ± 0.0002	514 ± 87	609 ± 103
	hexachlorobenzene	291.6 ± 29.5	0.0096 ± 0.001	4891 ± 847	4607 ± 797
	musk xylene	291.6 ± 29.5	0.065 ± 0.007	2601 ± 329	2529 ± 320
9	o-terphenyl	291.6 ± 29.5	0.016 ± 0.002	2086 ± 247	1992 ± 236
	Methoxychlor	291.6 ± 29.5	0.043 ± 0.004	1239 ± 142	1202 ± 138
	benzo[a]pyrene	291.6 ± 29.5	0.0038 ± 0.0004	271 ± 39	242 ± 35

Table C4 **The dietary uptake efficiency of the test chemical $E_{D,M}$ with standard error; the dietary uptake efficiency of a non-biotransformed substance of equivalent K_{OW} value $E_{D,N}$ with standard error; the biotransformation rate constant in the gastrointestinal content k_{GM} with 95% confidence interval; the wet weight biomagnification factors BMF with standard error; and the lipid-normalized biomagnification factor BMF_L with standard error.**

Test	Chemical	E_D ± SE	$E_{D,N}$ ± SE	k_{GM} (95% CI) (d^{-1})	BMF ± SE	BMF_L ± SE
	hexachlorobenzene	0.53 ± NA	0.53 ± 0.03	6E-11 (0 - 0.3)	0.47 ± 0.05	2.6 ± 0.3
	2,6 -dimethyldecane	.034 ± 0.009	0.53 ± 0.03	30 (17 - 53)	0.011 ± 0.001	0.059 ± 0.006
	2,3 -dimethylheptane	0.17 ± 0.04	0.53 ± 0.03	4 (2 - 9)	0.039 ± 0.004	0.22 ± 0.02
	1-methylphenanthrene	.021 ± 0.007	0.53 ± 0.03	51 (24 - 109)	0.0031 ± 0.0005	0.017 ± 0.003
	n-dodecane	0.1 ± 0.03	0.53 ± 0.03	9 (4 - 16)	0.032 ± 0.003	0.18 ± 0.02
1	n-nonane	0.13 ± 0.04	0.53 ± 0.03	7 (3 - 14)	0.02 ± 0.003	0.11 ± 0.02
	Phenanthrene	0.08 ± 0.02	0.53 ± 0.03	11 (5 - 21)	0.012 ± 0.002	0.069 ± 0.009
	2,2,4,6,6-pentamethylheptane	0.07 ± 0.02	0.53 ± 0.03	14 (7 - 25)	0.025 ± 0.003	0.14 ± 0.01
	trans-decalin	0.34 ± 0.09	0.53 ± 0.03	1 (0 - 3)	0.12 ± 0.01	0.65 ± 0.07
	1,3,5-trimethylbenzene	0.04 ± 0.02	0.53 ± 0.03	24 (9 - 57)	0.0039 ± 0.0007	0.022 ± 0.004
	1,3,5-trimethylcyclohexane	0.23 ± 0.06	0.53 ± 0.03	3 (0.8 - 6)	0.054 ± 0.006	0.3 ± 0.03
	hexachlorobenzene	0.53 ± NA	0.53 ± 0.03	7E-06 (0 - 0.3)	0.35 ± 0.05	1.2 ± 0.2
	3,5,5-trimethylcyclohexene	0.3 ± 0.1	0.53 ± 0.03	1 (0 - 6)	0.021 ± 0.005	0.07 ± 0.02
	2,4,6-trimethyl-3-heptene	0.14 ± 0.06	0.53 ± 0.03	6 (2 - 15)	0.024 ± 0.004	0.08 ± 0.01
	cis-1,1,3,5-tetramethylcyclohexane	0.4 ± 0.1	0.53 ± 0.03	0.8 (0 - 4)	0.12 ± 0.02	0.4 ± 0.06
	1-decene	0.07 ± 0.03	0.53 ± 0.03	15 (6 - 34)	0.011 ± 0.002	0.037 ± 0.007
	iso-butylcyclohexane	0.4 ± 0.1	0.53 ± 0.03	1 (0 - 4)	0.06 ± 0.01	0.19 ± 0.03
2	cis-bicyclo(4.3.0)nonane	0.3 ± 0.1	0.53 ± 0.03	2 (0 - 6)	0.023 ± 0.005	0.07 ± 0.02
	2,2,4,6,6-pentamethyl-3-heptene	0.09 ± 0.03	0.53 ± 0.03	10 (4 - 25)	0.014 ± 0.003	0.045 ± 0.009
	1-tert-butyl-4-methylbenzene	0.4 ± 0.2	0.53 ± 0.03	0.6 (0 - 4)	0.028 ± 0.006	0.09 ± 0.02
	1,2,3,4-tetramethylbenzene	0.2 ± 0.1	0.53 ± 0.03	3 (0 - 8)	0.015 ± 0.003	0.05 ± 0.01
	1-dodecene	0.11 ± 0.04	0.53 ± 0.03	7 (2 - 19)	0.02 ± 0.004	0.07 ± 0.01
	1,4-dimethyl-1,2,3,4-tetrahydronaphthalene	0.4 ± 0.1	0.53 ± 0.03	0.9 (0 - 4)	0.044 ± 0.008	0.14 ± 0.03
	1-hexadecene	0.3 ± 0.1	0.39 ± 0.03	0.7 (0 - 3)	0.17 ± 0.02	0.55 ± 0.08
	hexachlorobenzene	0.53 ± NA	0.53 ± 0.03	7E-06 (0 - 0.3)	0.16 ± 0.02	1 ± 0.1
3	Naphthalene	0.04 ± 0.02	0.53 ± 0.03	27 (8 - 85)	0.0007 ± 0.0002	0.005 ± 0.001
	2-methylnaphthalene	0.1 ± 0.05	0.53 ± 0.03	9 (2 - 29)	0.0015 ± 0.0004	0.01 ± 0.003

Test	Chemical	E_D ± SE	$E_{D,N}$ ± SE	k_{GM} (95% CI) (d^{-1})	BMF ± SE	BMF_L ± SE
	2,3-dimethylnaphthalene	0.12 ± 0.07	0.53 ± 0.03	7 (1 - 25)	0.0022 ± 0.0006	0.014 ± 0.004
	deuterated hexadecane	0.17 ± 0.07	0.36 ± 0.03	2 (0 - 6)	0.051 ± 0.007	0.33 ± 0.04
	Anthracene	0.02 ± 0.02	0.53 ± 0.03	47 (12 - 172)	0.0006 ± 0.0002	0.0038 ± 0.001
	9-methylanthracene	0.02 ± 0.01	0.53 ± 0.03	66 (16 - 266)	0.0005 ± 0.0001	0.0033 ± 0.0009
	Pyrene	0.02 ± 0.01	0.53 ± 0.03	67 (15 - 282)	0.0005 ± 0.0001	0.0034 ± 0.0009
	1-methylpyrene	0.02 ± 0.01	0.53 ± 0.03	60 (14 - 243)	0.0006 ± 0.0001	0.0036 ± 0.001
	1,2-benzoanthracene	0.02 ± 0.01	0.53 ± 0.03	57 (13 - 230)	0.0006 ± 0.0002	0.0038 ± 0.001
	benzo[a]pyrene	0.01 ± 0.006	0.53 ± 0.03	106 (31 - 355)	0.0005 ± 0.0001	0.0033 ± 0.0008
	dibenzo[a,h]anthracene	0.02 ± 0.01	0.52 ± 0.03	46 (14 - 140)	0.0009 ± 0.0002	0.006 ± 0.001
	hexachlorobenzene	0.53 ± NA	0.53 ± 0.03	7E-06 (0 - 0.3)	0.2 ± 0.03	1 ± 0.1
	1,1,3,3,5-pentamethylindan	0.23 ± 0.08	0.53 ± 0.03	3 (0.3 - 7)	0.026 ± 0.005	0.13 ± 0.02
	2,2,5,7-tetramethyltetraline	0.18 ± 0.07	0.53 ± 0.03	4 (0.7 - 11)	0.01 ± 0.002	0.05 ± 0.01
	6-n-butyl-2,3-dimethylnaphthalene	0.03 ± 0.01	0.53 ± 0.03	35 (16 - 76)	0.0024 ± 0.0005	0.012 ± 0.002
	2,3-dimethyl-5-(4-methylpentyl)naphthalene	0.3 ± 0.2	0.51 ± 0.02	0.9 (0 - 5)	0.009 ± 0.002	0.04 ± 0.01
	fichtelite (isopropylhydrophenanthrene)	0.12 ± 0.05	0.52 ± 0.03	7 (2 - 17)	0.049 ± 0.006	0.24 ± 0.03
4	Dehydroabietine	0.04 ± 0.02	0.52 ± 0.03	23 (10 - 49)	0.008 ± 0.001	0.041 ± 0.007
	9-n butylphenanthrene	0.04 ± 0.01	0.52 ± 0.03	29 (13 - 62)	0.0027 ± 0.0005	0.013 ± 0.003
	1-ethylpyrene	0.09 ± 0.04	0.53 ± 0.03	10 (4 - 25)	0.004 ± 0.0009	0.019 ± 0.004
	2,3,6,7-tetramethylanthracene	0.03 ± 0.01	0.52 ± 0.03	30 (14 - 67)	0.0032 ± 0.0006	0.016 ± 0.003
	Chrysene	0.03 ± 0.01	0.53 ± 0.03	31 (14 - 67)	0.003 ± 0.0006	0.015 ± 0.003
	1-octylpyrene	0.03 ± 0.01	0.15 ± 0.03	4 (1 - 13)	0.01 ± 0.001	0.049 ± 0.007
	benzo[b]chrysene	0.04 ± 0.02	0.52 ± 0.03	23 (9 - 58)	0.0033 ± 0.0007	0.016 ± 0.004
	hexachlorobenzene	0.53 ± NA	0.53 ± 0.03	6E-11 (0 - 0.3)	0.27 ± 0.05	1.2 ± 0.2
	3,5,5' - trimethyl hexene	0.2 ± 0.1	0.53 ± 0.03	3 (0 - 13)	0.013 ± 0.004	0.06 ± 0.02
	2-methyl-1-nonene	0.2 ± 0.2	0.53 ± 0.03	3 (0 - 15)	0.008 ± 0.006	0.03 ± 0.03
	2-methyldecane	0.3 ± 0.2	0.53 ± 0.03	2 (0 - 12)	0.008 ± 0.003	0.04 ± 0.01
5	c7 iso-alkyl benzene	0.5 ± 0.3	0.53 ± 0.03	0.1 (0 - 5)	0.012 ± 0.004	0.05 ± 0.02
	1-undecene	0.3 ± 0.2	0.53 ± 0.03	2 (0 - 10)	0.008 ± 0.003	0.04 ± 0.01
	2-methyl-1-undecene	0.2 ± 0.1	0.53 ± 0.03	3 (0 - 17)	0.007 ± 0.002	0.03 ± 0.01
	2-methyl-1-dodecene	0.12 ± 0.06	0.52 ± 0.03	7 (1 - 21)	0.012 ± 0.003	0.06 ± 0.01
	1-tridecene	0.2 ± 0.1	0.52 ± 0.03	4 (0 - 19)	0.021 ± 0.005	0.09 ± 0.02

Test	Chemical	E_D ± SE	$E_{D,N}$ ± SE	k_{GM} (95% CI) (d^{-1})	BMF ± SE	BMF_L ± SE
	n - tridecane	0.2 ± 0.1	0.52 ± 0.03	3 (0 - 11)	0.045 ± 0.009	0.2 ± 0.04
	2-butyl-1-decene	0.12 ± 0.06	0.51 ± 0.02	6 (1 - 20)	0.019 ± 0.004	0.08 ± 0.02
6	hexachlorobenzene	0.53 ± NA	0.53 ± 0.03	6E-11 (0 - 0.3)	0.14 ± 0.02	0.66 ± 0.1
	1,3 - dimethyladamantane	0.1 ± 0.04	0.53 ± 0.03	9 (3 - 23)	0.012 ± 0.002	0.06 ± 0.01
	1,3,5 - triisopropylbenzene	0.3 ± 0.1	0.52 ± 0.03	2 (0 - 6)	0.058 ± 0.009	0.28 ± 0.04
	2,6-di-isopropyldecalin	0.07 ± 0.03	0.52 ± 0.02	13 (5 - 32)	0.014 ± 0.002	0.07 ± 0.01
	hexadecahydropyrene	0.3 ± 0.1	0.53 ± 0.03	1 (0 - 6)	0.1 ± 0.02	0.49 ± 0.07
	hydrogenated 1-me-7-(1-me-ethyl)-phen.	0.13 ± 0.06	0.51 ± 0.02	6 (1 - 16)	0.038 ± 0.006	0.18 ± 0.03
	1,2,3,10b - tetrahydrofluoranthene	0.3 ± 0.2	0.53 ± 0.03	2 (0 - 8)	0.005 ± 0.001	0.022 ± 0.007
	4,5,9,10 - tetrahydropyrene	0.16 ± 0.09	0.53 ± 0.03	5 (0.2 - 18)	0.004 ± 0.001	0.018 ± 0.005
	1-methyl-7-(1-methylethyl)-phenanthrene	0.5 ± 0.2	0.52 ± 0.03	0.2 (0 - 4)	0.006 ± 0.002	0.027 ± 0.008
	3 - methylcholanthene	0.5 ± 0.2	0.52 ± 0.03	0.2 (0 - 4)	0.006 ± 0.002	0.028 ± 0.009
	indeno[1,2,3-cd]pyrene	0.08 ± 0.04	0.52 ± 0.03	11 (3 - 32)	0.0029 ± 0.0007	0.014 ± 0.004
	benzo[g,h,i]perylene	0.09 ± 0.04	0.52 ± 0.03	10 (3 - 29)	0.0032 ± 0.0008	0.015 ± 0.004
7	hexachlorobenzene	0.53 ± NA	0.53 ± 0.03	6E-11 (0 - 0.3)	0.24 ± 0.05	0.7 ± 0.2
	2,2,4,4,6,8,8-heptamethylnonane	0.03 ± 0.02	0.45 ± 0.02	21 (7 - 62)	0.02 ± 0.006	0.06 ± 0.02
	2-isopropyl decalin	0.19 ± 0.09	0.53 ± 0.03	4 (0.2 - 13)	0.018 ± 0.004	0.05 ± 0.01
	n-octyl benzene	0.2 ± 0.1	0.53 ± 0.03	5 (0 - 21)	0.009 ± 0.003	0.025 ± 0.008
	4-ethyl-1,1-biphenyl	0.5 ± 0.3	0.53 ± 0.03	0.1 (0 - 5)	0.009 ± 0.003	0.024 ± 0.009
	2,7-diisopropylnaphthalene	0.4 ± 0.3	0.53 ± 0.03	0.9 (0 - 10)	0.011 ± 0.004	0.03 ± 0.01
	Anthracene	0.3 ± 0.2	0.53 ± 0.03	1 (0 - 10)	0.008 ± 0.003	0.021 ± 0.008
	o-terphenyl	0.3 ± 0.1	0.53 ± 0.03	2 (0 - 10)	0.05 ± 0.01	0.15 ± 0.03
	Fluoranthene	0.3 ± 0.2	0.53 ± 0.03	1 (0 - 9)	0.009 ± 0.003	0.024 ± 0.009
	2,3-benzofluorene	0.4 ± 0.2	0.53 ± 0.03	0.9 (0 - 8)	0.01 ± 0.003	0.027 ± 0.01
	Triphenylene	0.5 ± 0.3	0.53 ± 0.03	0.01 (0 - 4)	0.009 ± 0.003	0.025 ± 0.009
	7-methylbenz[a]anthracene	0.2 ± 0.1	0.53 ± 0.03	4 (0 - 17)	0.004 ± 0.001	0.01 ± 0.004
	benzo[k]fluoranthene	0.2 ± 0.1	0.53 ± 0.03	3 (0 - 15)	0.004 ± 0.001	0.012 ± 0.004
8	hexachlorobenzene	0.53 ± NA	0.53 ± 0.03	6E-11 (0 - 0.3)	0.14 ± 0.03	0.6 ± 0.1
	perhydrochrysene	0.18 ± 0.1	0.53 ± 0.03	4 (0.1 - 14)	0.046 ± 0.009	0.21 ± 0.04
	fluoranthene	0.3 ± 0.2	0.53 ± 0.03	2 (0 - 10)	0.009 ± 0.003	0.04 ± 0.02
	benzo[b]fluorene	0.17 ± 0.1	0.53 ± 0.03	4 (0.1 - 17)	0.005 ± 0.002	0.023 ± 0.008

Test	Chemical	E_D ± SE	$E_{D,N}$ ± SE	k_{GM} (95% CI) (d^{-1})	BMF ± SE	BMF_L ± SE
	6-methylchrysene	0.3 ± 0.2	0.53 ± 0.03	1 (0 - 8)	0.008 ± 0.003	0.04 ± 0.01
	6-ethylchrysene	0.15 ± 0.09	0.52 ± 0.03	5 (0.2 - 20)	0.006 ± 0.002	0.03 ± 0.01
	hexachlorobenzene	0.53 ± NA	0.53 ± 0.03	6E-11 (0 - 0.3)	0.36 ± 0.03	1.05 ± 0.1
	musk xylene	0.38 ± 0.09	0.53 ± 0.03	0.8 (0 - 3)	0.14 ± 0.01	0.4 ± 0.04
9	o-terphenyl	0.3 ± 0.07	0.53 ± 0.03	2 (0.2 - 4)	0.087 ± 0.01	0.26 ± 0.03
	Methoxychlor	0.13 ± 0.03	0.53 ± 0.03	7 (3 - 12)	0.022 ± 0.003	0.064 ± 0.008
	benzo[a]pyrene	0.18 ± 0.06	0.53 ± 0.03	4 (1 - 10)	0.007 ± 0.001	0.02 ± 0.004

Table C5 Gastrointestinal exchange rate parameters. Rate constant for the fecal egestion of the gastro-intestinal content k_{GE}, rate constant for the chemical transfer from the gastrointestinal content to the fish body k_{GB}; the chemical partition coefficient between the gastrointestinal content and the fish body K_{GB}; and rate constant for the chemical transfer from the fish body to the gastrointestinal content k_{BG}.

Test	Chemical	k_{GE} (d^{-1})	k_{GB} (d^{-1})	K_{GB}	k_{BG} (d^{-1})
1	hexachlorobenzene	0.98	1.11	1.07	0.039
	Dimethyldecane	0.98	1.11	1.07	0.039
	2,3 dimethylheptane	0.98	1.11	1.06	0.039
	methylphenanthrene	0.98	1.11	1.06	0.039
	n-dodecane	0.98	1.11	1.07	0.039
	n-nonane	0.98	1.11	1.06	0.039
	Phenanthrene	0.98	1.11	1.06	0.039
	pentamethylheptane	0.98	1.11	1.07	0.039
	trans-decalin	0.98	1.11	1.06	0.039
	1,3,5-trimethylbenzene	0.98	1.11	1.06	0.039
	1,3,5-trimethylcyclohexane	0.98	1.11	1.06	0.039
2	hexachlorobenzene	0.98	1.11	0.82	0.017
	3,5,5-trimethylcyclohexene	0.98	1.11	0.82	0.017
	2,4,6-trimethyl-3-heptene	0.98	1.11	0.82	0.017
	cis-1,1,3,5-tetramethylcyclohexane	0.98	1.11	0.82	0.017
	1-decene	0.98	1.11	0.82	0.017
	iso-butylcyclohexane	0.98	1.11	0.82	0.017
	cis-bicyclo(4.3.0)nonane	0.98	1.11	0.82	0.017
	2,2,4,6,6-pentamethyl-3-heptene	0.98	1.11	0.82	0.017
	1-tert-butyl-4-methylbenzene	0.98	1.11	0.82	0.017
	1,2,3,4-tetramethylbenzene	0.98	1.11	0.82	0.017
	1-dodecene	0.98	1.11	0.82	0.017
	1,4-dimethyl-1,2,3,4-tetrahydronaphthalene	0.98	1.11	0.82	0.017
	1-hexadecene	0.98	0.63	0.82	0.010
3	hexachlorobenzene	0.98	1.11	1.13	0.020
	Naphthalene	0.98	1.11	1.13	0.020
	2-methylnaphthalene	0.98	1.11	1.13	0.020
	2,3-dimethylnaphthalene	0.98	1.11	1.13	0.020
	d34 hexadecane	0.98	0.55	1.13	0.010
	Anthracene	0.98	1.11	1.13	0.020
	9-methylanthracene	0.98	1.11	1.13	0.020
	Pyrene	0.98	1.11	1.13	0.020
	1-methylpyrene	0.98	1.11	1.13	0.020
	1,2-benzoanthracene	0.98	1.11	1.13	0.020

Test	Chemical	k_{GE} (d^{-1})	k_{GB} (d^{-1})	K_{GB}	k_{BG} (d^{-1})
	benzo[a]pyrene	0.98	1.11	1.13	0.020
	dibenzo[a,h]anthracene	0.98	1.07	1.13	0.019
	hexachlorobenzene	0.98	1.11	1.00	0.017
	1,1,3,3,5-pentamethylindan	0.98	1.11	1.00	0.017
	2,2,5,7-tetramethyltetraline	0.98	1.11	1.00	0.017
	6-n-butyl-2,3-dimethylnaphthalene	0.98	1.11	1.00	0.017
	2,3-dimethyl-5-(4-methylpentyl)naphthalene	0.98	1.02	1.00	0.016
	fichtelite (isopropylhydrophenanthrene)	0.98	1.07	1.00	0.017
4	Dehydroabietine	0.98	1.07	1.00	0.017
	9-n butylphenanthrene	0.98	1.07	1.00	0.017
	1-ethylpyrene	0.98	1.11	1.00	0.017
	2,3,6,7-tetramethylanthracene	0.98	1.07	1.00	0.017
	Chrysene	0.98	1.11	1.00	0.017
	1-octylpyrene	0.98	0.17	1.00	0.003
	benzo[b]chrysene	0.98	1.07	1.00	0.017
	hexachlorobenzene	0.98	1.11	0.97	0.016
	3,5,5' - trimethyl hexene	0.98	1.11	0.97	0.016
	2-methyl-1-nonene	0.98	1.11	0.97	0.016
	2-methyldecane	0.98	1.11	0.97	0.016
	c7 iso-alkyl benzene(c13)	0.98	1.11	0.97	0.016
5	1-undecene (c11)	0.98	1.11	0.97	0.016
	2-methyl-1-undecene (c12)	0.98	1.11	0.97	0.016
	2-methyl-1-dodecene (c13)	0.98	1.07	0.97	0.015
	1-tridecene (c13)	0.98	1.07	0.97	0.015
	n - tridecane (c13)	0.98	1.07	0.97	0.015
	2-butyl-1-decene (c14)	0.98	1.02	0.97	0.015
	hexachlorobenzene	0.98	1.11	1.00	0.011
	1,3 - dimethyladamantane	0.98	1.11	1.00	0.011
	1,3,5 - triisopropylbenzene	0.98	1.07	1.00	0.011
	di-isopropyldecalin	0.98	1.07	1.00	0.011
	hexadecahydropyrene	0.98	1.11	1.00	0.011
6	hydrogenated 1-me-7-(1-me-ethyl)-phen.	0.98	1.02	1.00	0.010
	1,2,3,10b - tetrahydrofluoranthene	0.98	1.11	1.00	0.011
	4,5,9,10 - tetrahydropyrene	0.98	1.11	1.00	0.011
	1-methyl-7-(1-methylethyl)-phenanthrene	0.98	1.07	1.00	0.011
	3 - methylcholanthene	0.98	1.07	1.00	0.011
	indeno[1,2,3-cd]pyrene	0.98	1.07	1.00	0.011
	benzo[g,h,i]perylene	0.98	1.07	1.00	0.011
7	hexachlorobenzene	0.98	1.11	0.75	0.010
	2,2,4,4,6,8,8-heptamethylnonane	0.98	0.80	0.75	0.007

Test	Chemical	k_{GE} (d^{-1})	k_{GB} (d^{-1})	K_{GB}	k_{BG} (d^{-1})
	2-isopropyl decalin	0.98	1.11	0.75	0.010
	n-octyl benzene	0.98	1.11	0.75	0.010
	4-ethyl-1,1-biphenyl	0.98	1.11	0.75	0.010
	2,7-diisopropylnaphthalene	0.98	1.11	0.75	0.010
	Anthracene	0.98	1.11	0.75	0.010
	o-terphenyl	0.98	1.11	0.75	0.010
	Fluoranthene	0.98	1.11	0.75	0.010
	2,3-benzofluorene	0.98	1.11	0.75	0.010
	Triphenylene	0.98	1.11	0.75	0.010
	7-methylbenz[a]anthracene	0.98	1.11	0.75	0.010
	benzo[k]fluoranthene	0.98	1.11	0.75	0.010
8	hexachlorobenzene	0.98	1.11	0.98	0.013
	perhydrochrysene	0.98	1.11	0.98	0.013
	fluoranthene	0.98	1.11	0.98	0.013
	benzo[b]fluorene	0.98	1.11	0.98	0.013
	6-methylchrysene	0.98	1.11	0.98	0.013
	6-ethylchrysene	0.98	1.07	0.98	0.012
9	hexachlorobenzene	0.98	1.11	0.78	0.017
	musk xylene	0.98	1.11	0.78	0.017
	o-terphenyl	0.98	1.11	0.78	0.017
	methoxychlor	0.98	1.11	0.78	0.017
	benzo[a]pyrene	0.98	1.11	0.78	0.017

Table C6 **Comparison of lipid normalized residues in rainbow trout exposed to contaminated diet in experiment #1 with chronic target lipid body burdens estimated using the target lipid model (*3*) and resulting chronic toxic units.**

Test Substance	Mol Wt. g/mol	Cdiet µg/g dry	$Cdiet_{norm}$ µM/g lipid	k_{BT} 1/day	E_D g/g	$Cfish_{end\ uptake}$ µM/g lipid	Chronic Toxic Units
hexachlorobenzene	284	100	2.26	0.08	0.53	0.58	0.125
dimethyldecane	170	96.3	3.63	0.22	0.034	0.03	0.003
2,3 dimethylheptane	128	78.9	3.95	0.29	0.17	0.15	0.012
methylphenanthrene	192	130	4.34	0.45	0.021	0.01	0.002
n-dodecane	128	100	5.01	0.22	0.1	0.14	0.011
n-nonane	128	88.1	4.41	0.43	0.13	0.09	0.007
phenanthrene	178	105	3.78	0.47	0.08	0.04	0.008
pentamethylheptane	170	93.7	3.53	0.19	0.07	0.08	0.006
trans-decalin	138	100	4.65	0.2	0.34	0.48	0.037
1,3,5-trimethylbenzene	120	101	5.40	0.8	0.04	0.02	0.002
1,3,5-trimethylcyclohexane	126.00	80.10	4.08	0.29	0.23	0.21	0.021
		1170.7	47.8			2.3	0.3

Chronic critical target lipid body burdens for rainbow trout:
1) Baseline compounds (aliphatic hydrocarbons) = 13 µM/g lipid
2) Monoaromatic hydrocarbons = 10.1 µM/g lipid
3) Polyaromatic hydrocarbons = 5.8 µM/g lipid
4) Hexachlorobenzene = 4.6 µM/g lipid

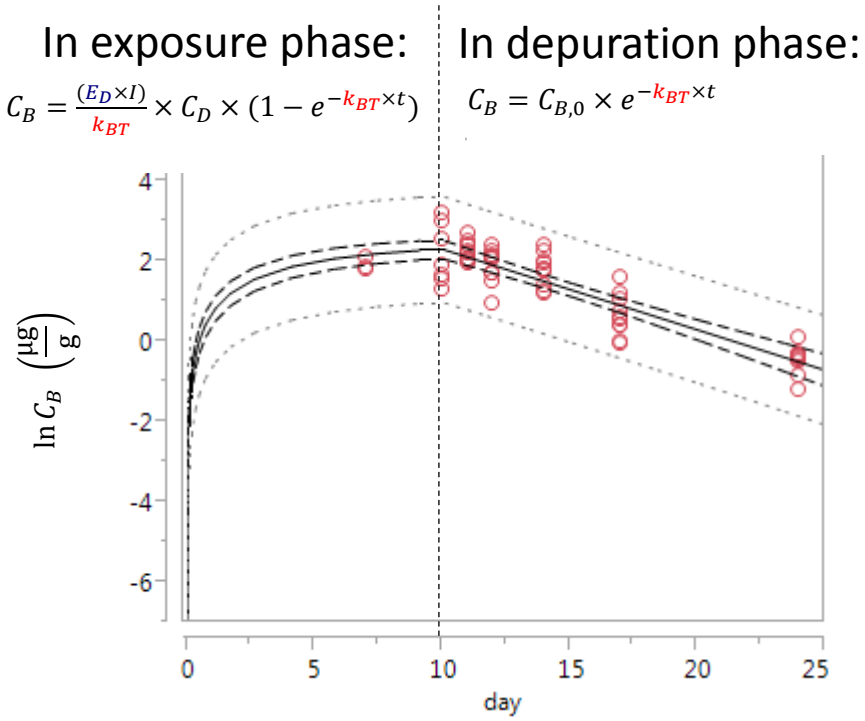


Figure C1 **Concentrations of a test chemical in the fish body throughout the uptake and depuration phase of a dietary bioaccumulation experiment. The predicted solid line is a 2 part piecewise regression mode (equations above). The dashed lines represent the confidence interval of the mean concentrations, while the dotted lines represent the prediction interval for individual observations.**

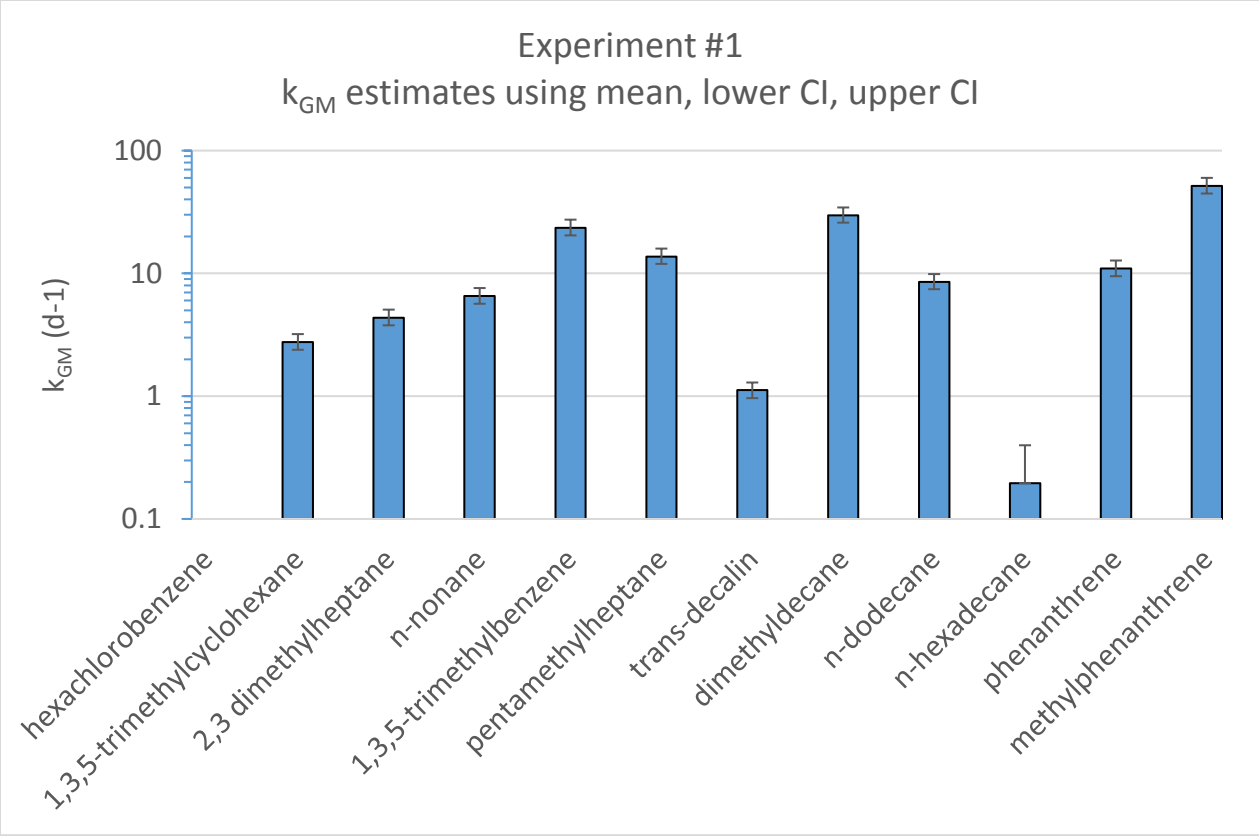
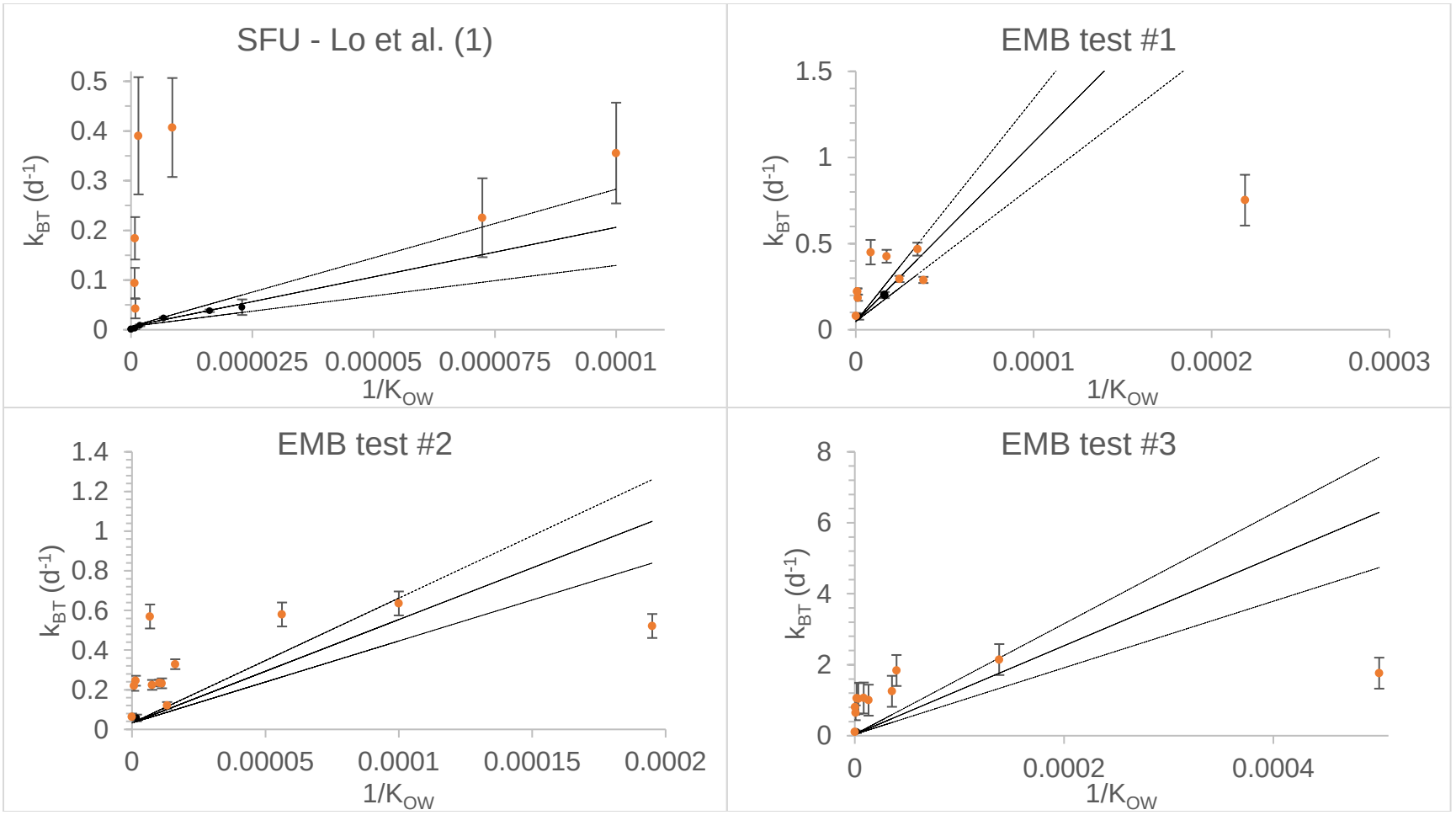
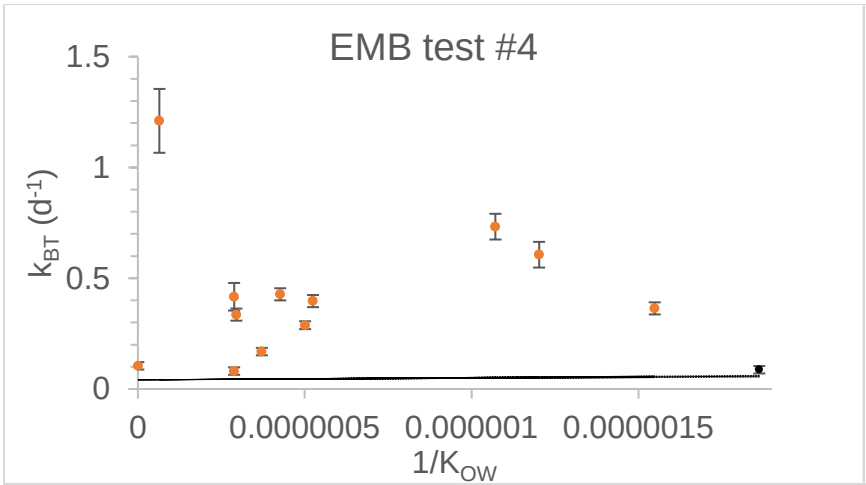
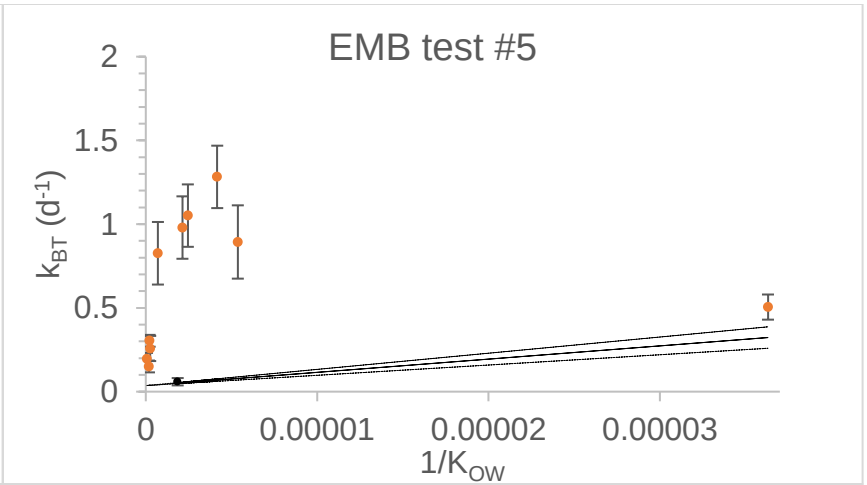
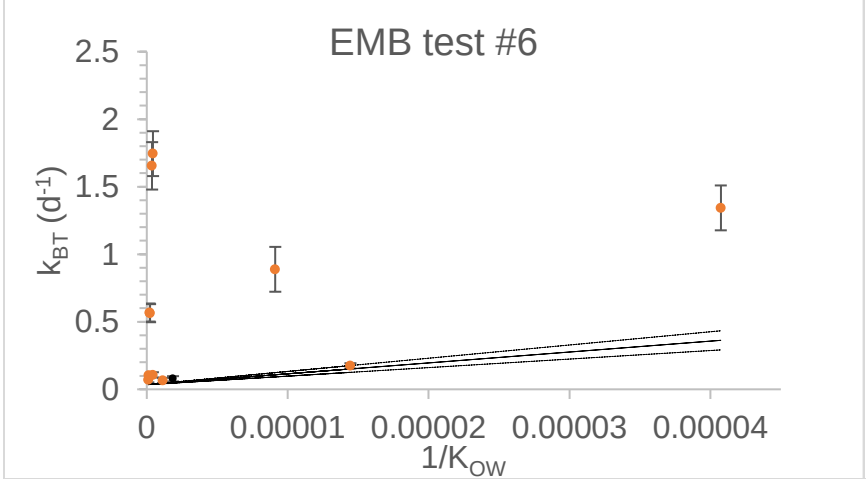


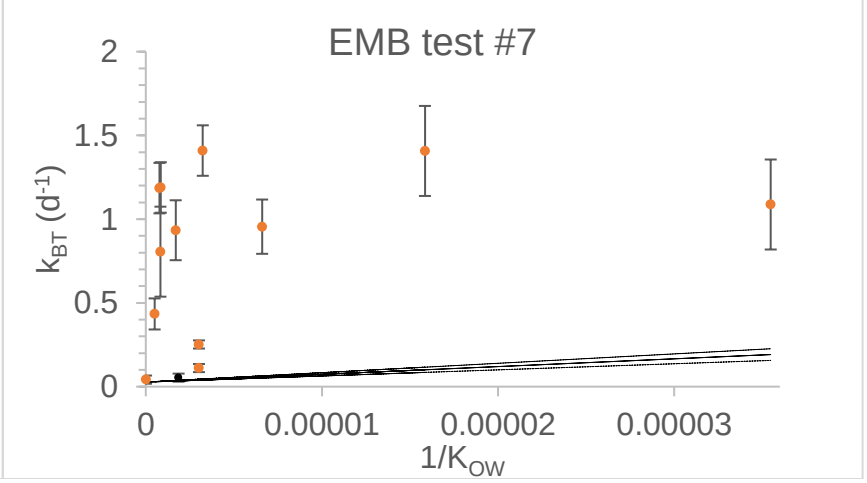
Figure C2 ***k*GM estimates of several test chemicals in experiment #1. The error bars reflect the variation in k_{GM} estimates resulting from normalizing the dietary uptake efficiency of the reference chemical hexachlorobenzene to the mean, lower, and upper 95% confidence interval estimates of the dietary uptake efficiency $E_{D,N}$ of hexachlorobenzene determined in Lo et al. (*1*).**


SFU - Lo et al. (1)
k_{BT} (d^{-1})
0.5
0.4
0.3
0.2
0.1
0
0
0.000025
0.00005
0.000075
0.0001
$1/K_{OW}$
EMB test #1
k_{BT} (d^{-1})
1.5
1
0.5
0
0
0.0001
0.0002
0.0003
$1/K_{OW}$
EMB test #2
k_{BT} (d^{-1})
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.00005
0.0001
0.00015
0.0002
$1/K_{OW}$
EMB test #3
k_{BT} (d^{-1})
8
6
4
2
0
0
0.0002
0.0004
$1/K_{OW}$


EMB test #4
1.5
1
0.5
0
k_{BT} (d^{-1})
0
0.0000005
0.000001
0.0000015
$1/K_{OW}$


EMB test #5
2
1.5
1
0.5
0
k_{BT} (d^{-1})
0
0.00001
0.00002
0.00003
$1/K_{OW}$


EMB test #6
2.5
2
1.5
1
0.5
0
k_{BT} (d^{-1})
0
0.00001
0.00002
0.00003
0.00004
$1/K_{OW}$


EMB test #7
2
1.5
1
0.5
0
k_{BT} (d^{-1})
0
0.00001
0.00002
0.00003
$1/K_{OW}$

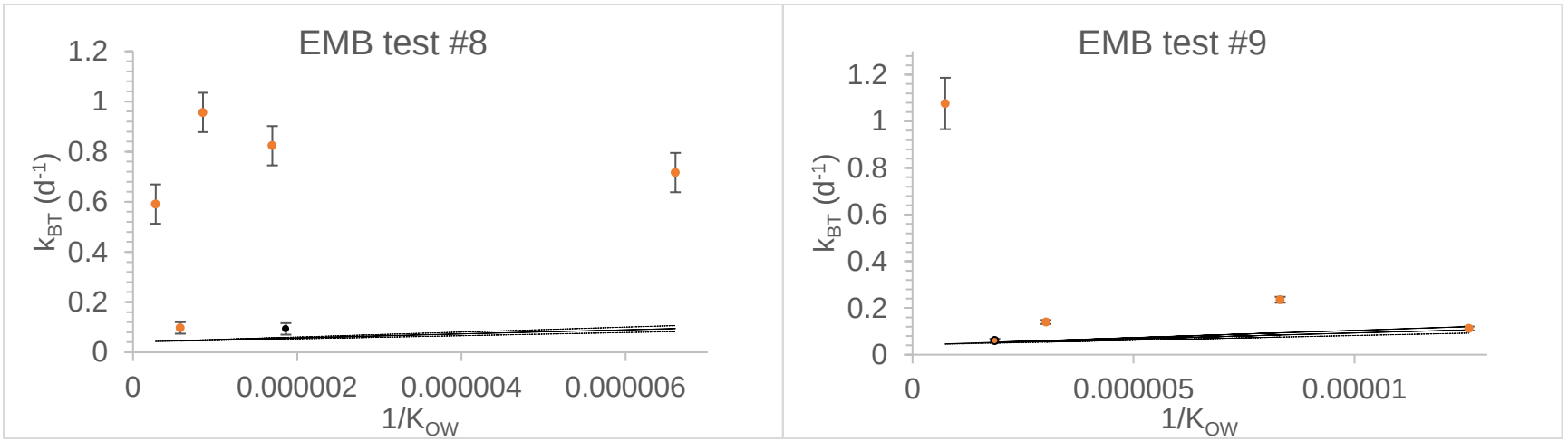


Figure C3 **Total elimination rate constants in the body for chemicals from each experiment. Error bars indicate the standard error of the mean. The line describes the predicted total elimination rate constant for non-metabolizable reference chemicals ($k_{BT,R}$) through multiple linear regression, parameterized to the fish from all ten experiments (Equation 5.12). Dotted lines represent the 95% confidence interval of predicted $k_{BT,R}$.**

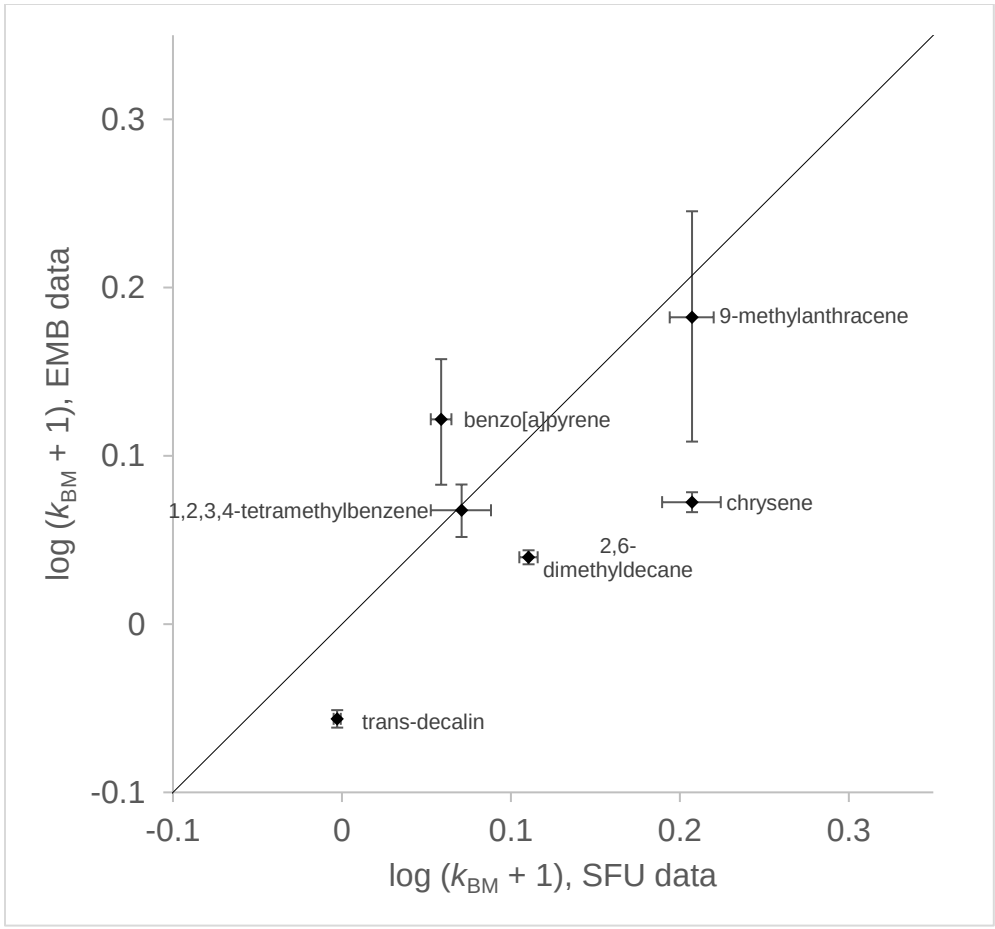


Figure C4 **Log (k_{BM}+1) data from present study (EMB data) compared to a previous study (SFU data) for 6 test chemicals (*1*). Data is normalized to 10 g fish, using the function $k_{BM,10g} = k_{BM,Xg} \cdot (W_{B,X} / 10)$ from Arnot et al. (*7*). Error bars represent the standard error of the point estimates. Line represents a 1:1 relationship.**

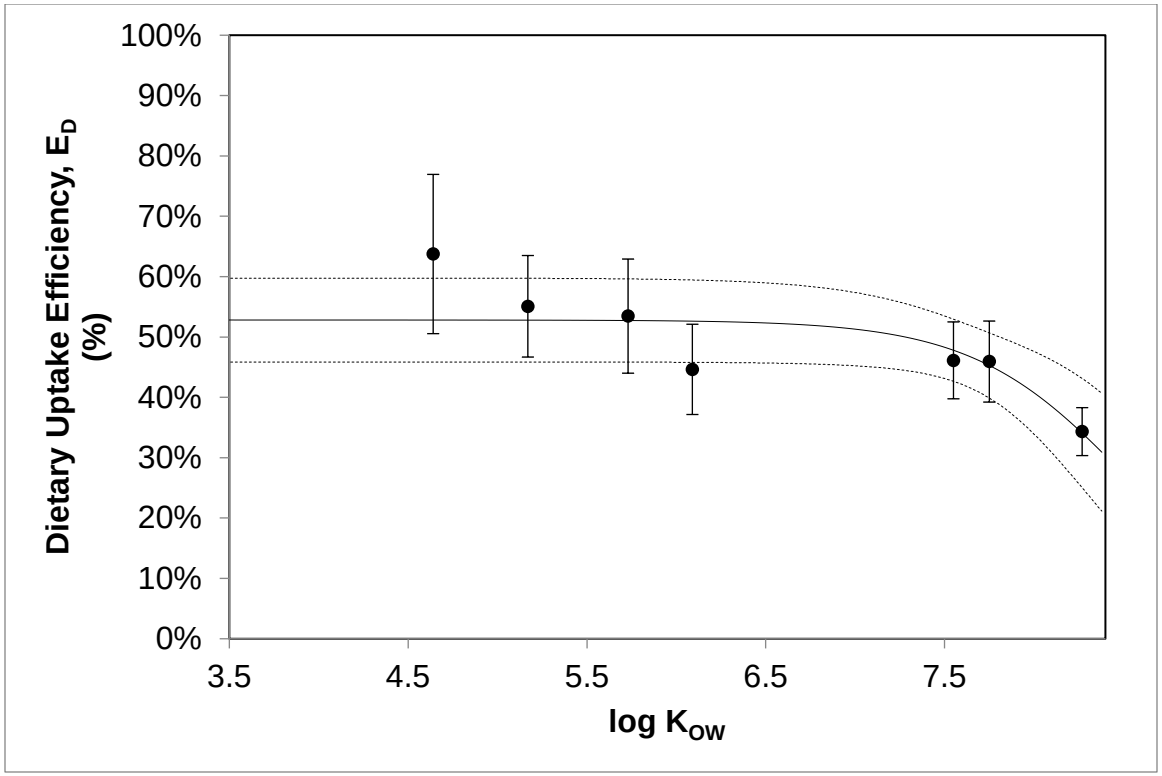


Figure C5 **Dietary uptake efficiencies of 7 reference chemicals versus K_{OW} (standard errors illustrated in error bars). The line (Equation 5.13) represents a non-linear weighted regression fit to reference chemical data (95% confidence intervals for the predicted mean illustrated by the dotted lines).**

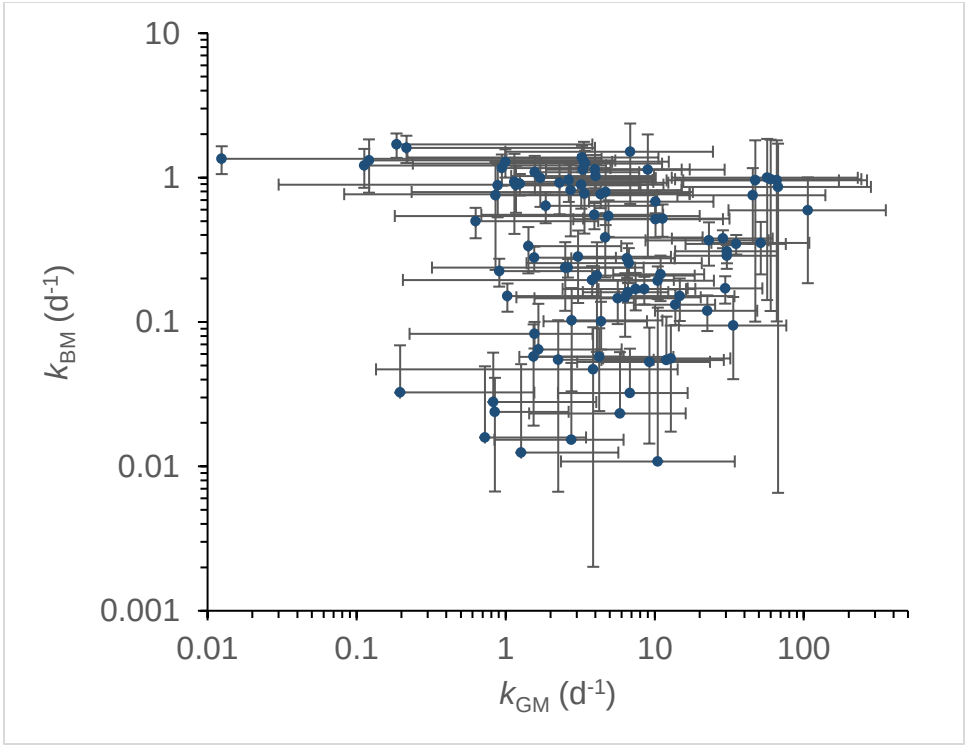


Figure C6 **Somatic biotransformation rate constants k_{BM} plotted against gastro-intestinal biotransformation rate constants k_{GM}. Estimates below 0.001 are not included in the plot. Error bars represent the confidence intervals of the mean.**

References

1. Lo, J. C.; Campbell, D. A.; Kennedy, C. J.; Gobas, F. A. P. C. Somatic and gastro-intestinal in-vivo biotransformation rates of hydrophobic chemicals in fish. *Environ. Toxicol. Chem.* 2015, *34* (10), 2282-2294.
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Appendix D

Supplemental Information for Chapter 6

Supplemental information for “In Vitro to In Vivo Extrapolation of Biotransforming Hydrophobic Chemicals in the Fish Body”*

Table D1 Details of in vitro and in vivo biotransformation studies in rainbow trout referred to in this study.

Study type	Source	Location	Average fish size (g)	Fish source	Water temperature (ºC) (range)	Mean feeding rate (%bw·d^{-1})
in vitro S9 experiments	Lo et al. (*1*)	SFU	386	Miracle Springs Inc.	13.5 (12.5-14.5)	1
in vitro S9 experiments (with contaminated diet)	Present study (Chapter 6)	SFU	581	Miracle Springs Inc.	13.1 (12-13.9)	1
in vivo dietary bioaccumulation experiments	Lo et al. (2)	SFU	30	Miracle Springs Inc.	12.6 (11.4-13.3)	1.5
in vivo dietary bioaccumulation experiments	Lo et al. (3)	ExxonMobil Biomedical Sciences Inc.	1.5	Thomas Fish Co.	13.6 (12.9-14.3)	3

Table D2 **Concentration of protein (mg.ml^{-1}) in the incubation medium (C_{S9}); volume of the incubation mixture (V_{inc}), volume of S9 used in incubation assay ($V_{S9,inc}$); volume of supernatant S9 fraction collected after the centrifugation of the liver extract (V_{hom}); volume of liver used in the preparation of the liver S9 (W_H); maximum in vitro biotransformation rate constants ($k_{dep,C\rightarrow 0}$), and corresponding Michaelis constant (K_M) for rainbow trout in vitro liver S9 biotransformation bioassays involving multiple solvent delivery based experiments and sorbent phase dosing experiments (in bold type face) in experiments with pre-exposed and non-pre-exposed fish livers. SE refers to the standard error of the mean.**

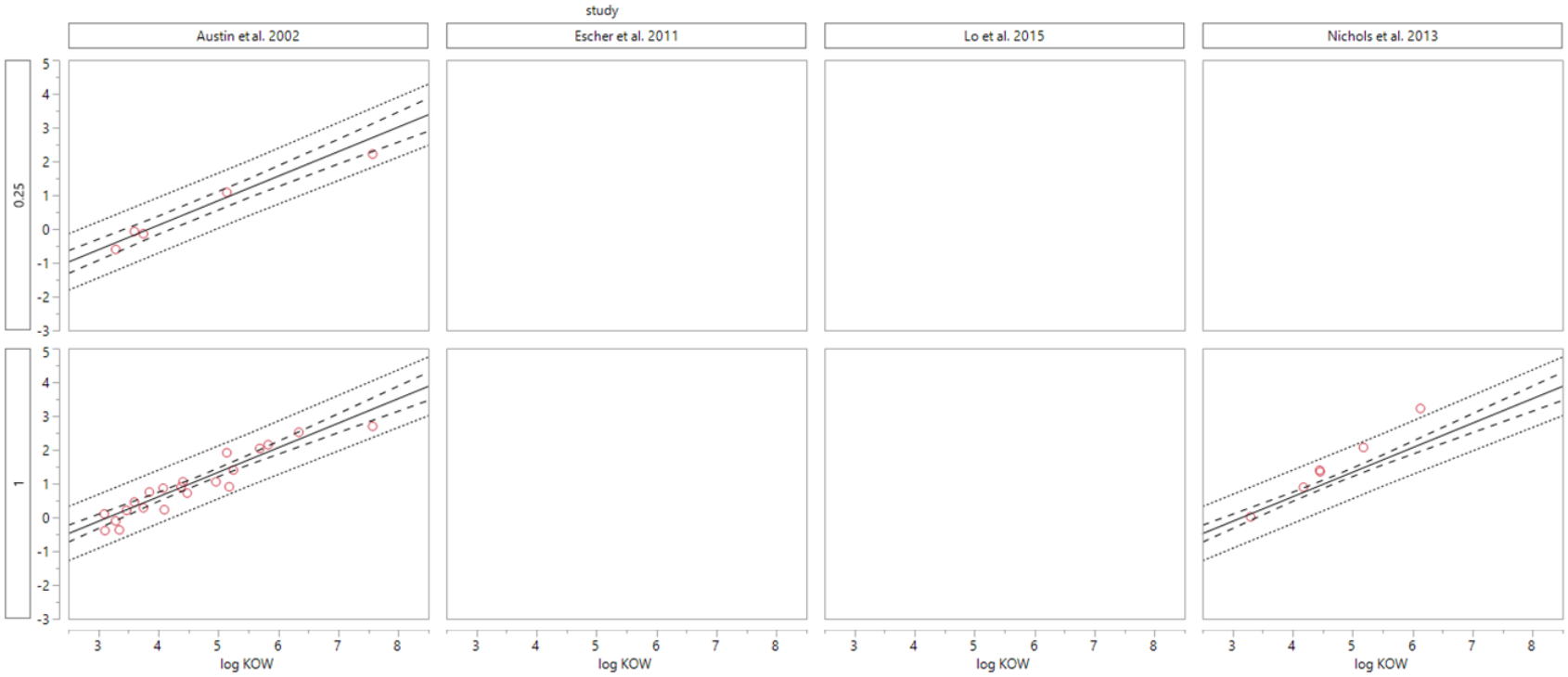
						$k_{dep,C\rightarrow 0}$ (min^{-1}), [K_M (µM)]			
Study	**C_{S9} (mg·mL^{-1})**	**V_{inc} (mL)**	**$V_{S9,inc}$ (mL)**	**V_{hom} (mL)**	**W_H (g)**	**9-methyl anthracene log K_{OW} = 5.07**	**Pyrene log K_{OW} = 5.18**	**Chrysene log K_{OW} = 5.81**	**Benzo[*a*]pyrene log K_{OW} = 6.13**
in vitro S9 experiments using non pre-exposed liver S9 (Lo et al. (1))	3.5 (SE 0.1)	0.5	0.2	28.9	5.3	0.017 (SE 0.001), [1.6 (SE 0.4)] **0.013 (SE 0.002)**	0.09 (SE 0.01), [0.31 (SE 0.08)] **0.07 (SE 0.01)**	0.049 (SE 0.008), [0.14 (SE 0.05)] **0.12 (SE 0.02)**	0.09 (SE 0.02), [0.18 (SE 0.08)] **0.12 (SE 0.03)**
in vitro S9 experiments using pre-exposed liver S9 (present study, Chapter 6)	2.9 (SE 0.2)	0.5	0.2	24.5	4.6	0.014 (SE 0.001), [1.2 (SE 0.2)]	0.097 (SE 0.008), [0.21 (SE 0.04)]	0.048 (SE 0.005), [0.12 (SE 0.02)]	0.12 (SE 0.01), [0.05 (SE 0.01)]

Table D3 **Mean fish weight (W_B) during the depuration phase of in vivo dietary studies and somatic in vivo biotransformation rate constants (k_{BM}) of four test chemicals in in vivo dietary bioaccumulation bioassays.**

Test	W_B (g)	Source	k_{BM} (d^{-1})			
			9-methyl anthracene	Pyrene	Chrysene	Benzo[*a*]pyrene
1	62 (SE 4)	Lo et al. (*2*)	0.38 (SE 0.03)	N/A	0.38 (SE 0.04)	0.09 (SE 0.01)
2	0.88 (SE 0.05)	Lo et al. (3)	0.9 (SE 0.4)	0.8 (SE 0.4)	N/A	0.6 (SE 0.2)
3	1.12 (SE 0.04)	Lo et al. (3)	N/A	N/A	0.31 (SE 0.03)	N/A
4	1.6 (SE 0.2)	Lo et al. (3)	N/A	N/A	N/A	1.0 (SE 0.1)

Table D4 **Left: *Somatic* biotransformation rate constants normalized to 10 g fish ($k_{BM,IVIVE,10g}$) derived from in vitro experiments using sorbent phase dosing and multiple solvent delivery dosing experiments in pre-exposed and non-pre-exposed liver S9 extracts ($k_{BM,IVIVE,10g}$ (d^{-1})); Right: *Somatic* biotransformation rate constants normalized to 10 g fish ($k_{BM,IVIVE,10g}$) derived from in vivo dietary bioaccumulation studies reported in Lo et al. (*2, 3*).**

Chemical	$k_{BM,IVIVE,10g}$ (d^{-1})			$k_{BM,in\text{-}vivo,10g}$ (d^{-1})			
	Sorbent Phase-dosing method	Multiple solvent delivery dosing method, non-pre-exposed fish	Multiple solvent delivery dosing method, pre-exposed fish	Lo et al. (2) Test 1	Lo et al. (3) Test 2	Lo et al. (3) Test 3	Lo et al. (3) Test 4
9-methyl anthracene	0.066	0.084	0.064	0.6 (SE 0.2)	0.5 (SE 0.2)	N/A	N/A
Pyrene	0.35	0.44	0.42	N/A	0.4 (SE 0.2)	N/A	N/A
Chrysene	0.36	0.15	0.14	0.6 (SE 0.2)	N/A	0.18 (SE 0.02)	N/A
Benzo[*a*]pyrene	0.31	0.23	0.29	0.14 (SE 0.05)	0.3 (SE 0.1)	N/A	0.66 (SE 0.07)


study
Austin et al. 2002
Escher et al. 2011
Lo et al. 2015
Nichols et al. 2013
0.25
1
log KOW

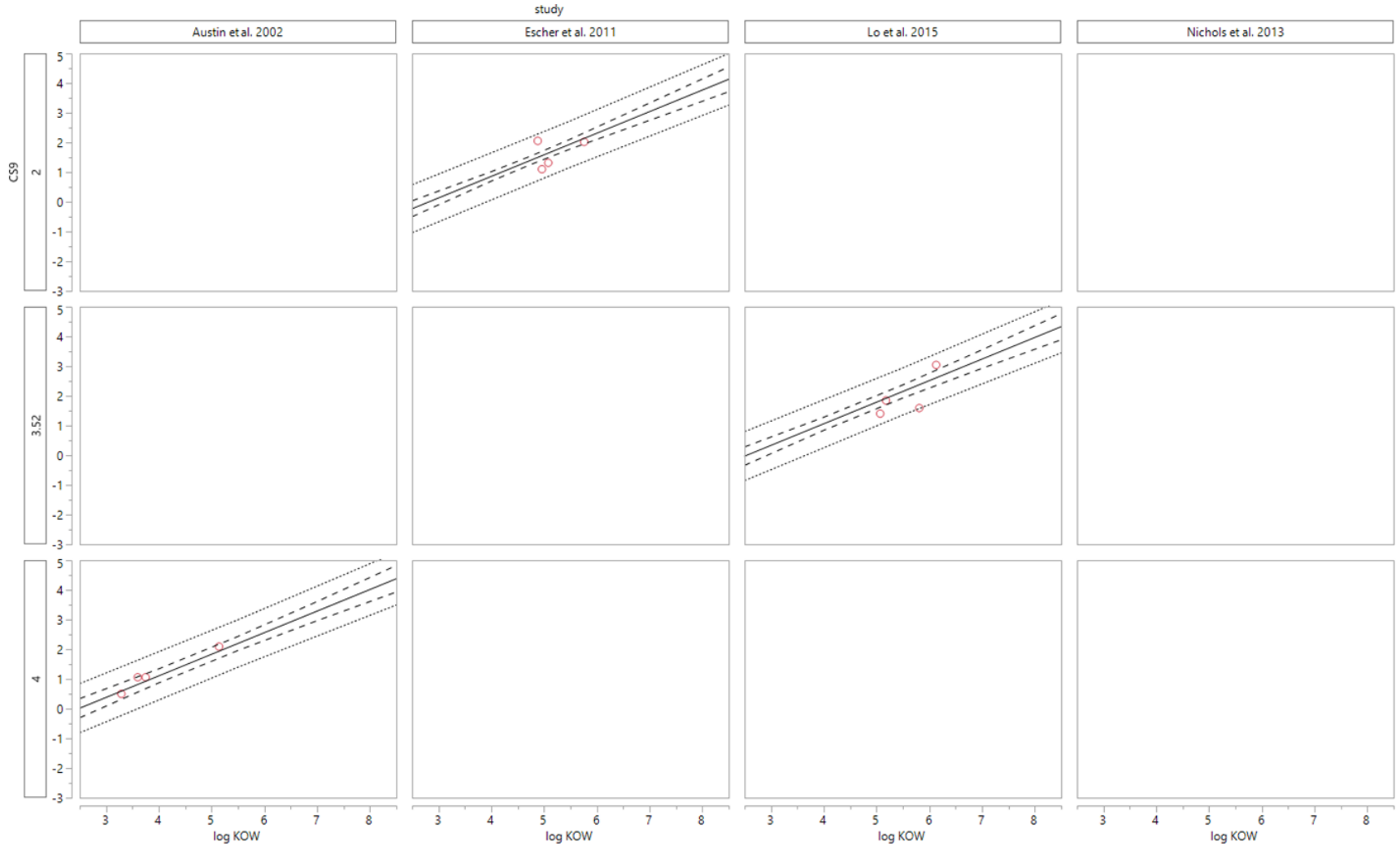

study
Austin et al. 2002
Escher et al. 2011
Lo et al. 2015
Nichols et al. 2013
CS9
2
3.52
4
log KOW

Figure D1 **Multiple linear regression of the log ratio of fraction bound to fraction unbound, log[(1 – $f_{u,inc}$)/$f_{u,inc}$] (y-axis), to the logarithm of K_{OW} (x-axis) and the logarithm of the concentration of S9 protein in the incubation medium, C_{S9}. The solid lines represent the fit of the linear regression model to the empirical data. Dashed lines represent the 95% confidence interval of the mean model fit, and the dotted lines represents the 95% prediction interval.**

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