Development and Application of an *In Vivo* Test for Estimating Biotransformation Rate Constants and Bioconcentration Factors of Hydrophobic Organic Chemicals in Fish

by

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Abstract

Bioconcentration factors (BCFs) are the most commonly used metric by regulatory agencies to assess the bioaccumulation of chemicals in fish. However, due to logistical and economic constraints to laboratory testing, there is limited empirical BCF data. In addition, there are no accepted *in vivo* methods to measure biotransformation rates of hydrophobic organic chemicals (HOCs) in fish. This study presents a method for measuring *in vivo* biotransformation rate constants and BCFs of HOCs in aqueous bioconcentration tests. BCF tests were conducted for the test chemicals; methoxychlor, pyrene, cyclohexyl salicylate and 4-n-nonylphenol using a sorbent phase as a dosing reservoir. A co-exposure using non-biotransformed reference chemicals was used to derive biotransformation rates of the test chemicals. The tests were successful for measuring depuration and biotransformation rate constants (k_T, k_M), and BCFs in fish that will contribute empirical data for evaluating predictive models (e.g., *in vitro* to *in vivo* extrapolation; IVIVE) and *in vitro* k_Ms.

Keywords: bioaccumulation; bioconcentration factor; metabolic biotransformation; rainbow trout; hydrophobic organic chemicals; octanol-water partition coefficient

Dedication

This report is dedicated to my family and friends. You have shown immense and unwavering support throughout the entire process, from when I first applied to the program to the final stages of this report. I could not have done this without you.

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List of Acronyms

µg L^{-1}	Micrograms per liter
1,2,4,5-TClBz	1,2,4,5-Tetrachlorobenzene
1,3,5-TClBz	1,3,5-Trichlorobenzene
4NP	4-n-nonylphenol
ACN	Acetonitrile
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
B	Bioaccumulative
BAF	Bioaccumulation Factor (L/kg-lipid)
BCF	Bioconcentration Factor (L/kg)
CEPA	Canadian Environmental Protection Act
CS	Cyclohexyl salicylate
CYP	Cytochrome
d12-Chrysene	Deuterated Chrysene
d8-Naphthalene	Deuterated Naphthalene
DCM	Dichloromethane
DO	Dissolved Oxygen
DSL	Domestic Substances List
ECD-GC	Electron Capture Detection- Gas Chromatography
US EPA	USA Environmental Protection Agency
EPI Suite	Estimation Program Interface Suite
EU	European Union
EVA	Ethylene-Vinyl Acetate
GC/MS	Gas Chromatography/Mass Spectrometry
GPH	Gallons Per Hour
HexaClBz	Hexachlorobenzene
IS	Internal Standard
iT	Inherently Toxic
IVIVE	*In vitro* to *in vivo* extrapolation model
K_{EW}	EVA-Water Partition Coefficient
k_M	Whole body biotransformation Rate Constant (d-1)
K_{OW}	Octanol-Water Partition Coefficient

K_{SW}	Sorbent phase-Water Partition Coefficient
k_T	Depuration rate constant of Test Chemicals (d-1)
$k_{T,R}$	Depuration Rate Constant of Reference Chemicals (d-1)
LC_{50}	Lethal concentration that causes death of 50% of the population
LLE	Liquid-Liquid Extraction
Ln	Natural Logarithm
LOD	Limit of Detection
LOQ	Limit of Quantitation
LRT	Long Range Transport
m/z	Mass-to-Charge Ratio
MC	Methoxychlor
ME	Matrix Effect
MoA	Mode of Action
MS-222	Tricaine Methane Sulfonate
NOEC	No Observed Effect Concentration
OECD	Organization for Economic Cooperation and Development
P	Persistent
PAH	Polycyclic Aromatic Hydrocarbon
PBT	Persistent, Bioaccumulative, Toxic
PCB	Polychlorinated Biphenyl
PentaClBz	Pentachlorobenzene
POP	Persistent Organic Pollutant
PYR	Pyrene
QSAR	Quantitative Structure-Activity Relationship
RCF	Relative Centrifugal Force
REACh	Registration, Evaluation, Authorisation, and Restriction of Chemical Substances
SD	Standard Deviation
SE	Standard Error
SFU	Simon Fraser University
Tukey HSD	Tukey’s Honestly Significant Difference
ww	Wet Weight

Glossary

Bioaccumulation	The process in which a chemical substance is absorbed in an organism by all routes of exposure (i.e., dietary and environmental sources)
Bioaccumulation Factor	The degree to which bioaccumulation occurs can be expressed as a bioaccumulation factor (BAF) and is typically measured under field conditions
Bioconcentration	The process by which a chemical substance is absorbed by an organism from the ambient environment through its respiratory and dermal surfaces (i.e., chemical absorbed through diet is not included)
Bioconcentration Factor	The degree to which bioconcentration occurs, measured under controlled laboratory conditions in which dietary uptake is not included
Biotransformation	The enzymatic conversion of a chemical form to a metabolite
Contaminant	Any substance entering a system where it is not normally found
Depuration	Processes that result in elimination of a chemical from an organism
Depuration rate constant (k_T)	Numerical value that defines the rate of reduction of the concentration of the test substance in the test fish following the transfer of the test fish from a medium containing the test substance to a medium without the test substance
Hydrophobicity	The physical property of a molecule that is repelled from a mass of water
Octanol-water partition coefficient	The ratio of the concentrations of a chemical in 1-octanol and in water
Organic compound	A compound that contains one or more C-H bonds, or a compound that contains carbon
Pollutant	A contaminant that adversely effects the properties of the environment

Polychlorinated biphenyls (PCBs)	A class of organic compounds with 1-10 chlorine atoms attached to a biphenyl (a molecule with two benzene rings)
Polycyclic aromatic hydrocarbon (PAHs)	Chemical compounds that consist of two or more fused aromatic rings
Toxicokinetics	Processes of uptake, elimination, distribution and biotransformation of potentially toxic substances
Uptake rate constant (k_1)	The numerical value defining the rate of increase in the bioconcentration of a test substance in a test fish when the fish is exposed to that chemical
Xenobiotic	Chemicals that are foreign to a biological system

Chapter 1. Introduction

Several thousand tonnes of chemicals are manufactured and released into the environment annually (Gobas, De Wolf, Burkhard, Verbruggen, & Plotzke, 2009; ONU, 2009; Weisbrod et al., 2007). The potential impacts of these chemicals are reason for global concern, particularly for those chemicals considered toxic, persistent and bioaccumulative. The persistence (P) of a chemical refers to its ability to resist degradation in the environment and is measured by the chemical's half-life ($t_{1/2}$). If a chemical occurs in its parent form for ≥ 2 days in air, and ≥ 6 months in water or soil it is considered persistent by Health Canada and Environment and Climate Change Canada according to the Canadian Environmental Protection Act (CEPA 1999) (Canada, 2013). The inherent toxicity (iT) of a chemical refers to its immediate or long-term harmful effects on the environment and the hazard a chemical presents to the health of the environment (Canada, 2013). A chemical is said to be inherently toxic if it has toxic effects at concentrations below 1 mg/L, based on its toxicity to non-human, aquatic organisms (Canada, 2013). The bioaccumulation (B) potential of a chemical is the ability of a substance to accumulate in an organism over time at concentrations that exceed those in the environment. Some of the criteria for bioaccumulation are summarized in Table 1. Chemicals that have PBT characteristics are of particular concern due to their potential to cause harm to humans and wildlife (ONU, 2009; Weisbrod et al., 2007). As a result, national and international efforts exist to assess both existing and new commercial substances for PBT characteristics (Canada, 2013; ONU, 2009).

1.1. Current regulations for hydrophobic organic chemicals

Currently, there are approximately 150,000 existing chemicals in commerce worldwide and 1,000-2,000 new substances being manufactured annually (Gobas et al., 2009; ONU, 2009; Weisbrod et al., 2007). Regulatory agencies exist to evaluate chemicals for their potential risks to human and environmental health. In Canada, chemicals are regulated under the Canadian Environmental Protection Act (CEPA,1999) (Canada, 2013). All new chemicals (i.e., not listed on the Domestic Substances List, DSL) must go through a screening level risk assessment (SLRA) before they are imported, manufactured or used in Canada. PBTs and high-exposure chemicals listed on

the DSL are also subject to a screening level risk assessment. If a substance meets or exceeds the criteria outlined in Section 64 of CEPA 1999, as determined from the SLRA, the chemical is deemed “toxic” and added to the Toxic Substances list and regulatory control ensues. Otherwise, chemicals may be placed on the Priority Substances List for a more in-depth risk assessment (Canada, 2013). Chemicals not requiring immediate attention are put aside for no further action at the time (Canada, 2013).

While most countries have their own regulatory agencies and criteria to evaluate substances (e.g., CEPA in Canada, Registration, Evaluation and Authorization of Chemicals (REACh) in the European Union, and the US Environmental Protection Agency’s Toxic Substances Control Act (TSCA)), their approaches are similar (Canada, 2013; ECHA, 2017; ONU, 2009). A common strategy for evaluating the thousands of commercial substances is to screen them for their potential to be inherently Toxic (iT), Persistent (P) and Bioaccumulative (B) (Arnot & Gobas, 2006; Gobas et al., 2009). Substances that meet the criteria for P B and iT are considered to be PBT substances and undergo a risk assessment to determine whether they could pose significant harm to humans and the environment (Gobas et al., 2009). By first screening chemicals for PBT, the number of chemicals requiring extensive and costly risk assessments is reduced.

A chemical is deemed bioaccumulative if it exceeds the criteria designated by various regulatory agencies listed in Table 1. However, empirical bioaccumulation data are often unavailable or reliant on predictive models (Arnot & Gobas, 2006; Arnot, Pawlowski, & Champ, 2018; Laue et al., 2014). For example, of the 11,300 organic substances that were reviewed by Environment Canada, less than 4% have available measured values (Arnot & Gobas, 2006).

Table 1. A summary of regulatory bioaccumulation assessment endpoints and criteria.

Regulatory Agency	Bioaccumulation Endpoint	Criterion (log transformed)	Program
Environment and Climate Change Canada	K_{OW}	≥ 100,000 (5.0)	CEPA (1999) *
Environment and Climate Change Canada	BCF	≥ 5,000 L/kg (3.7)	CEPA (1999)
Environment and Climate Change Canada	BAF	≥ 5,000 L/kg (3.7)	CEPA (1999)
European Union 'Bioaccumulative'	BCF	> 2,000 L/kg (3.3)	REACh §
European Union 'Very Bioaccumulative'	BCF	> 5,000 L/kg (3.7)	REACh
European Union	BMF	> 1.0	REACh
European Union	TMF	> 1.0	REACh
United States 'Bioaccumulative'	BCF	1,000 - 5,000 L/kg (3.3 - 3.7)	TSCA †, TRI
United States 'Very Bioaccumulative'	BCF	≥ 5,000 L/kg (3.7)	TSCA, TRI
United Nations Environment Programme	K_{OW}	≥ 100,000 (5.0)	Stockholm Convention ‡
United Nations Environment Programme	BCF	≥ 5,000 L/kg (3.7)	Stockholm Convention

* Canadian Environmental Protection Act (CEPA), 1999 (Government of Canada 1999, 2000)
§ Registration, Evaluation, Authorization and Restriction of Chemicals (REACh) Annex XIII (European Commission 2001)(ECHA, 2017).
† US Environmental Protection Agency, Toxic Substances Control Act (TSCA) and Toxic Release Inventory (TRI) programs (US EPA 1976).
‡ Stockholm Convention on Persistent Organic Pollutants (UNEP 2001).

Bioaccumulation is most often assessed using bioconcentration factors (BCFs) in fish or octanol-water partition coefficients (K_{OW}) (Arnot & Gobas, 2006). The K_{OW} of organic substances can be obtained relatively easily using computer modelling or lab-based measures. However, K_{OW} values should not be relied on solely since they only describe the passive chemical partitioning and the variety of methods to obtain K_{OW} values may incur significant error (Weisbrod et al., 2007, 2009). Certain physiological processes are not considered by K_{OW} such as active uptake/loss of chemicals from gills and elimination via fecal egestion and biotransformation (Cowan-Ellsberry et al., 2008). To reduce reliance on K_{OW} and K_{OW}-based bioaccumulation models, *in vitro* methods and extrapolation models for *in vitro* to *in vivo* data exist. Alternatively, bioconcentration factors are used to assess bioaccumulation potential in aquatic organisms. Bioconcentration refers to the process in which hydrophobic organic chemicals (HOCs) are absorbed by fish from surrounding water and accumulated in the fat tissue of the

organism (Gobas & MacKay, 1987). The bioconcentration of a chemical is controlled by its partitioning behavior and can be related to the K_{OW} of a chemical (Gobas & MacKay, 1987). Bioconcentration factors (BCFs) are obtained from laboratory experiments in which the uptake and elimination rates of a chemical are measured by exposing fish to a concentration of chemical in water during the uptake phase and clean (i.e., chemical-free) water during the elimination phase (Adolfsson-Erici, Åkerman, & Mclachlan, 2012; OECD, 2012). Fish are sampled throughout the uptake and depuration phases and analysed in the lab for chemical residues (Adolfsson-Erici et al., 2012; OECD, 2012). A common standardized method of deriving fish BCF values is the OECD 305 BCF flow-through bioconcentration test (OECD, 2012; Schlechtriem, Fliedner, & Schäfers, 2012). Despite the BCF being a preferred metric by regulatory agencies, BCF tests incur high costs, are lengthy and require large numbers of test animals for each substance. Therefore, with thousands of chemicals needed to be tested, alternative methods to estimate BCF's and biotransformation rate constants in fish that reduce efforts and animal use have been developed such as *in vitro* tests and *in vitro* to *in vivo* extrapolation models (Adolfsson-Erici et al., 2012; Arnot & Gobas, 2006; Arnot, Mackay, & Bonnell, 2008; Trowell, Gobas, Moore, & Kennedy, 2018).

Traditionally, K_{OW} or K_{OW}-based bioaccumulation models are used to assess the bioaccumulation potential of chemicals in fish when empirical *in vivo* data are unavailable (Arnot & Gobas, 2006). More recently, models include important uptake, elimination and metabolism processes (Arnot & Gobas, 2003, 2004; Arnot et al., 2008; Gobas, 1993; Lo, Campbell, Kennedy, & Gobas, 2015; Lo, Letinski, Parkerton, Campbell, & Gobas, 2016). However, despite improvements, available K_{ow} and K_{ow}-based models do not provide *a priori* estimates of biotransformation rates. The lack of empirical biotransformation information and the limitations of predicting metabolic transformation rates has reduced the success in predicting BCF values for highly biotransformed substances (Dimitrov et al., 2005; Trowell et al., 2018).

Biotransformation is an important elimination process that can reduce the degree of passive bioaccumulation of hydrophobic chemicals from aqueous routes of exposure and is shown to significantly impact the corresponding BCF (de Wolf, de Bruljn, Selner, & Hermans, 1992; Laue et al., 2014; South, Keffer, & Beauchamp, 1980). Consequently, the bioaccumulation classification of a chemical can be affected based on whether biotransformation is considered or not (Arnot & Gobas, 2004; Laue et al., 2014). BCFs

can be successfully estimated for chemicals that do not undergo substantial biotransformation, however for substances that are significantly biotransformed, BCFs, and thus the bioaccumulation potential of high K_{OW} chemicals, may be overestimated if biotransformation is not considered in the estimates (Kuo & Di Toro, 2013). Therefore, biotransformation rates of hydrophobic chemicals are crucial for determining the bioaccumulation potential and subsequent classifications of chemicals. Incorporating whole organism biotransformation rates based on *in vivo* data to models could reduce error in bioaccumulation estimates and contribute important information to the weight-of-evidence (WoE) approach to bioaccumulation assessments (Arnot & Gobas, 2006; J. Nichols et al., 2007; Weisbrod et al., 2009).

To prevent incorrectly classifying chemicals as bioaccumulative, methods for assessing chemical biotransformation rates are required. Currently, reliable and accepted methods for determining biotransformation rates of commercial chemicals experimentally *in vivo* only exist for dietary studies (Cowan-Ellsberry et al., 2008; Lo et al., 2015, 2016; J. W. Nichols, Huggett, Arnot, Fitzsimmons, & Cowan-Ellsberry, 2013; Uchea, Sarda, Schulz-Utermoehl, Owen, & Chipman, 2013; Weisbrod et al., 2009).There are several methods being developed to estimate biotransformation rates in whole organisms. For example, Quantitative Structure-Activity Relationship (QSAR) models are used to predict biotransformation rates and BCFs based on chemical structure (Arnot et al., 2009; Papa, van der Wal, Arnot, & Gramatica, 2014). Other approaches include measuring biotransformation rates *in vitro* using subcellular liver preparations (S9) (Han, Nabb, Yang, Snajdr, & Mingoia, 2009; Laue et al., 2014; Lee et al., 2014) or hepatocytes (Han, Nabb, Mingoia, & Yang, 2007; Trowell et al., 2018). The biotransformation rates determined *in vitro* are then used to estimate biotransformation rates in whole organisms using *in vitro* to *in vivo* extrapolation (IVIVE) models (J. W. Nichols, Fitzsimmons, & Burkhard, 2007; J. W. Nichols et al., 2013).

IVIVE models and *in vitro* testing have the potential to reduce the time, costs and animal requirements of bioaccumulation assessments (Cowan-Ellsberry et al., 2008; Eisenbrand et al., 2002; Laville, Ait-Aissa, Gomez, Casellas, & Porcher, 2004). However, these *in vitro* approaches still require evaluation (Weisbrod et al., 2009). Therefore, as part of the efforts to move towards *in vitro* and subsequent IVIVE techniques to assess bioaccumulation of hydrophobic substances, whole fish *in vivo* biotransformation rates determined in aqueous studies are required. Furthermore, aqueous exposure-based

biotransformation rates are required to measure BCFs requested by regulatory agencies (Gobas & Lo, 2016).

BCF testing for hydrophobic organic chemicals (HOCs) is challenging due to the hydrophobic nature of high log K_{OW} substances. To try and achieve constant exposure, passive dosing methods for HOCs have been developed for *in vitro* and *in vivo* experiments (Mayer & Holmstrup, 2008; Smith, Dom, Blust, & Mayer, 2010; Smith, Oostingh, & Mayer, 2010; Trowell et al., 2018). Passive dosing is sometimes referred to as partitioning-based dosing (Bandow et al., 2009), partition-controlled delivery (Brown et al., 2001) or partition driven administration (Reichenberg & Mayer, 2006).

Passive dosing using a sorbent-phase polymer as a reservoir for the analyte of interest have been investigated using ethylene-vinyl-acetate (EVA) or silicone (Birch, Gouliarmou, Lützhoft, Mikkelsen, & Mayer, 2010; Butler et al., 2013; Smith, Oostingh, et al., 2010). This method of passive dosing may overcome certain limitations of other methods of dosing by eliminating the need of a carrier solvent and reducing the potential for the release of undissolved portions of chemical in testing tanks since only the portion of chemicals dissolved in methanol is added to the EVA beads prior to the tests (Butler et al., 2013; Smith, Oostingh, et al., 2010). Furthermore, the continuous partitioning of HOCs from a loaded reservoir (EVA) eliminates having to dose with co-solvents and can better define the exposure concentrations (Smith, Oostingh, et al., 2010). However, in some cases, analytes have been shown to deplete before the end of testing (Smith, Dom, et al., 2010). Overall, this method has proven useful for dosing with hydrophobic chemicals and can potentially be used for an i*n vivo* bioconcentration test for fish (Mayer & Holmstrup, 2008; Smith, Oostingh, et al., 2010).

1.1.1. OECD guidelines for testing of chemicals: Bioaccumulation in fish: Aqueous exposure

Methods for measuring BCFs in fish is outlined in the Organization for Economic and Cooperative Development (OECD) 305 test guidelines (OECD, 2012). The minimized aqueous test consists of two phases, an exposure phase and depuration phase. The testing protocol requires a 28-day uptake phase or an uptake phase that is long enough to achieve steady-state. Chemicals that do not reach steady state within the 28-day uptake period require a lengthier uptake phase of up to 60 days. Sampling (n=4)

occurs at a minimum of five occasions during the uptake phase and four occasions during the depuration phase for each test substance, however, the test advises to sample more frequently. The OECD test No. 305 Minimized Aqueous Bioconcentration Test for fish requires a minimum of 36 fish, generally extends over a 3-6 month period and costs approximately $125,000 per chemical (OECD, 2012; Weisbrod et al., 2007). Despite improvements compared to the full length BCF test, the minimized design still requires significant effort and, like the full test, does not provide a method to measure biotransformation rate constants in fish (k_M, d^{-1}). This highlights the need for an *in vivo* test that reduces costs, animals and effort for hydrophobic organic chemicals and that includes measurements for biotransformation rate constants. It also highlights the importance of evaluating alternative methods to acquire BCF and biotransformation data that generally reduce testing efforts required for *in vivo* tests, such as *in vitro* assays and IVIVE modelling techniques (Cowan-Ellsberry et al., 2008; Eisenbrand et al., 2002; Laville et al., 2004).

1.1.2. Biotransformation of test chemicals

The test chemicals chosen for this study are methoxychlor, pyrene (PYR), cyclohexyl salicylate (CHS) and 4-n-nonylphenol (4NP). All four chemicals have been reported to undergo biotransformation at a range of rates and were recently included in an OECD ring-trial to support the development of test guidelines for determining *in vitro* clearance using rainbow trout hepatocytes and liver S9 subcellular fractions (Fay et al., 2015; J. Nichols et al., 2018).

Methoxychlor

Methoxychlor [1,1,1-trichloro-2,2-bis (p-methoxyphenyl) ethane], is an organochlorine pesticide originally developed as a substitute for DDT and was widely used to control various insect pests (Krisfalusi, Eroschenko, & Cloud, 1998; Versonnen, Roose, Monteyne, & Janssen, 2004). While methoxychlor has a low toxicity towards mammals and lower persistence than DDT, it is bioaccumulative in the environment and is shown to have estrogenic effects on most organisms (Eroschenko, Amstislavsky, Schwabel, & Ingermann, 2002; Green, Mwatibo, & Swartz, 1997; Krisfalusi et al., 1998; Versonnen et al., 2004). Methoxychlor is ubiquitous in the environment due to widespread human use and is reported to have adverse effects on non-target aquatic

organisms such as fish (Krisfalusi et al., 1998). Despite bioaccumulation factors up to 8,000 in certain species, there are generally low residues in human and animal tissue due to its fairly rapid biotransformation and readily excretable degradation products (Versonnen et al., 2004).

Pyrene

Pyrene belongs to a widely studied group of chemicals called polycyclic aromatic hydrocarbons (PAHs) (Ikenaka et al., 2013). PAHs are ubiquitous in aquatic ecosystems and many are toxic to humans and other organisms. Pyrene is often used in environmental toxicology research since it is one of the most abundant PAHs found in the environment (Vives, Grimalt, Ventura, & Catalan, 2005). Hydrocarbons, like pyrene and its metabolites, are of particular interest due to their carcinogenic and mutagenic properties (Black, 1988; Lehr & Jerina, 1977; Varanasi, Stein, Nishimoto, Reichert, & Collier, 1987; Vives et al., 2005).

Cyclohexyl salicylate

Cyclohexyl salicylate is an aromatic compound used in fragrance ingredients, a diverse group of chemicals consisting of over 2000 commonly used compounds (Laue et al., 2014). In a previous study, the biotransformation rate of cyclohexyl salicylate in fish was measured from an *in vitro* assay using trout liver S9 fractions (Laue et al., 2014). The authors found cyclohexyl salicylate to be more rapidly biotransformed compared to other fragrance compounds used in the study (Laue et al., 2014). While existing BCF data indicates that cyclohexyl salicylate is non-bioaccumulative as per US EPA bioaccumulation criteria, models without biotransformation estimate higher BCF's than is expected if biotransformation is included, thus categorizing cyclohexyl salicylate as bioaccumulative (Laue et al., 2014). Cyclohexyl salicylate is an example of the importance of biotransformation and its implications for regulatory agencies. It highlights the need for a method to measure *in vivo* biotransformation rates to compare and evaluate current bioaccumulation metrics.

4-n-nonylphenol

4-n-nonylphenol (4NP) is one of many isomeric compounds that make up Nonylphenol (NP), a chemical widely used for synthesizing non-ionic surfactants (i.e., nonylphenol-polyethoxylates, NP-PEs) (Zalko, Costagliola, Dorio, Rathahao, & Craved,

2003). 4NP contamination in the environment primarily results from the degradation of NP-PEs, though it can also be directly released from plastics (Zalko et al., 2003). 4NP is an endocrine disrupting chemical shown to induce estrogenic effects in fish both in vivo and in vitro (Luo et al., 2017; Thibaut et al., 2000). In rainbow trout, metabolic studies reveal two major biotransformation pathways for 4NP (Zalko et al., 2003). First, the conjugation of the phenol group by glucuronic acid and second, via the oxidation of the alkyl side chain (Thibaut, Debrauwer, Rao, & Cravedi, 1999, 1998).

Recently, all four test chemicals were included in an international ring trial to determine the reliability of *in vitro* methods used to measure intrinsic clearance of hydrophobic organic chemicals by rainbow trout (J. Nichols et al., 2018). This study may therefore contribute to efforts to evaluate *in vitro* methods by providing empirical *in vivo* biotransformation rate information for rainbow trout.

1.2. Research gaps and limitations

Currently, empirical BCFs exist for relatively few chemicals due to the significant effort required to derive these metrics from whole organisms experimentally (Arnot & Gobas, 2006; Fernández et al., 2012). Aqueous methods for measuring BCF values for hydrophobic organic chemicals in fish are limited to costly and time intensive tests. Furthermore, aqueous tests for high log K_{OW} substances are limited due to their hydrophobic nature and test requirements to satisfy constant concentrations in water throughout the exposure period (OECD, 2012; Parkerton et al., 2008). Chemicals that are lipophilic in nature are difficult to use in aqueous testing due to their low solubility in water (i.e., 0.01-0.1 mg/L) (OECD, 2012). Constant aqueous concentrations of highly hydrophobic chemicals are difficult to maintain due to possible sorption to exposure tanks, fish feed and feces. Also aqueous concentrations of such chemicals are often in the same range or below limits of detection (OECD, 2012). Therefore, dietary exposure studies are generally favored for high log K_{OW} substances.

1.3. Hypothesis and research objectives

The main objective of this study is to develop and examine a simplified aqueous *in vivo* BCF test for hydrophobic organic chemicals (HOCs) that measures kinetic BCFs, depuration rate constants (k_T, d^{-1}) and whole-body biotransformation rate constants (k_M,

d^{-1}) in fish. This study uses a sorbent-phase (i.e., ethylene-vinyl-acetate, EVA) as a dosing reservoir for HOCs in aqueous bioconcentration tests. Furthermore, the study employs a technique that uses inert reference chemicals to determine whole organism biotransformation rate constants (k_M, d^{-1}) in rainbow trout (Lo et al., 2015, 2016). Reference chemicals were selected for their non-biotransformative (k_M is approximately 0) and hydrophobic (log K_{OW} > 3) properties. The study also aims to identify differences, if any, on the k_Ms and BCFs of the test chemicals in single treatments and in mixture treatments in the presence of reference chemicals. Lastly, the study will be used to explore the application of a sorbent phase-water partition coefficient K_{SW} for each reference and test chemicals to reduce error in the derivation of the k_M.

Chapter 2. Theory

2.1. Bioconcentration of non-ionic organic chemicals in fish

Chemical uptake from water by gill respiration is a major pathway by which hydrophobic organic chemicals (HOC) can accumulate in fish. To describe the accumulation of a chemical in an aquatic organism compared to the environment in which it occurs, bioconcentration factors (BCF) are used. To derive BCFs and whole organism biotransformation rate constants from aqueous bioconcentration tests, the experimental design for this study was based on methods used in previous studies involving the bioconcentration of contaminants in fish and lipid-water partitioning of hydrophobic organic chemicals (HOCs). The study also follows some of the principle components of the OECD test guideline No. 305 for determining bioconcentration factors in fish (Lo et al., 2016; OECD, 2012). Each BCF test includes an uptake and depuration phase, the application of a control group under the same conditions (without test chemicals), and the measurement of depuration and biotransformation rate constants (k_M and k_T, respectively) as well as kinetic bioconcentration factors (OECD, 2012).

2.1.1. Deriving the Bioconcentration Factor (BCF)

Bioconcentration of contaminants in fish is conceptualized by chemical uptake and removal pathways including uptake from water via the gills and the skin and removal from gills and skin to the water; elimination via fecal matter; metabolic biotransformation; and a pseudo-elimination process through growth dilution. The following differential equation (dC_f/dt) describes the rate at which concentrations of chemicals in fish change using a first-order kinetic bioaccumulation model (Gobas, 1993; OECD, 2012):

$$\frac{dC_f}{dt} = k_1 * C_w - (k_2 + k_E + k_G + k_M) * C_f \quad (1)$$

Where, C_W (g chemical L^{-1}) and C_f (g chemical kg $fish^{-1}$) are the concentrations of chemicals in water and fish; k_1 (L kg fish $bodyweight^{-1}$ d^{-1}) is the chemical uptake rate constant from water; k_2 (d^{-1}) is the respiratory elimination rate constant; k_E (d^{-1}) is the rate constant for elimination by fecal egestion; k_G (d^{-1}) is the pseudo-elimination via growth

dilution rate constant; and k_M (d^{-1}) is the biotransformation rate constant of the chemical in fish.

The bioconcentration factor (L kg^{-1}) in fish can be calculated either as the ratio of the concentrations of chemicals in fish and water at steady state, or as the ratio of the uptake (k_1) and depuration (k_T) rate constants (Gobas, Mackay, Shiu, Lahittete, & Garofalo, 1988).

$$BCF = \frac{C_f}{C_W} = \frac{k_1}{k_T} \quad (2)$$

Where, BCF is the bioconcentration factor (L kg-1); and k_T (d^{-1}) is the total depuration rate constant. The uptake (k_1) and elimination processes described in the first-order bioaccumulation model (Equation 1) and the derivation of the BCF (Equation 2) is detailed in Sections 2.1.2, 2.2.1 and 2.1.1.

The kinetic BCF in fish on a wet weight basis can be derived under the assumption that the uptake and depuration of chemicals in fish act approximately according to first-order kinetic processes (OECD, 2012):

$$BCF_{k,ww} = \frac{k_1}{k_T} \quad (3)$$

Where, $BCF_{k, ww}$ is the kinetic BCF in fish on a wet weight basis (L kg^{-1}).

The BCF was derived using two methods:

First, the BCF was derived as follows:

$$\frac{dC_f}{dt} = k_1 C_w - k_T C_f \quad (4)$$

If C_w is constant during the uptake phase, then Equation 4 can be integrated to give:

$$C_f = \frac{k_1}{k_T} * C_w * (1 - e^{-k_T t}) \quad (5)$$

Throughout the depuration phase $C_w = 0$, so Equation 5 becomes:

$$\frac{dC_f}{dt} = -k_T C_f \tag{6}$$

Which can be integrated to give:

$$C_f = C_{f,t=0} * e^{-k_T t} \tag{7}$$

Which can then be transformed to natural logarithm and re-arranged to give:

$$\begin{aligned} LnC_f &= LnC_{f,t=0} - k_T t \\ k_T t &= LnC_{f,t=0} - LnC_f \end{aligned} \tag{8}$$

At t = 7 (end of uptake period) we can find k_1 as:

$$k_1 = \frac{C_{f,t=7} * k_T}{C_w * (1 - e^{-k_T * t})} \tag{9}$$

Then, the BCF follows as:

$$BCF = \frac{k_1}{k_T} \tag{10}$$

The BCF was also derived using an alternative method as follows:

$$\frac{dC_f}{dt} = k_1 C_w - k_T C_f \tag{11}$$

In this case, C_w is not constant during the uptake phase. If we can assume that:

$$C_w = C_{w,t=0} * e^{-k_W t} \tag{12}$$

Then Equation 11 becomes:

$$\frac{dC_f}{dt} = k_1 C_{w,t=0} * e^{-k_W t} - k_T C_f \tag{13}$$

Which can be integrated to give:

$$C_{f,t=7} = k_1 * C_{w,t=0} * \frac{(e^{-k_W t} - e^{-k_T t})}{(k_T - k_w)} \quad (14)$$

This can then be re-arranged to give:

$$k_1 = \frac{(C_{f,t=7} * (k_T - k_w))}{C_{w,t=0} * (e^{-k_w t} - e^{-k_T t})} \quad (15)$$

k_T is found as before. Hence:

$$BCF = \frac{k_1}{k_T} \quad (16)$$

2.1.2. Determining the depuration rate constants, k_T (day^{-1})

The depuration rate constant (k_T, d^{-1}) is the sum of the elimination rate constants, k_2, k_E, k_G and k_M, described in Equation 1. The depuration rate constant is derived as in to the OECD 305 test guidelines from the linear regression of the natural logarithm of concentrations of chemical in fish during the depuration phase of an aqueous bioconcentration test versus time, using the equation:

$$k_T = \frac{Ln\left(\frac{C_{f,\ t=0}}{C_f(t)}\right)}{t} \quad (17)$$

Where, $C_{f,\ t=0}$ is the concentration in fish tissue at the beginning of the depuration phase; and C(t) is the concentration in fish tissue at time *t* (OECD, 2012).

2.1.3. Determining the uptake rate constant (k_1, day^{-1})

The first order differential equation describing the concentration of a chemical in an organism (Equation 1) can be integrated to fit the uptake and depuration data obtained from an aqueous BCF test (Miller et al., 2016; OECD, 2012). This method, the Levenberg-Marquardt algorithm, allows for the simultaneous estimation of the uptake (k_1) and depuration (k_T) rate constants from the fitted curve. If the concentration of chemical in the water (C_w) and k_T are constant, Equation 5 can be rearranged to estimate k_1 (Gobas & Morrison, 1999; OECD, 2012). Where, k_T is estimated using

Equation 17 and C_f (t) and C_w (t) are the concentrations of chemicals in fish and water at time t, respectively (OECD, 2012).

In this study's minimised aqueous BCF design, in which only concentrations of chemicals in water are collected during the uptake phase, the uptake rate constant (k_1, d^{-1}) can be calculated from the results of the test using one of two approaches:

i) The first method applies if the concentration of chemical in water is constant throughout the exposure phase:

$$k_1 = \frac{C_{f,t=7} * k_T}{C_w * (1 - e^{-k_T \cdot t})} \quad (18)$$

ii) The second method applies if the concentration of the chemical in water follows an exponential decline. The change in concentration of chemical in water is thus described by a water depletion rate constant (k_W, d^{-1}) calculated from the concentrations at the beginning and at the end of the exposure phase (day 0 and day 7):

$$k_1 = \frac{C_{f,t=7} \cdot (k_T - k_W)}{C_{w,t=0} \cdot (e^{-k_W \cdot t} - e^{-k_T \cdot t})} \quad (19)$$

Where, $C_{f,\ t=7}$ is the concentration of chemical in fish at the beginning of the depuration phase (day 7 of exposure phase); C_W is the mean concentration of chemical in water at the beginning and end of the exposure phase (i.e., day 0 and day 7) (Equation 18); $C_{w,\ t=0}$ is the concentration of chemical in water at the beginning of the exposure phase (day 0) (Equation 19).

The water depletion rate constant (k_W) describes the rate at which the concentration of chemical in water declines throughout the exposure phase:

$$k_W = \frac{Ln\left(\frac{C_{w,\ t=0}}{C_{w,\ t=7}}\right)}{7} \quad (20)$$

Where, $C_{w,\ t=0}$ and $C_{w,\ t=7}$ are the concentrations of chemical in water at the beginning (day = 0) and end (day = 7) of the exposure phase, respectively.

Calculating standard error for k_1

The standard error of the update rate constant (k_1) is calculated using the two following methods, corresponding to the two methods used to describe the uptake and elimination of the chemical in the fish.

The first method assumes that the concentration of chemical in water is constant throughout the exposure phase (Equation 18).

Given estimates:

- $C_f \pm SE(C_f)$
- $C_w \pm SE(C_w)$
- $k_T \pm SE(k_T)$

Note that k_T and $SE(k_T)$ are derived from a simple linear regression:

$$lnC_f = b_0 + b_1 \cdot t \quad (21)$$

Where,

- $k_T = -b_1$
- $SE(k_T) = SE(b_1)$

Assuming t is a constant and $C_{f,t=7}$, C_w, and k_T are independent variables, the standard error of k_1 can be approximated based on the rule of error propagation:

$$SE(k_1) = \sqrt{\left[\left(\frac{\partial k_1}{\partial C_f}\right) \cdot SE(C_f)\right]^2 + \left[\left(\frac{\partial k_1}{\partial C_w}\right) \cdot SE(C_w)\right]^2 + \left[\left(\frac{\partial k_1}{\partial k_T}\right) \cdot SE(k_T)\right]^2} \quad (22)$$

Where,

$\frac{\partial k_1}{\partial C_f}$ is the partial derivative of k_1 with respect to C_f,

$$\frac{\partial k_1}{\partial C_f} = \frac{k_T}{C_w \cdot (1 - e^{-k_T \cdot t})} \quad (23)$$

$\frac{\partial k_1}{\partial C_w}$ is the partial derivative of k_1 with respect to C_w,

$$\frac{\partial k_1}{\partial C_w} = -\frac{C_f \cdot k_T}{{C_w}^2 \cdot (1 - e^{-k_T \cdot t})} \quad (24)$$

$\frac{\partial k_1}{\partial k_T}$ is the partial derivative of k_1 with respect to k_T.

$$\frac{\partial k_1}{\partial k_T} = \frac{C_f \cdot e^{k_T \cdot t} \cdot \left(e^{k_T \cdot t} - k_T \cdot t - 1\right)}{C_w \cdot (e^{k_T \cdot t} - 1)^2} \quad (25)$$

The second method assumes that the concentration of chemical in water is not constant and instead changes throughout the exposure phase according to a water depletion rate constant as shown in Equation 19.

Given estimates:

- C_f ± SE (C_f)
- $C_{w,t=0}$ ± SE ($C_{w,t=0}$)
- k_T ± SE (k_T)
- k_W ± SE (k_W)

Assuming that t is a constant and that C_f, $C_{w,t=0}$, k_T, and k_W are independent variables, the standard error of k_1 can be approximated based on the rule of error propagation:

$$SE(k_1) = \sqrt{\left[\left(\frac{\partial k_1}{\partial C_f}\right) \cdot SE\left(C_f\right)\right]^2 + \left[\left(\frac{\partial k_1}{\partial C_{w,t=0}}\right) \cdot SE\left(C_{w,t=0}\right)\right]^2 + \left[\left(\frac{\partial k_1}{\partial k_T}\right) \cdot SE(k_T)\right]^2 + \left[\left(\frac{\partial k_1}{\partial k_W}\right) \cdot SE(k_W)\right]^2} \quad (26)$$

Where,

$\frac{\partial k_1}{\partial C_f}$ is the partial derivative of k_1 with respect to C_f,

$$\frac{\partial k_1}{\partial C_f} = \frac{k_T - k_W}{C_{w,t=0} \cdot (e^{-k_W \cdot t} - e^{-k_T \cdot t})} \quad (27)$$

$\frac{\partial k_1}{\partial C_{w,t=0}}$ is the partial derivative of k_1 with respect to $C_{w,t=0}$,

$$\frac{\partial k_1}{\partial C_{w,t=0}} = -\frac{C_f \cdot (k_T - k_W)}{{C_{w,t=0}}^2 \cdot (e^{-k_W \cdot t} - e^{-k_T \cdot t})} \quad (28)$$

$\frac{\partial k_1}{\partial k_T}$ is the partial derivative of k_1 with respect to k_T,

$$\frac{\partial k_1}{\partial k_T} = \frac{C_f}{C_{w,t=0} \cdot (e^{-k_W \cdot t} - e^{-k_T \cdot t})} - \frac{C_f \cdot t \cdot (k_T - k_W) \cdot e^{-k_T \cdot t}}{C_{w,t=0} \cdot (e^{-k_W \cdot t} - e^{-k_T \cdot t})^2} \quad (29)$$

$\frac{\partial k_1}{\partial k_W}$ is the partial derivative of k_1 with respect to k_W,

$$\frac{\partial k_1}{\partial k_W} = \frac{C_f \cdot t \cdot (k_T - k_W) \cdot e^{-k_T \cdot t}}{C_{w,t=0} \cdot (e^{-k_W \cdot t} - e^{-k_T \cdot t})^2} - \frac{C_f}{C_{w,t=0} \cdot (e^{-k_W \cdot t} - e^{-k_T \cdot t})} \quad (30)$$

2.1.4. Determining the growth-corrected depuration rate constant (k_{TG}, d^{-1})

The mass of chemical in fish can be determined from the concentration in fish multiplied by the fish's body weight.

$$M_f = C_f * W_f \quad (31)$$

Where, M_f is the mass of chemical in fish (g); C_f (g kg body wt^{-1}) is the concentration in an individual fish, and W_f is the corresponding fish body weight (kg).

The mass of fish throughout the depuration phase can then be determined as the following:

$$\frac{dM_f}{dt} = k_{1G} * M_w - k_{TG} * M_f \quad (32)$$

$$k_{TG} = Ln\frac{(\frac{M_{f,t=0}}{M(t)})}{t} \quad (33)$$

Where, M_f is the mass of chemical in fish; M_w is the mass in water; k_{1G} is the growth corrected uptake rate constant; and k_{TG} is the growth-corrected depuration rate constant.

2.2. Biotransformation of contaminants in fish

Biotransformation refers to a combination of chemical transformations reflecting a chemical's ability to undergo multiple enzyme-catalyzed transformations at various rates. The biotransformation of a chemical determines the ultimate accumulation and elimination rates of chemicals and can reduce bioaccumulation in fish (J. Nichols et al., 2007; Tierney, Farrel, & Brauner, 2014). The accumulation and elimination rates of chemicals affect the persistence and body burden, which in turn determine the toxicity of a substance. Therefore, biotransformation affects the bioaccumulation, persistence and toxicity of some chemicals in fish (Kleinow, Melancon, & Lech, 1987). The extent of biotransformation of a chemical in fish is likely dependent on the lipid content of the organism, the hydrophobicity of a chemical and the activity of metabolizing compounds (J. Nichols et al., 2007).

Biotransformation of xenobiotic substances often involves the cytochrome P-450 monooxygenase system, various conjugating enzymes and other enzymes that catalyze hydrolytic and reduction reactions. Such reactions may result in a metabolite of the parent compound that is more soluble in water and thus can be more easily eliminated (Kleinow et al., 1987; Van der Linde, Jan Hendriks, & Sijm, 2001). Biotransformation reactions are generally divided into two groups of reactions, Phase I and Phase II. Phase I or modification reactions include oxidation, reduction and hydrolysis. Phase II consists of conjugation reactions, during which chemical compounds (i.e., glucuronic acid) are added, forming a more hydrophilic and more easily excretable compound (Ikenaka et al., 2013). Various physiological factors such as temperature, age and gender, can affect xenobiotic biotransformation by altering the metabolizing enzyme activities in fish (Buhler & Williams, 1988; Förlin, 1980; Förlin & Haux, 1990; Kleinow et al., 1987). Simultaneous exposure to multiple chemicals can affect biotransformation rates through mechanisms such as competitive inhibition and enzyme reduction (Buhler

& Wang-Buhler, 1998; Buhler & Williams, 1988). For example, in an *in vitro* study using fish liver S9 fractions by Lee and colleagues (2014), competitive inhibition was observed to affect the biotransformation rates of benzo[a]pyrene, chrysene and 9-methylanthracene (Lee et al., 2014). Particularly in the environment, chemical induction of monooxygenase activity can affect the biotransformation of a chemical and, in turn, affect the toxicity of a chemical (Kleinow et al., 1987).

Substances can be modified into a wide variety of metabolites (Tierney et al., 2014). Most often, biotransformation reactions result in the detoxification of a substance into more hydrophilic metabolites that are more easily excreted than the parent chemical. On the other hand, substances can be transformed into highly reactive metabolites that can exhibit more toxic effects relative to their parent compounds (Buhler & Williams, 1988).

2.2.1. Determining the biotransformation rate constants, k_M (day^{-1})

The *in vivo* biotransformation rate constants (k_M, d^{-1}) in fish can be determined using the depuration rate constants measured from the concentrations of chemicals in fish throughout the depuration phase of each treatment. First, measured depuration rate constants of the reference chemicals ($k_{T,R}$, d^{-1}) are used to develop a relationship between $k_{T,R}$ and $1/K_{OW}$ or $1/K_{EW}$:

$$\text{a)}\quad k_{T,R} = A * \left(\frac{1}{K_{OW}}\right) + B$$
$$\text{b)}\quad k_{T,R} = C * \left(\frac{1}{K_{EW}}\right) + D \qquad (34)$$

Where, A, B, C and D are linear regression coefficients obtained from the linear regression of the relationship between the depuration rate constants (k_T, d^{-1}) of non-biotransformed reference chemicals and the log K_{OW} or log K_{EW} (Equation 34). More specifically, coefficient A and C describe depuration of the chemical to water via the respiratory route, whereas coefficients B and D describe the combined other depuration processes that are mostly independent of K_{OW} (i.e., growth dilution and fecal egestion) (Gobas & Lo, 2016). This equation applies to most chemicals with Log K_{OW}'s greater than approximately 2 or 3.

Then, the biotransformation rate constants of the test chemical (k_M) are derived from the experimental depuration rate constants of the test chemicals (k_T) subtracted from the non-biotransformed reference chemicals ($k_{T,R}$, assuming $k_M = 0$) as follows:

$$k_M = k_T - k_{T,R} \quad (35)$$

The biotransformation rate constants derived using this method do not include the effects of growth dilution and are thus independent of fish growth.

Chapter 3. Methods

The methods section is divided into three parts, describing (i) the experimental methods, (ii) the analytical methods used for chemical analysis, and (iii) data analysis and statistical design.

3.1. Summary of BCF tests and analysis

Simplified *in vivo* bioconcentration tests were carried out using juvenile rainbow trout (*Oncorhynchus mykiss*) for which a passive dosing design was developed. This design involved adding chemicals to ethylene-vinyl-acetate beads, placing the beads in a wire mesh pouch that was then inserted into a tank filter in lieu of a carbon filter. Using this dosing method, four BCF tests were conducted in which fish were exposed to test chemicals (Table 2) for 7 days, followed by a 14-day depuration period during which fish were transferred to clean (chemical-free) flow-through tanks.

During each experiment, water and fish samples were collected. Chemicals were extracted from each water sample using a liquid-liquid extraction (LLE) technique (see Sample processing) and, whole fish sample extractions and clean-up used a modified "Quick, Easy, Cheap, Effective, Rugged, and Safe" (QuEChERS) method (Molina-Ruiz, Cieslik, Cieslik, & Walkowska, 2014). All sample extractions were analysed using gas chromatography paired with mass spectrometry (GC/MS) or electron capture detection (ECD-GC).

Throughout each experiment, the concentrations of chemicals in three relevant media, the water, EVA beads, and fish were measured. To determine the concentrations of chemical in each medium (e.g., water, EVA, fish), calibration curves were constructed (Appendix A). Linear regression of the calibration curves was used to determine the concentrations of chemicals in the different media during the exposure and depuration phase of each experiment.

Chemicals

The test chemicals, methoxychlor (MC), cyclohexyl salicylate (CS), pyrene (PYR) and 4-n-nonylphenol (4NP) were selected based on their diverse chemical classes, the

existence of measured bioaccumulation and biotransformation data in fish and their range of hydrophobicity (Log K_{OW} 4.7-5.76). Furthermore, the test chemicals were chosen on the basis that they are expected to undergo varying rates of biotransformation in fish. The biotransformation rate constants of the test chemicals were estimated in an OECD ring-trial using *in vitro* assays derived from trout liver, providing the opportunity to compare the *in vitro* estimated biotransformation rates to empirical *in vivo* biotransformation rates (Fay et al., 2015). Table 2 shows information pertaining to each test chemical including the chemical structure, log K_{OW}, molecular weight (MW) purity and ions (m/z). MC and PYR were purchased from Sigma-Aldrich (Oakville, ON). CS was obtained from Vigon International (Stroudsburg, PA) and 4-n-nonylphenol was purchased from Alfa Aesar (Haverhill, MA). The chemical purity of all test chemicals was greater than 95%.

The reference chemicals (Table 3), 1,2,4,5-tetrachlorobenzene, pentachlorobenzene and hexachlorobenzene were purchased from Sigma-Aldrich. 1,3,5-trichlorobenzene was purchased from Chromatographic Specialties (Brockville, ON). The chemical purity of the reference chemicals was greater than 98%.

A range of internal standards were selected for precision and accuracy of the analytical techniques (Table 4). PCB-52 was purchased from AccuStandard (New Haven, CT) and d12-chrysene, 8-naphthalene and 4-tert-octylphenol were purchased from Sigma-Aldrich (St. Louis, MO).

Table 2. Structural formula, log K_{ow}, molecular weight (MW), purity and detection ions of test chemicals used in four aqueous BCF tests.

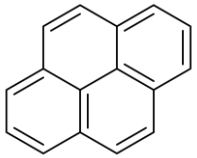
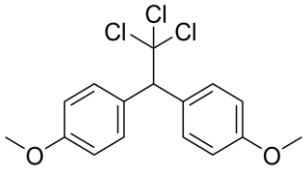
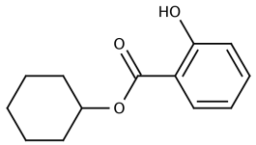
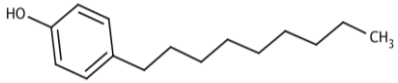
Test Chemicals [CASN]	Structure	Log K_{ow}	MW (g/mol)	Purity (%)	Ions (m/z)
Pyrene [129-00-0]		4.68, 5.08, 5.67 (US EPA, 2012; Hansch & Hoekman, 1995; Howard, 2017)	202.25	98	202
Methoxychlor [72-43-5]		4.88, 4.93 (US EPA, 2012; Hansch & Hoekman, 1995)	345.65	95	227,
Cyclohexyl salicylate [25485-88-5]		4.7, 4.87 (US EPA, 2012; Laue et al., 2014)	220.26	99	120, 138
4-n-nonylphenol [104-40-5]		5.76, 5.99, 6.1 (US EPA, 2012; Adolfsson-Erici et al., 2012)	220.35	99	179

Table 3. Structural formula, log K_{ow}, molecular weight, purity and detection ions of reference chemicals used in four aqueous BCF tests.

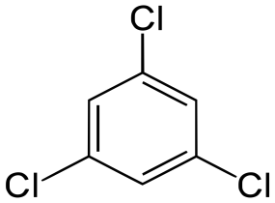
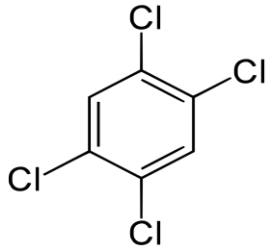
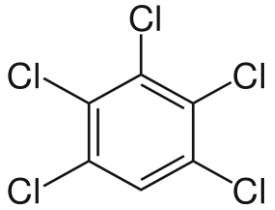
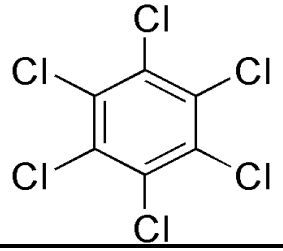
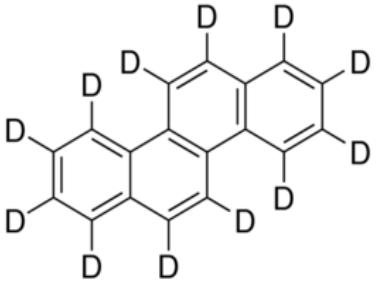
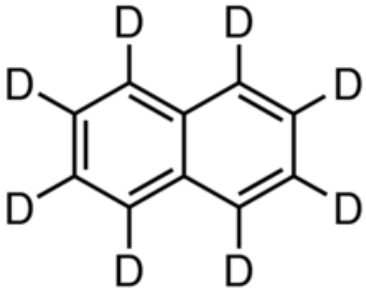
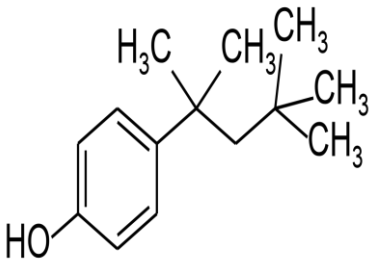
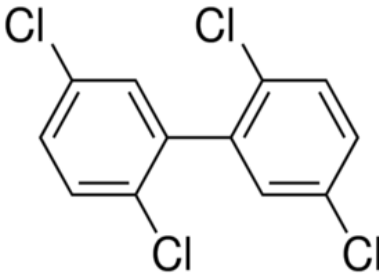
Reference Chemicals [CASN]	Structure	Log K_{ow}	MW (g/mol)	Purity (%)	Ions (m/z)
1,3,5-Trichlorobenzene [108-70-3]		3.93, 4.19 (US EPA, 2012; Hansch & Hoekman, 1995)	181.45	98	180
1,2,4,5-Tetrachlorobenzene [95-94-3]		4.57, 4.64 (US EPA, 2012; Hansch & Hoekman, 1995)	215.89	98	216
Pentachlorobenzene [608-93-5]		5.17, 5.22 (US EPA, 2012; Hansch & Hoekman, 1995)	250.34	98	250
Hexachlorobenzene [118-74-1]		5.73, 5.86 (US EPA, 2012; De Bruijn, Busser, Seinen, & Hermens, 1989)	284.78	98	284

Table 4. Structural formula, molecular weight (MW), purity and detection ions of chemicals used as internal standards in four aqueous BCF tests.

Chemicals used as internal standards [CASN]	Structure	MW (g/mol)	Purity (%)	Ions (m/z)
d12-chrysene [1719-03-5]		240.36	98	240
d8-naphthalene [1146-65-2]		136.22	98	136
4-tert-octylphenol [140-66-9]		206.32	97	207
PCB-52 [35693-99-3]		291.99	98	292

3.2. Fish

Juvenile rainbow trout (*Oncorhynchus mykiss*, approximately 10-20 g body weight) were purchased from Miracle Springs Hatchery and Trout Farm and Sun Valley Trout Park (Mission, BC). Fish were acclimatized in standard holding tanks supplied with municipal de-chlorinated water in a cold lab (approximately 12 ^{o}C) at Simon Fraser University (SFU) in Burnaby for at least 1-week prior to commencing the experiment. During the exposure phase of each BCF test, fish were housed in three 52-L semi-static tanks supplied with municipal de-chlorinated water at a temperature of 12 ^{o}C (SD = 2), with lighting controlled for a 12-hour light/dark period. Fish were fed commercial fish chow at a daily rate of 1% of the pre-experiment mean fish body weight. Fish were not fed on the first day of exposure and depuration due to potential stress from tank transfer. Fish food (1.5 mm EWOS Pacific Complete Feed for Salmonids, Cargill, Surrey, BC) contained 18.6% lipids, 46.6% protein, 32.4% of non-digestible organic materials, and 2.4% water. Average body weights of the fish during the depuration phase and lipid content for each experiment are summarized in Table 10.

3.3. Experimental methods

3.3.1. Dosing design

Preparation of sorbent

The chemical dosing reservoir for the exposure phase of each experiment consisted of poly (ethylene co-vinyl acetate) (EVA) beads ([24937-78-8], Sigma-Aldrich Canada Co.). Initially, 100 g of the beads were weighed out in a 500 mL Erlenmeyer flask. The beads were then dosed by adding stock solutions of the test and reference chemicals in methanol to the Erlenmeyer flask containing the EVA beads, stirring the mixture using a stir bar and stir plate for 1 hour and subsequently evaporating the methanol (Fisher Chemical) under a steady stream of nitrogen at approximately 10 psi. Due to low solubility in methanol, some of the reference chemicals (i.e., pentachlorobenzene and hexachlorobenzene) required additional manipulation to dissolve in methanol, therefore the chemical-methanol mixtures were subject to low heat using a hot plate (40 ^{o}C) and sonication using a sonicator (Branson 5510, 60-70 sonics at room temperature) until the chemicals were in solution for approximately 1-hour. Once

dissolved, the chemical solution was added to the beads in a glass Erlenmeyer flask. The Erlenmeyer flask was placed in a fume hood and covered with aluminum foil and stirred for 24-48 hours to allow partitioning of the chemicals to the EVA beads. Finally, the methanol was evaporated under a steady stream of nitrogen (approximately 10 psi) and the dry beads were then inserted into a metal mesh pouch. For experiments with the test chemicals cyclohexyl salicylate and 4-n-nonylphenol, 250 g of EVA was measured out instead of 100 g and divided into two wire mesh bags for the exposure phase.

Aqueous Pilot Study

To test the feasibility of the EVA dosing system, a pilot study was performed to examine the ability of the test and reference chemicals to partition from the EVA to the water at levels above GC/MS limits of detection (LOD) and below toxicity for rainbow trout. A wire mesh bag containing 100 g of EVA beads containing the test and reference chemicals were inserted in a Tetra 26312 Whisper EX 45 (30-45 Gallon) filter with a flow rate of 240 gallons per hour (GPH) on the side of a 52 L glass aquarium containing 40 L of ultrapure water at room temperature. Water samples were collected at thirteen different time points throughout the 14-day pilot study including a sample collected prior to adding the beads and filter to serve as a control for background contamination of test chemicals. Thirteen water samples were collected by transferring 400 mL of the tank water to a 500 mL glass volumetric flask using a 1 L glass beaker. Water was replaced to the 40 L mark of the tank after each sampling point. Water samples were recorded before the filter was inserted as a control for background contamination. After adding the filter, water samples were recorded at different time points for 14 days (i.e., days 0, 1, 3, 5, 7, 8, 11 and 13). Water samples were extracted using techniques outlined under Analytical methods and analyzed by GC/MS to determine the concentrations of the chemicals in the water over 14 days.

Sorbent phase loading and concentrations for BCF tests

The concentrations of chemicals required for the aqueous bioconcentration test were determined based on existing EVA-water partition coefficients (K_{EW}) from the literature and K_{EW}'s determined from the pilot experiment (Table 5) (George, Vlahos, Harner, Helm, & Wilford, 2011; Harner, Farrar, Shoeib, Jones, & Gobas, 2003). A loading efficiency of the chemicals into the EVA beads (0.33%) was measured from the pilot study and used to inform the dosing level requirements for the BCF tests to obtain

concentration of chemicals in fish above limits of detection (LOD) and below toxicity thresholds for rainbow trout (Appendix C). Furthermore, dosing concentrations were determined under the assumption that fish would absorb the hydrophobic chemicals rapidly from the water. Therefore, higher concentrations would be required to maintain constant concentrations in the water to compensate for removal of chemical by uptake in fish. Lastly, the dosing concentrations were estimated by incorporating approximate uptake and elimination parameters of the test and reference chemicals from literature into a bioaccumulation model (Gobas, 1993). The concentration estimates were then compared to information about the solubility and toxicity of chemicals to mitigate any adverse effects to fish that would render the tests invalid.

3.3.2. Experimental set-up & laboratory conditions

The EVA dosing methodology developed in the aqueous pilot study was applied to an experiment using juvenile female rainbow trout of approximately 10-20 g. Both the exposure and depuration phase were carried out in a cold room (approximately 12 ^{o}C) at Simon Fraser University with a 12-hour light/dark period. The exposure set-up consisted of twelve 52-L glass aquaria, each containing approximately 40 rainbow trout (Figure 1). Each tank was filled with 40-L of de-chlorinated water and aerated with two submerged air stones. Each tank was equipped with one Tetra Whisper EX 45 filter containing a mesh pouch with the EVA beads. In all tests, glass wool was added to the filter at the beginning of the exposure to act as a medium to aid in water quality maintenance. In four of the tanks, the filters contained beads dosed with one of the test chemicals (methoxychlor, pyrene and cyclohexyl salicylate, 4-n-nonylphenol), in another four tanks, the filters contained beads dosed with a mixture of one of the test chemicals and the four reference chemicals (Table 2) and the final four tanks contained filters holding clean EVA beads to serve as a control to identify potential differences in health and behavior between test and control fish.

Each experiment was carried out in two phases: exposure/uptake and depuration phases. During the exposure phase, fish were exposed for 7-days to test and reference chemicals using the EVA dosing design. For the depuration phase, the fish were transferred to clean flow-through tanks during which sampling (n=3 at each timepoint) took place over 14-days.

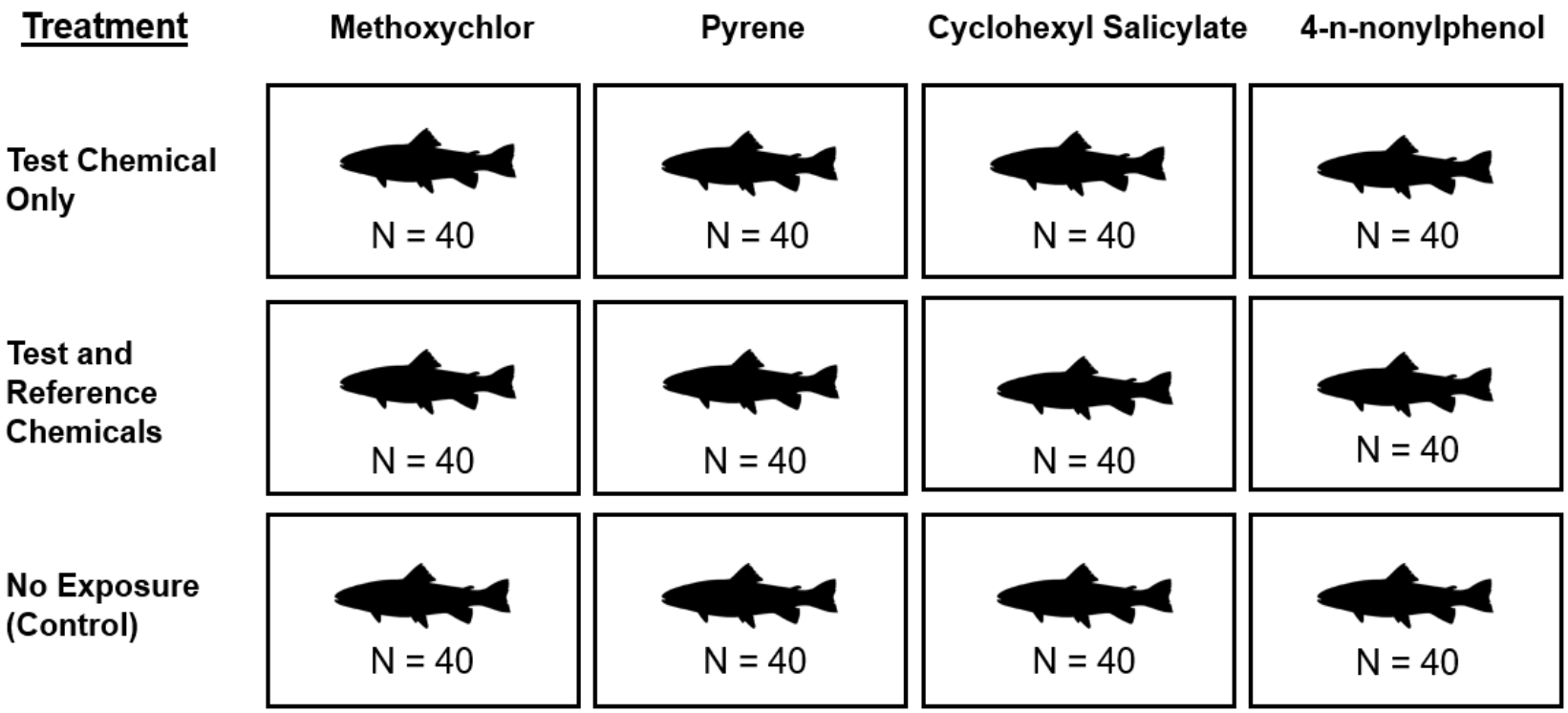


Figure 1. **A conceptual diagram illustrating the experimental set-up of the exposure phase for four BCF tests. The diagram shows the tanks used for each treatment (test only, test and reference chemical), as well at the control tanks. Each tank held approximately 40 rainbow trout during the exposure phase.**

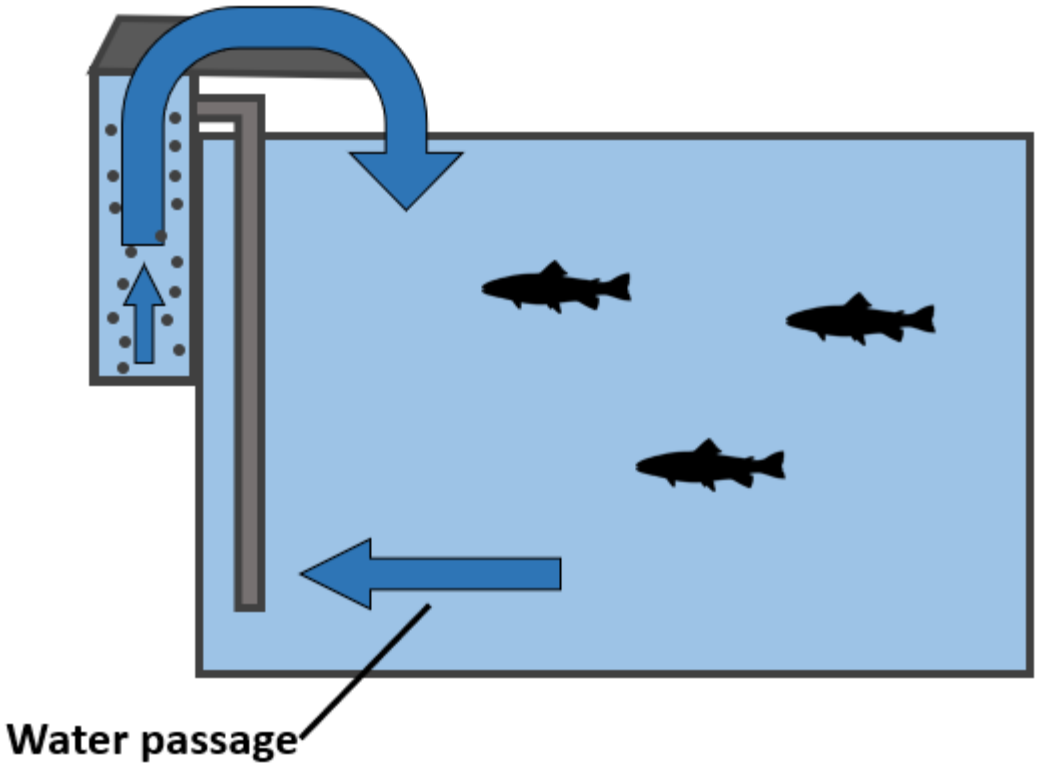


Figure 2. **A conceptual diagram describing the passive dosing system used during the exposure phase of each BCF test. Arrows indicate direction of water flow.**

3.3.3. Chemical exposure & sampling

Chemical exposure

After the water in the test tanks equilibrated with the test chemicals (i.e., approximately 48-hours after the dosed EVA beads were added to the filters), rainbow trout were transferred from holding tanks to the exposure and control tanks. Fish were randomly chosen and approximately 40 individuals were transferred to each tank.

Water temperature and water chemistry (pH, dissolved oxygen, and ammonia levels) were monitored daily throughout the exposure phase to ensure environmental conditions remained stable. To reduce ammonia levels, fecal waste was siphoned out of each tank daily, and the water removed from siphoning was replaced to the 40-L mark.

Water samples were collected to describe the concentrations of chemicals during the uptake portion of the tests. Water samples were collected (400 mL, n=3) at the beginning (day 0) and end of the exposure phase (day 7), then processed and analysed by GC/MS to determine the concentrations of chemicals in water at the two timepoints. On day 7, the exposure was terminated, and fish were transferred to their respective flow-through depuration tanks. Fish were sampled and then weighed at each sampling point throughout the depuration phase of each experiment.

Aqueous BCF tests for hydrophobic organic chemicals are challenging due to the difficulty maintaining constant concentrations of chemicals in water throughout the exposure (uptake) phase of the tests (Smith, Oostingh, et al., 2010). To address this limitation and test the feasibility of the passive dosing method, it was necessary to measure the concentrations of chemicals in water throughout the exposure phase. In addition, the initial concentrations of chemicals in water were used to determine the EVA-water partition coefficients for all test and reference chemicals.

Chemical depuration

During the depuration phase of the experiment, the rainbow trout were transferred from the exposure and control tanks to clean 52-L glass aquaria equipped with a de-chlorinated flow-through filter system. To better capture the depuration rates of the test chemicals, the sampling points occurred more frequently at the beginning of the depuration phase. At each sampling point, the fish were randomly sampled in triplicate (n=3) and removed from the experimental and control tanks. Fish were euthanized, according to SFU Animal Care Standards using an aqueous solution containing equal parts of Ethyl 3-aminobenzoate methane sulfonate (MS-222, Sigma-Aldrich Canada Co.) and sodium bicarbonate ($NaHCO_3$, Fisher Chemical) in 10 L of de-chlorinated water (0.3 g/L). Each fish was weighed, its length recorded and then wrapped in aluminum foil and frozen at -18 °C until being processed.

3.3.4. Analytical methods

All extracts were analyzed for test and reference chemicals using an Agilent 6890 gas chromatograph (GC) attached to an Agilent 5973N mass spectrometer (MS) detector (Agilent, Mississauga, ON). The MS was equipped with a programmable cool-

on-column injection port, a HP-5MS 5% phenyl methyl siloxane-coated column (30 m x 250 µm film thickness), and a fused-silica deactivated guard column (5 m x 530 µm x 250 µm film thickness) (Agilent, Mississauga, ON). The injection volume was 1 µL and the GC was programmed to inject at a temperature of 45ºC which was held for 1.5 minutes. Subsequently, a temperature ramp of 15 ºC/min brought the GC oven to a temperature of 150 ºC. The temperature was held at 150 ºC for 8.5 min, followed by a temperature ramp of 10 ºC/min for 27 min to a temperature of 285 ºC. The MS quantified the compounds of interest using selective ion monitoring (Table 2, Table 3 and Table 4) using an ion energy of 70 eV and an ion source temperature of 230 ºC.

Calibration curves for test and reference chemicals were created in hexanes, water and fish tissue and were used to measure concentrations in these media during the bioconcentration tests (Appendix A). To calculate the concentration of the chemicals detected by the GC/MS linear regression of the data points was used. Linear regression follows the equation:

$$y = b0 + bx \quad (36)$$

Where y is the peak area ratio (test chemical/internal standard); b0 and b are the intercept and slope of the regression line, respectively; and x is the concentration of the test chemical in the samples of interest in µg L^{-1} or µg kg^{-1}. The calibration standards were run with every analysis to account for instrument fluctuation and are summarized in Appendix A.

3.3.5. Sample processing

All sample preparation, extraction, clean-up and analysis were performed in a laboratory at Simon Fraser University (SFU) on Burnaby campus. To analyze the samples, the chemicals had to first be extracted from the medium in which they occurred (e.g. water, EVA, fish tissue). Different methods of extraction were used for different media. All fish tissue samples were stored in airtight Teflon-taped glass vials in a freezer (-18 ºC) and all water and EVA samples were stored in airtight glass bottles in a refrigerator (1.6 ºC). Stock solutions of internal standards, test and reference chemicals made up in Toluene were tightly capped and Teflon taped to reduce evaporation and stored in a refrigerator (1.6 ºC).

Glassware

All glassware and spatulas were detergent washed and either air dried or dried in an oven at 60 ºC (Fisher Scientific, Isotemp Oven, Model 655F), then rinsed three times with reagent grade hexanes (ACP chemicals) and dichloromethane (DCM, EMP Millipore Corporation) and air dried prior to sample processing.

Water samples

All water samples were extracted using a liquid-liquid extraction (LLE) procedure to isolate the hydrophobic organic compounds from aqueous samples in preparation for GC/MS analysis. Each water sample (400 mL) was transferred to a clean 500 mL volumetric flask and 20 µL of internal standard solution d8-naphthalene and d12-chrysene [100 ppm] made up in methanol were added. Samples were mixed and left to stand for 5 minutes and subsequently, 75 mL of reagent grade hexanes was added and left to stand for another 15 minutes at room temperature. Samples were extracted by shaking each volumetric flask by hand for 5 minutes and then alternating shaking and sonicating for another 5 minutes. Samples were placed in a fridge (1.6 °C) overnight to allow time for separation of the hexane and the water phase. The next day, the hexane layer was transferred to round bottomed flasks using Pasteur pipettes. The extracts were evaporated to approximately 5 mL using a rotary evaporator (model RE-47, Yamato Scientific Co., LTD) and blown down to approximately 1 mL under a steady stream of nitrogen (approximately 10 psi). Samples were quantitatively transferred to amber 2 mL glass vials with Teflon-coated screw caps (Agilent Technologies, Santa Clara, CA) and analyzed by GC/MS.

EVA extraction and analysis

Three samples (0.5 g) of EVA beads dosed for each tank were transferred to clean glass scintillation vials at the beginning of each experiment to determine the concentrations of the chemicals in the EVA and the dosing efficiency. DCM (8 mL) was added to each scintillation vial to dissolve the EVA. The vials were mixed by Vortex for 10 seconds and allowed to stand for 15 minutes. The vials were mixed again for 10 seconds and a 50 µL aliquot of the DCM was transferred to a new glass scintillation vial. Hexane (10 mL) was added to the vial and shaken on a vortex mixer (Baxter Scientific Products) for 5 seconds. One mL of the final solution was transferred to a 2 mL amber glass vial (Agilent Technologies) and 10 µL of internal standard (IS) solution made up of

d8-naphthalene, d12-chrysene, PCB-52 or 4-tert-octylphenol in hexane [100 µg/mL] was added prior to being analyzed by GC/MS.

Fish tissue extraction and clean-up

Sample preparation

To prepare samples for extraction, clean-up and lipid content analysis, frozen fish samples were thawed for 20 minutes prior to homogenization. Whole fish were individually blended in hexane and DCM rinsed 500 mL or 250 mL glass Bernardin canning jars (Walmart) using an Oster® blender and further homogenized using a polytron for 1-2 minutes. Between samples, the polytron homogenizer and blender blades were detergent washed, and rinsed with hexanes (3x) then DCM (3x) to reduce contamination. Following homogenization samples were placed in a freezer (-18 ^{o}C) prior to sample extraction.

Sample extraction and clean-up

All fish sample extraction and clean-up followed a QUEChERs method adapted from Molina Ruiz et al (Molina-Ruiz et al., 2014). Homogenized fish were thawed for 45-60 minutes at room temperature in a dark area. A sample (1.0 g) of homogenate was transferred to a 50 mL Falcon^{TM} conical centrifuge tube (Fisher Scientific). Internal standard solution (40 µL of 100 ppm d8-naphthalene, d12-chrysene and PCB-52 in hexane) made up in hexane was added to each sample except procedural blanks and mixed in a vortex shaker for 30 seconds. Milli-Q water (5 mL) and 10 mL of acetonitrile (HPLC grade ACN, Fisher Chemical) was added to each sample and each tube was shaken vigorously by hand for 1 minute. The samples were then left to stand for 15 minutes at room temperature.

For the extraction phase, 6.5 grams of a salt mixture (8 parts anhydrous magnesium sulfate ($MgSO_4$, Fisher Chemical), 2 parts sodium chloride (NaCl, ACP Chemicals, Montreal, QC), 2 parts sodium citrate dihydrate ($Na_3C_6H_5O_7$: H_2O, Sigma-Aldrich),1-part sodium citrate dibasic sesquihydrate ($Na_2HCit:3H_2O$, Sigma-Aldrich) was added to each centrifuge tube for salt-induced phase partitioning and shaken by hand for 1 minute immediately after salt addition to avoid salt agglomeration. Reagent grade chloroform ($CHCl_3$, 1 mL, ACP Chemicals Inc. Toronto, ON) was added to each tube and then the samples were mixed by Vortex for 2 minutes. Samples were then centrifuged

using a Beckman Allegro 64R for 15 minutes at 8,700 relative centrifugal force (RCF). Six mL of the upper ACN layer was transferred to 15 mL falcon centrifuge tubes containing clean-up sorbents (1 g $MgSO_4$, 150 mg primary secondary amine (PSA) SPE bulk sorbent, 50 mg SPE bulk sorbent, silica, SAX and 50 mg Amino NH_2 SPE bulk sorbent (Agilent Technologies Inc) and the centrifuge tubes containing the sorbents and sample in ACN were shaken for 1 minute and then centrifuged for 5 minutes at 5,000 RCF. The upper 4 mL ACN layer was transferred to 15 mL Falcon™ conical centrifuge tubes (Fisher Scientific) containing 1 mL $CHCl_3$ and 100 mg of C_{18} and shaken for 1 minute before centrifuging again for 5 minutes at 5,000 RCF. The upper 4 mL of the samples were then transferred to 10 mL glass screw cap vials and acidified with 40 µL of 5% formic acid in ACN. The samples were shaken by hand for 1 minute and evaporated to dryness under a steady stream of N_2 (approximately 10 psi) at 40 °C using a heating block. The samples were reconstituted in 1 mL of hexanes and mixed by Vortex for 10 seconds before placing them in a freezer overnight.

The next day, 0.5 mL of hexane was transferred to autosampler vials and run through a microcolumn. The microcolumn was made up by inserting 0.5 g of glass wool to the bottom of a Pasteur pipette and adding 100 mg of C_{18} and 50 mg of the sorbent mixture. Each microcolumn was rinsed with 1 mL hexane and then 0.5 mL of each sample was poured through the microcolumn and eluted with 1 mL of hexane. The final 1.5 mL was evaporated to approximately 1 mL using N_2 and analyzed using GC/MS.

Derivatization

Due to the polarity of 4-n-nonylphenol, this test chemical required additional derivatization to be suitable for GC/MS analysis. 4-n-nonylphenol was analyzed for its trimethylsilyl (TMS) derivative. In this study, the derivatization technique employed was silylation using *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA), where the derivatization reaction replaces any active hydroxyl groups with a TMS group which is less polar and more favorable for GC/MS detection (Bowden, Colosi, Mora-Montero, Garrett, & Yost, 2009; Luo et al., 2017). To carry out the derivatization, the extracted sample was evaporated to dryness under a steady stream of N_2. Once dried, 100 µL of BSTFA was added to each dried extract, mixed by vortex and capped. The samples were placed on a heating block at 70 °C for 1-hour. The derivatized extracts were removed from heat and cooled to room temperature for 5 minutes. The samples were

evaporated to dryness under a steady stream of N_2 and the dried derivatized chemical residues were reconstituted in 0.5 mL of hexane and 1 µL analyzed by GC/MS.

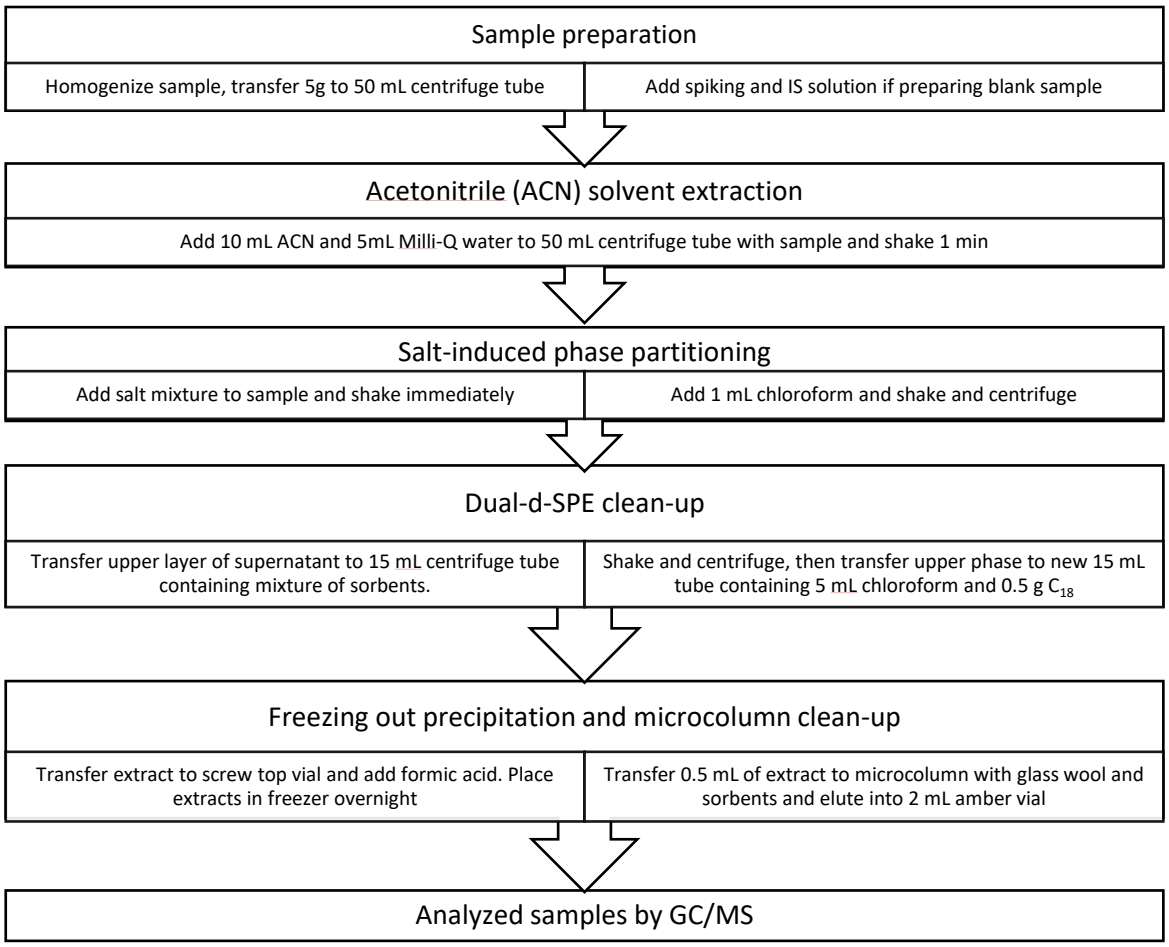


Figure 3. Flow chart of QUEChERs protocol for fish sample extraction and clean-up following a method adapted from Molina Ruiz et al. (Molina-Ruiz et al., 2014).

Determination of lipid content

A Bligh and Dyer assay (Bligh & Dyer, 1959) was used to gravimetrically measure the lipid content of whole trout homogenates. Lipid content (total lipid/ g tissue) was calculated as:

$$\%\ \text{lipid content} = (\text{total lipid (g)}/\text{tissue sample mass (g)}) * 100 \qquad (37)$$

Where,

total lipid (g) = weight of lipid in aliquot * volume of lipid layer/volume of aliquot

For the lipid analysis, 1-4 g of frozen fish homogenate was thawed and transferred to a 50 mL Falcon™ conical centrifuge tube. Once thawed, a $CHCl_3$ solution

with 50 mg/L butylated hydroxy toluene (BHT) was added and homogenized with a tissue homogenizer for 30 seconds. 1 x volume of $CHCl_3$, 2 x volume of methanol, 1 x volume of $CHCl_3$ containing BHT and 1 x volume of distilled H_2O was added. The samples were then homogenized for 1 minute and let stand for 5 minutes before centrifuging at 1500 rotations per minute (RPM) for 20 minutes. After centrifugation, the top alcohol layer was aspirated using a Pasteur pipette and discarded. The $CHCl_3$ layer was carefully removed using a Pasteur pipette and transferred to a graduated cylinder. An aliquot of the lipid-$CHCl_3$ layer was transferred (0.3-1.0 mL) to three disposable glass test tubes and the sample was evaporated to dryness under a gentle stream of N_2. The tubes were then weighed to determine the amount of lipid leftover.

3.3.6. Quality assurance and control

Extraction efficiency tests were conducted for each medium (e.g. EVA, water and fish homogenate). The extraction efficiency in each medium was determined by spiking test/reference chemicals and internal standards with 20 µL of 100 ppm to samples that were extracted under the same conditions as experimental samples. The samples were then analyzed by GC/MS. Test and reference chemicals introduced into 1 mL of hexane served as the standard of the matrix effect (n=3) which is defined as the difference between the response of the analyte in solvent compared to that in a biological matrix of equal concentration (Kwon, Lehotay, & Geis-Asteggiante, 2012).

Samples were randomly chosen and processed in batches of 8-10 fish including 1-2 procedural blanks (control fish). Procedural blanks followed the same extraction, clean-up and analysis procedures as the tissue and water samples. These blanks served as a measure of background contamination.

The limit of detection (LOD), defined as the mean blank level plus three standard deviation of the mean, was determined based on a standard curve made up in hexanes and is summarized for each chemical in Appendix C.

The limit of detection for fish tissue was 0.05 ppm for the reference chemicals and 0.025 ppm for the test chemicals. The limit of detection based on a standard curve extracted from water was approximately 0.0625 ppm. To account for chemical losses during sample extraction and analysis the process efficiency was calculated. Overall, the

process efficiency was greater than 90% for all the reference chemicals, and therefore no correction factor was applied to the concentration data (Appendix C). Concentrations for all chemicals in the control water and fish were below the limits of detection and quantitation and therefore, no corrections for background contamination was necessary.

Extraction efficiencies for test and reference chemicals were not significantly different from 100%, therefore no correction factors were applied when determining measured concentrations of chemicals from depuration experiments (Appendix C).

3.4. Calculations and Statistical Analyses

The following metrics were derived in this study from the pilot study and aqueous bioconcentration factor test:

- The sorbent-phase-water partition coefficient K_{EW} (using ethylene vinyl acetate, EVA) of reference chemicals and test chemicals.
    - K_{EW} was then compared to K_{OW} values found in the literature to identify if error could be reduced in k_M measurements.
- The depuration rate constants (k_T, d^{-1}) of the test and reference chemicals.
    - Depuration rate constants of test and reference chemicals were compared to determine whether the presence of conservative reference chemicals affects the depuration rate constants of the test chemicals.
- The biotransformation rate constants (k_M, d^{-1}) of four test chemicals.
- The kinetic BCFs and lipid normalized BCFs of four test and four reference chemicals.
- The growth-rate constants of the fish.
    - Growth rate constants were compared in treatment and control tanks to identify any treatment effects on fish growth.

All statistical analyses were conducted in JMP 13.1.0 (2017) and R 3.4.3 (R core team 2016).

3.4.1. Measuring and calculating the EVA-Water partition coefficient (K_{EW})

The concentrations of chemicals in EVA for each treatment were determined using methods outlined in section 3.3.5 under EVA extraction and analysis. The EVA-water partition coefficients for each chemical were determined using the log-transformed concentrations of the chemicals in water in each treatment tank at the beginning of each exposure (t=0) and the log of the concentration of chemicals in EVA beads using the following equation.

$$LogK_{EW} = LogC_{EVA} - LogC_w \quad (38)$$

Where, C_{EVA} and C_W are the mean concentrations of chemicals in EVA (µg/L) and water (µg/L) (n = 3) at the beginning of the exposure phase (Day 0), respectively. The standard error of the mean log K_{EW} is calculated as follows:

$$SE_{Log\ KEW} = \sqrt{((SE_{LogCEVA})^2 + (SE_{LogCw})^2)} \quad (39)$$

Where, SE_{KEW} is the standard error of the mean log K_{EW} and $SE_{LogCEVA}$ and SE_{LogCW} are the standard errors of the mean log concentration of chemicals in EVA and log concentration of chemicals in water (µg/L). Concentrations of chemicals in EVA were converted from µg/kg to µg/L by considering the density of the EVA beads (0.987 g/mL).

3.4.2. Determining the depuration (k_T) and biotransformation rate constants (k_M)

The depuration of the test and reference chemicals from the fish were assumed to follow first-order kinetics. Total depuration rate constants (k_T, d^{-1}) were derived from the linear regression of the natural logarithm of concentrations of chemicals in fish during the depuration phase versus time, using Equation 17 (Gobas & Lo, 2016; OECD, 2012).

Where, $C_{f,t=0}$ is the concentration of the chemical in fish at the beginning of the depuration phase (day 0) and C_f (t) is the concentration of the chemical in fish at any time point during the depuration phase. The depuration rate constants (k_T) were compared using an analysis of covariance (ANCOVA). A Tukey's Honestly Significant Difference (HSD) test was then used to determine whether the k_Ts were different between treatments ($p < 0.05$). A mass-based method was used to growth correct the

depuration rate constants (k_{TG}, d^{-1}). The concentrations of chemicals of each individual fish were multiplied by the corresponding fish weight to obtain the mass of test chemical per fish throughout the depuration phase. Then the natural logarithm of the mass was plotted against time for the depuration phase (Equation 33).

Metabolic biotransformation rate constants for the test chemicals (k_M in d^{-1}) were determined according to methods outlined in Gobas and Lo 2016 (Gobas & Lo, 2016). First, a linear regression was performed using the depuration rate constants of the non-biotransformed reference chemicals ($k_{T,R}$) versus $1/K_{EW}$ or $1/K_{OW}$ (Equation 34). The biotransformation rate constant of the test chemical was then found as the difference between k_T of the test chemical and the $k_{T,R}$ derived from the linear regressions using K_{OW} or K_{EW} (Equation 34), assuming that for non-biotransformable reference chemicals, $k_M = 0$.

3.4.3. Determining the Bioconcentration Factor (BCF)

The kinetic BCF's were calculated using two methods outlined in the theory section and then corrected for lipid-content.

In this study, steady-state conditions were expected to be achieved, as is the case in most tests using hydrophobic chemicals (OECD, 2012). Therefore, it is acceptable to use the uptake and elimination rate constants k_1 and k_T to derive the BCF_k, when steady-state conditions are not achieved during the uptake phase (OECD 305).

The BCF_K was calculated under the assumption that the chemicals behave according to first order kinetic processes (OECD, 2012). This method is acceptable whether or not a steady state concentration is achieved during the uptake phase (OECD, 2012).Steady-state is not necessary since the BCF is calculated at the ratio of the uptake rate constant (k_1) to the depuration rate constant (k_T) rather than using the ratio of the concentration of the test substance in the fish's tissue to the concentration of the test substance in the water as required to calculate the BCF at steady state (OECD, 2012). The kinetic method of calculating the BCF is only valid if uptake and depuration act approximately by first-order kinetics (OECD, 2012). Intermediate sampling points were measured throughout the depuration phase to assess the first-order kinetics assumption.

The kinetic bioconcentration factor (BCF_K) using methods from Gobas and Lo (2016) and the OECD 305 test guidelines for an aqueous BCF test (Gobas & Lo, 2016; OECD, 2012). The uptake rate constant (k_1, d^{-1}) was calculated using two methods outlined in the theory section (Equations 18 and 19). The calculated k_1 was then used to determine the BCF for each test and reference chemical.

Determining the growth dilution rate constant (k_G)

Growth dilution rate constants k_G (d^{-1}) were determined from measured fish weights over time as the slope of the linear regression of the natural logarithm of 1/weight (g) versus time (day) (OECD, 2012) (Appendix B).

Determining the lipid normalized BCF (BCF_{KL})

Lipid analysis was conducted on fish samples removed at the beginning (day 0 of depuration), middle (day 7 of depuration) and end of the depuration phase (day 14) of each experiment. At least 30 fish were analyzed for lipid content per BCF test. A mean lipid content value was determined from each BCF test and then used to normalize the BCF. To detect changes in lipid content throughout the depuration phase, the lipid content of fish was measured gravimetrically using a Bligh and Dyer assay (Bligh & Dyer, 1959) at the beginning middle and end of the depuration phase and calculated according to Equation 37. The lipid normalized BCF_{KL} can be derived from BCF_K and the measured lipid content of the fish as:

$$BCF_{kL} = BCF_k / L_n \quad (40)$$

Where, BCF_{KL} is the lipid-normalized kinetic BCF_k (L kg^{-1}) and L_n is the mean wet weight-based lipid content in fish. All BCF estimates were compared to identify the differences between each method of estimating BCF's.

Chapter 4. Results and discussion

4.1. Pilot experiment of EVA sorbent-phase dosing

The pilot experiment revealed that the dosing system was successful in generating approximately constant concentrations in chemicals, well above limits of detection (LOD), throughout a two-week period and reached equilibrium quickly (less than two days). Therefore, the EVA beads, prepared as described in 3.3.1, act sufficiently as a reservoir for hydrophobic organic chemicals in the absence of fish in the tanks.

Chemicals partitioning from the EVA beads to the water produced aqueous concentrations that satisfied necessary requirements to proceed with the planned study (Figure 4 and 5). First, the concentrations of chemicals in water were well above LOD and below effects levels for rainbow trout (Appendix D). Then, for all chemicals, the chemicals in the EVA and water reached an apparent equilibrium within approximately 48 hours after the dosed EVA was added to the tank. This confirmed that the dosing system is effective and hydrophobic organic test chemicals partition adequately into the water from the EVA as illustrated in Figure 4 and Figure 5.

The mean aqueous concentrations of methoxychlor, pyrene, cyclohexyl salicylate and 4-n-nonylphenol, from day 2 to the end of the experiment (day 14) were 5.6 µg/L (SE = 0.08), 2.1 µg/L (SE = 0.04), 146 µg/L (SE = 2.9) and 1.47 µg/L (SE = 0.1), respectively. The mean aqueous concentrations of the reference chemicals for 1,3,5-trichlorobenzene, 1,2,4,5-tetrachlorobenzene, pentachlorobenzene and hexachlorobenzene, from day 2 to the end of the experiment (day 14) were 8.7 µg/L (SE = 0.9), 11.9 µg/L (SE = 0.7), 7.1 µg/L (SE = 0.4) and 0.83 µg/L (SE = 0.03), respectively. The pilot study revealed that the concentrations of some chemicals in water (i.e., 1,3,5-trichlorobenzene and 1,2,4,5-tetrachlorobenzene) decreased slightly towards the end of the pilot study, indicating that the concentrations of chemicals in EVA beads may deplete over time. The depletion of concentrations of chemicals in EVA over time was considered in the determination of dosing concentrations for each exposure treatment.

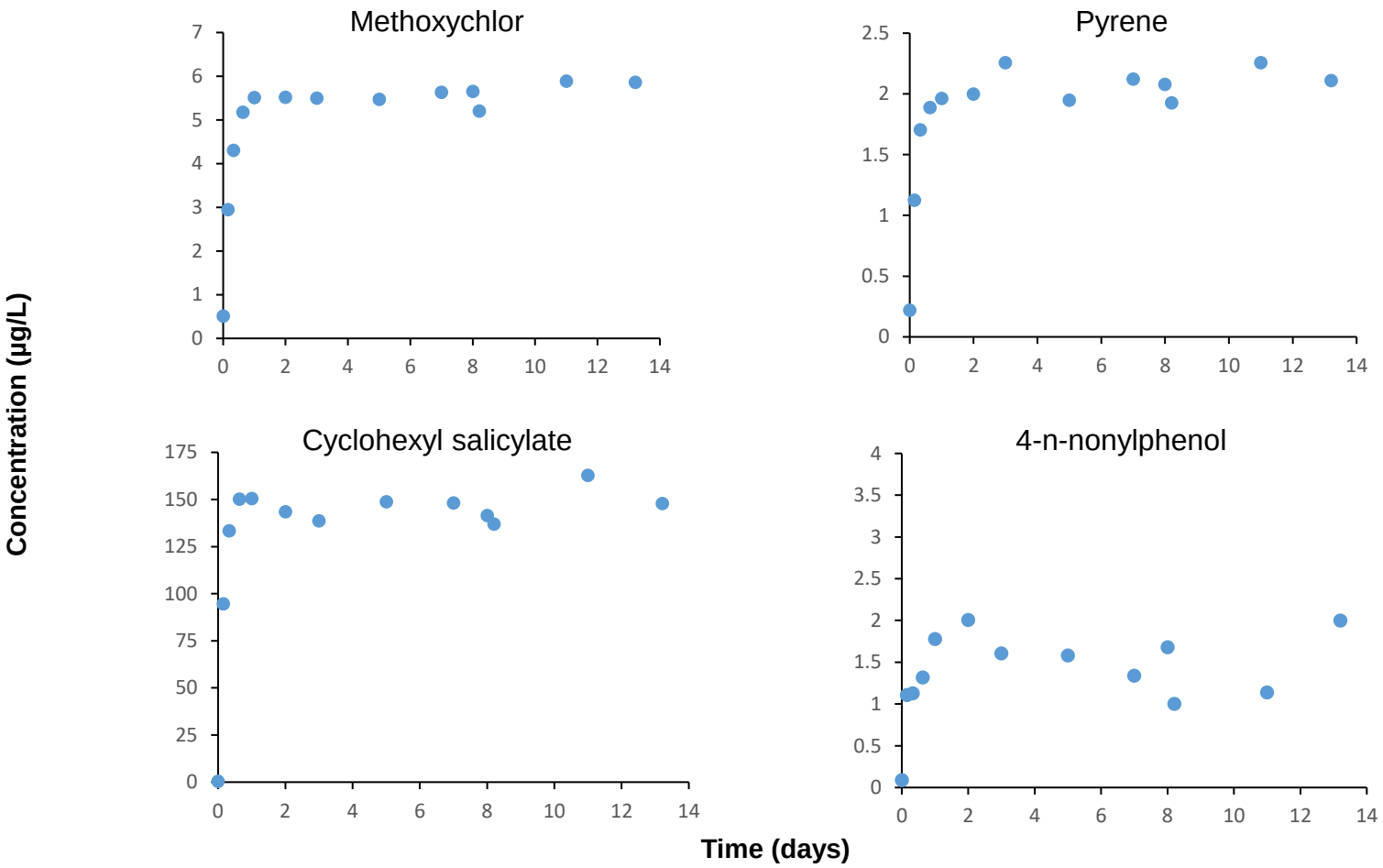


Figure 4. **Concentrations (µg/L) of test chemicals in water throughout the 14-day pilot study.**

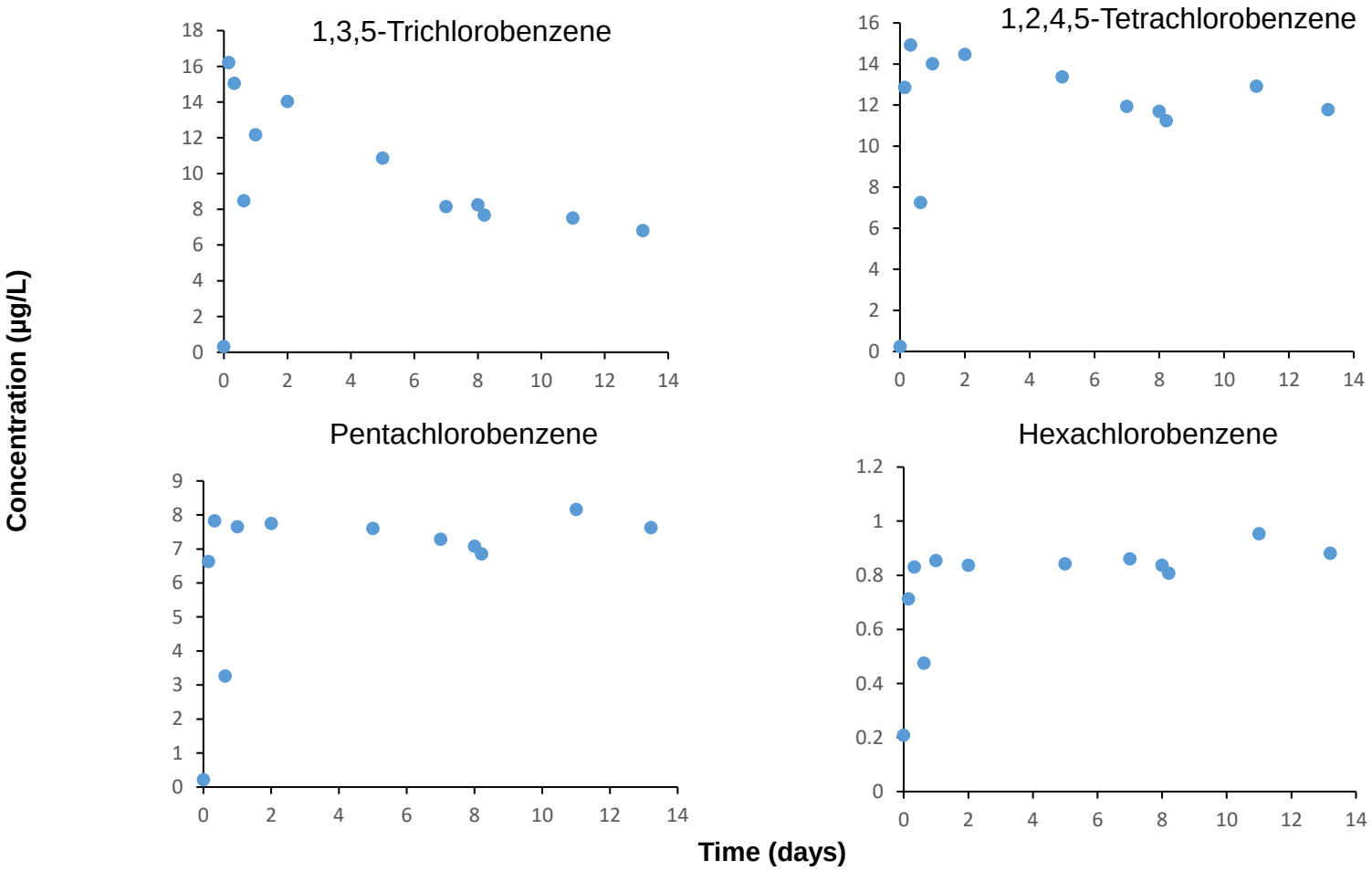


Figure 5. Concentrations (µg/L) of reference chemicals in water throughout the 14-day pilot study.

4.1.1. Passive dosing: partitioning of HOCs from EVA

The measured EVA-Water partition coefficients from the pilot experiment are summarized in Table 5. The Log K_{EW} values for almost all the chemicals mostly aligned with their respective peer-reviewed Log K_{OW} range (Table 5), except for cyclohexyl salicylate. An investigation to determine why cyclohexyl salicylate is an outlier is necessary to identify why the log K_{OW} of cyclohexyl salicylate is much greater than log K_{EW}. However, this is to be determined in future studies.

Typically, octanol-water partition coefficients (K_{OW}) are used to measure the hydrophobicity of chemicals. The higher the Log K_{OW}, the more hydrophobic a substance. However, the selection of values for the K_{OW} tends to contain substantial error due to differences in methodology among studies and experimental errors. Therefore, a major limitation of many bioaccumulation studies is using the appropriate K_{OW} values to determine a chemical's potential to bioaccumulate. In the determination of k_M, K_{OW} values may contain significant error due to reasons stated above. Alternatively, the K_{EW} and BCF can be measured simultaneously and may therefore reduce error in the k_M determination. This study provides measurement of a proxy for K_{OW}, i.e. the sorbent phase-water partition coefficient or EVA-water partition coefficient K_{EW}. The use of K_{EW} may reduce error in calculating biotransformation rate constants by using K_{EW} instead of K_{OW} (Equation 34b). In this test, the log K_{EW} values calculated from the pilot study were used to determine the amount of chemical required for dosing the beads in the fish experiment.

Table 5. Mean of the log concentrations of test and reference chemicals in EVA and in water measured during the pilot study. Respective log K_{EW}s and Log K_{OW}s from literature studies. The standard error of the mean is reported.

Chemical	Mean log C_{EVA} (± SE) µg/L	Mean log C_w (± SE) µg/L	Log K_{EW} (± SE)	Log K_{OW}
Methoxychlor	5.72 (0.02)	0.75 (0.01)	4.97 (0.02)	4.68, 5.08, 5.67 (US EPA, 2012; Hansch & Hoekman, 1995; Howard, 2017)
Pyrene	5.31 (0.06)	0.32 (0.01)	4.99 (0.06)	4.88, 4.93 (US EPA, 2012; Hansch & Hoekman, 1995)
Cyclohexyl salicylate	5.32 (0.06)	2.17 (0.01)	3.15 (0.06)	4.7, 4.87 (US EPA, 2012; Laue et al., 2014)
4-n-nonylphenol	4.99 (0.005)	0.15 (0.05)	4.84 (0.05)	5.76, 5.99, 6.1 (US EPA, 2012; Adolfsson-Erici et al., 2012)
1,3,5-trichlorobenzene	5.37 (0.04)	0.91 (0.03)	4.47 (0.05)	3.93, 4.19 (US EPA, 2012; Hansch & Hoekman, 1995)
1,2,4,5 tetrachlorobenzene	5.56 (0.07)	1.08 (0.01)	4.47 (0.07)	4.57, 4.64 (US EPA, 2012; Hansch & Hoekman, 1995)
Pentachlorobenzene	5.72 (0.06)	0.87 (0.01)	4.85 (0.06)	5.17, 5.22 (US EPA, 2012; Hansch & Hoekman, 1995)
Hexachlorobenzene	5.27 (0.05)	0.06 (0.01)	5.34 (0.05)	5.73, 5.86 (US EPA, 2012; De Bruijn, Busser, Seinen, & Hermens, 1989)

4.2. Bioconcentration Experiments

4.2.1. Water quality in exposure tanks

Water quality measurements are summarized in Tables 6-9. Only slight variations in water temperature, pH and dissolved oxygen were observed in all treatment and control tanks throughout the 7-day exposure phase. The mean temperature, pH and DO for the bioconcentration tests were 12.4 ºC (SD = 0.77), 6.8 (SD = 0.75) and 7.5 mg/L (SD = 0.75), respectively. Among all four BCF tests, the water temperature varied 4 ºC. However, within each treatment and control tank the water temperature varied less

than ± 2 ºC throughout the exposure and the concentration of dissolved oxygen remained above 60% saturation. Ammonia levels increased throughout the exposure, despite efforts to control levels such as siphoning out waste and replacing glass wool to tank filters. The average ammonia throughout the exposure phase was 3.6 ppm (SD =1.9) which is below BC water quality criteria for freshwater fish at a pH of approximately 6.8 and a temperature of approximately 12 º C (e.g., 22.5 ppm NH_3) (Ministry of Environment & Climate Change, 2018). A one-way analysis of variance (ANOVA) was performed followed by a Tukey-Kramer HSD test (95% confidence) to compare the means between the water quality parameters in the treatment and control tanks. The mean water temperatures (ºC) in the treatment tanks were slightly higher than in control tanks (Tukey Kramer HSD test: p = 0.025). Alternatively, there was no evidence of a difference between the mean pH (Tukey Kramer HSD test: p = 0.65) in treatment and control tanks. Finally, there was no evidence of a difference between the mean dissolved oxygen (DO) (mg/L) (Tukey Kramer HSD test: p = 0.11) in the treatment and control tanks. Based on requirements of the OECD 305 aqueous BCF testing protocols, the water quality measurements recorded throughout the exposure phase in this study were satisfactory (OECD, 2012).

Table 6. Water quality parameters in control and treatment tanks throughout the exposure phase (days = 7) of the methoxychlor BCF test. Mean and standard deviation of each parameter (± SD) are reported.

Tank	Temperature ± SD (◦C)	pH ± SD	Dissolved Oxygen ± SD (mg/L)	Ammonia (ppm)
Methoxychlor	11.9 (0.4)	5.8 (0.5)	8.1 (0.6)	4.1 (1.5)
Methoxychlor + Reference Chemicals	11.9 (0.4)	5.9 (0.4)	7.7 (0.6)	4.25 (1.6)
Control	10.9 (0.4)	5.3 (0.5)	8.5 (0.8)	3 (1.4)

Table 7. Water quality parameters in control and treatment tanks throughout the exposure phase (days = 7) of the pyrene BCF test. Mean and standard deviation of each parameter (± SD) are reported.

Tank	Temperature ± SD (◦C)	pH ± SD	Dissolved Oxygen ± SD (mg/L)	Ammonia (ppm)
Pyrene	12.8 (0.5)	7.1 (0.2)	6.8 (0.5)	3.3 (1.8)
Pyrene + Reference chemicals	12.6 (0.5)	7.0 (0.2)	6.8 (0.4)	3.3 (1.8)
Control	12.3 (0.5)	7.0 (0.2)	6.8 (0.4)	3.3 (1.8)

Table 8. Water quality parameters in control and treatment tanks throughout the exposure phase (days = 7) of the cyclohexyl salicylate BCF test. Mean and standard deviation of each parameter (± SD) are reported.

Tank	Temperature ± SD (◦C)	pH ± SD	Dissolved Oxygen ± SD (mg/L)	Ammonia (ppm)
Cyclohexyl Salicylate	13.1 (0.4)	7.2 (0.2)	7.1 (0.3)	2.4 (1.1)
Cyclohexyl Salicylate + Reference chemicals	13.1 (0.4)	7.1 (0.2)	6.7 (0.2)	2.4 (1.1)
Control	13.0 (0.2)	7.2 (0.2)	7.4 (0.4)	1.9 (1.1)

Table 9. Water quality parameters in control and treatment tanks throughout the exposure phase (days = 7) of the 4-n-nonylphenol BCF test. Mean and standard deviation of each parameter (± SD) are reported.

Tank	Temperature ± SD (◦C)	pH ± SD	Dissolved Oxygen ± SD (mg/L)	Ammonia (ppm)
4-n-nonylphenol	12.7 (0.6)	7.5 (0.2)	8.0 (0.4)	5.1 (2.2)
4-n-nonylphenol + Reference chemicals	12.7 (0.7)	7.3 (0.1)	7.9 (0.3)	4.9 (2.4)
Control	12.4 (0.8)	7.4 (0.1)	8.1 (0.3)	5.0 (2.2)

4.2.2. Fish

No differences in feeding behavior, or other effects of the treatments on behavior or external appearance were observed in the fish of the exposure and control groups in all BCF tests. There were three mortalities, two in the control tank and one in the treatment tank during the cyclohexyl salicylate exposure, though this was likely attributed to the transfer of fish from the acclimatization tanks to the exposure and control tanks. Overall, the mortality in both control and treated fish was less than 10% at the end of the test, satisfying the less than 10% mortality requirement for a valid BCF test, as per the OECD 305 protocol (OECD, 2012).

There was no evidence of a difference in growth rates or lipid content between the treatment and control fish (Table 10). Growth rate constants (k_G) were calculated using the OECD 305 method (OECD, 2012) as the slope of the natural logarithm of 1/weight (g) over time and are summarized in Table 10. The average weight and lipid content of treatment fish in all four BCF tests were 19.33 (SD = 13.4) and 4.4% (SD = 1.2). The average weight and lipid content of control fish in all four BCF tests were 18.49 (SD = 11.5) and 4.2% (SD = 0.9). There was no difference in mean weights of treatment

and control fish across all four BCF tests (Tukey's HSD test: $p = 0.58$). Similarly, there was no difference in mean lipid content of treatment and control fish across all four BCF tests (Tukey's HSD test: $p = 0.25$). Finally, there was no evidence of a difference between k_G, derived from the linear regression of fish weights over the depuration phase in test and control fish (ANCOVA: $p < 0.01$). The k_G's of the test and control tanks were compared to evaluate statistical significance (Tukey HSD Test: $p < 0.05$).

Table 10. The average weights of treatment and control fish (W_B), fish body lipid content (ΦBL), and growth dilution rate constants of test and control fish (k_G, d^{-1}) with corresponding p values from the linear regression of growth over the duration of the depuration phase of each BCF test. Standard deviation of the means are reported for W_B and ΦBL and standard error of the mean is reported for k_G.

Test	W_B (±SD) g	W range g	ΦBL (±SD) %	k_G (±SE) d^{-1}	*p* value for k_G
Methoxychlor					
Treatment	39.2 (18)	55	5.39 (1.5)	0.0025 (0.001)	0.07
Control	42.1 (15)	48	4.39 (1.1)	0.0047 (0.002)	0.07
Pyrene					
Treatment	21.8 (4.4)	18	4.79 (0.89)	0.0027 (0.0005)	<0.0001
Control	23.5 (4.5)	19	4.92 (0.81)	0.0011 (0.0006)	0.10
Cyclohexyl Salicylate					
Treatment	11.6 (2.4)	10	4.20 (1.0)	0.0029 (0.00095)	0.0034
Control	11.7 (2.6)	10	4.24 (0.6)	0.0018 (0.002)	0.27
4-n-nonylphenol					
Treatment	10.2 (2.5)	10.7	3.54 (0.8)	0.01 (0.006)	0.11
Control	10.1 (2.1)	8.5	3.46 (0.6)	0.0067 (0.008)	0.42

4.2.3. Concentrations of chemicals in water

For most chemicals in the four BCF tests, the concentrations of the chemicals in water at day 7 were lower than that at day 0 (Table 11 and Table 12, Figure 6 and Figure 7). The decline of concentrations of chemicals in water from the beginning to the end of the exposure phase is likely due to the addition of fish to the treatment tanks. The test and reference chemicals are hydrophobic in nature, therefore, the chemical uptake by fish may be faster than the chemical partitioning from the EVA into the water. Thus, a limiting aspect of this method is the delivery rate of the chemicals partitioning from the beads to the water. The decline of concentrations of chemicals in water throughout the exposure phase is an important consideration for aqueous studies using hydrophobic chemicals. For example, a primary assumption in standard protocols for calculating aqueous bioconcentration factors; is that the concentrations of chemicals in water remain constant throughout the exposure phase (OECD, 2012).

Table 11. Mean concentrations of test chemicals in water (C_W, µg/L) (n = 2) in test chemical (A) and test and reference chemical (B) treatment tanks at the beginning (t = 0) and the end (t = 7) of the exposure phase. Standard errors (±SE) of the mean concentrations of chemicals in water are reported.

Chemical	Initial C_W, t=0 (±SE) µg/L	Final C_W, t=7 (±SE) µg/L
Methoxychlor	A) 1.1 (0.02) B) 1.2 (0.07)	A) 0.62 (0.01) B) 0.61 (0.04)
Pyrene	A) 7.4 (0.08) B) 3.8 (0.1)	A) 0.53 (0.08) B) 0.05 (0.01)
Cyclohexyl salicylate	A) 488 (88) B) 509 (86)	A) 35.1 (1.9) B) 47.0 (8.1)
4-n-nonylphenol	A) 16.85 (0.9) B) 15.41 (1.3)	A) 4.07 (0.02) B) 4.12 (0.05)

Table 12. Mean concentrations of reference chemicals in water (C_W, µg/L) (n = 4) in four BCF tests at the beginning (t = 0) and the end (t = 7) of the exposure phase. Standard errors (±SE) of the mean concentrations of chemicals in water are reported.

Chemical	Initial C_W, t=0 (±SE) µg/L	Final C_W, t=7 (±SE) µg/L
1,3,5-Trichlorobenzene	1- 13.9 (0.6) 2- 36.2 (8.7) 3- 39.2 (2.0) 4- < 0.025	1- 3.24 (1.0) 2- 1.9 (0.09) 3- 2.0 (0.2) 4- < 0.025
1,2,4,5-Tetrachlorobenzene	1- 6.3 (0.05) 2- 6.3 (0.07) 3- 5.2 (1.8) 4- 20.8 (12.8)	1- 1.62 (0.11) 2- 0.46 (0.03) 3- 0.65 (0.05) 4- 39.8 (16.2)
Pentachlorobenzene	1- 2.2 (0.04) 2- 1.7 (0.02) 3- 1.7 (0.4) 4- 36.2 (12.4)	1- 0.66 (0.03) 2- 0.11 (0.01) 3- 0.45 (0.02) 4- 8.0 (0.4)
Hexachlorobenzene	1- 0.35 (0.01) 2- 1.62 (0.02) 3- 0.62 (0.02) 4- 4.55 (0.17)	1- 0.25(0.0) 2- 0.20 (0.0) 3- 0.38 (0.004) 4- 4.93 (0.93)

Numbered by BCF test (i.e., 1- Methoxychlor, 2- Pyrene, 3- Cyclohexyl salicylate, 4- 4-n-nonylphenol).

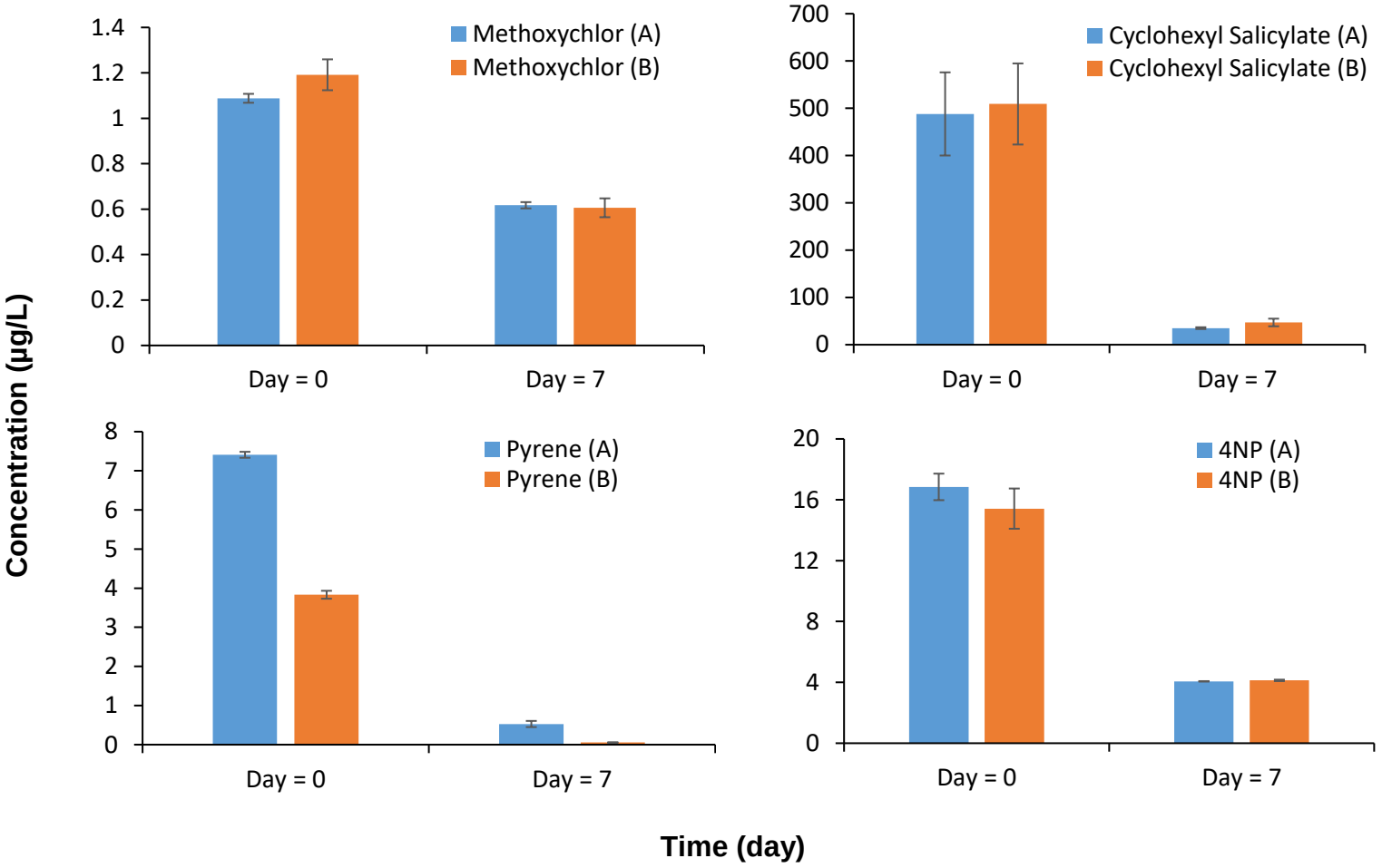


Figure 6. **Mean concentrations of test chemicals in water at the beginning and end of the exposure phase (day 0, day 7) for (A) test chemical and (B) test + reference chemical treatments (µg/L). The standard error of the mean concentrations of chemicals in water is reported (± SE).**

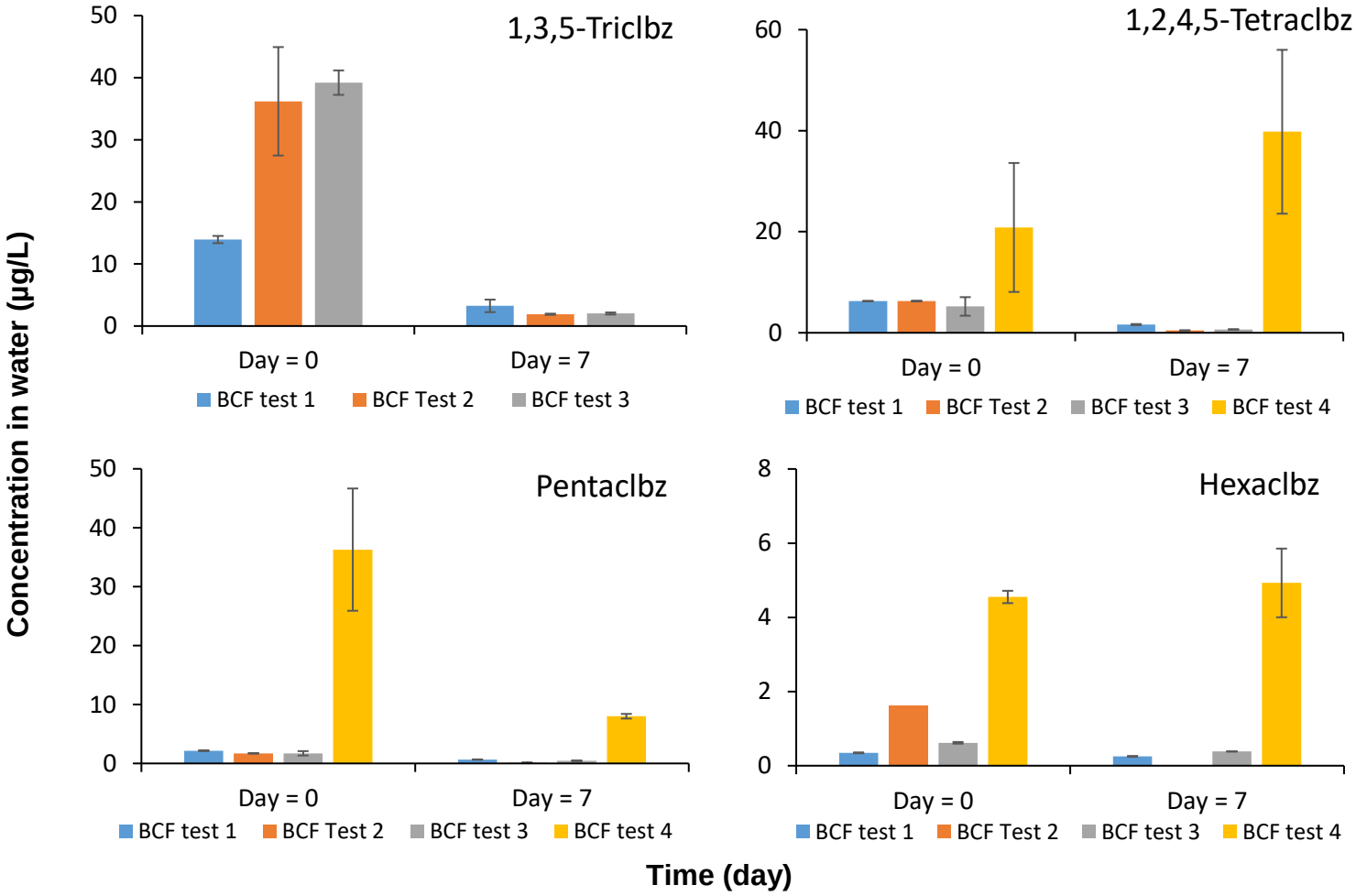


Figure 7. **Mean concentrations of reference chemicals in water (µg/L) at the beginning and end of the exposure phase (day 0, day 7) for each BCF test. The standard error of the mean concentrations of chemicals in water is reported (± SE).**

The minimized design of the current study only required water samples to be collected at the beginning and end of the exposure phase. It was assumed that the concentration of chemical in water (C_W) throughout the uptake phase was constant, and was therefore calculated as the mean of the initial and final concentration of chemical in water ($C_{W,\,t=0}$ and $C_{W,t=7}$, respectively). In an alternative approach, it was assumed that the concentrations of hydrophobic organic chemicals in water deplete according to a depletion constant (k_W). This concentration-time course is reasonably well described by an exponential decline described in Equation 20. For hexachlorobenzene (Log K_{OW} = 5.73), the concentrations of chemicals in water remained nearly constant throughout the exposure phase (Figure 8). Whereas for 4-n-nonylphenol (Log K_{OW} = 5.76), the concentration followed an exponential decline. Thus, for some hydrophobic chemicals, fish rapidly deplete the concentration of chemical in water initially, then establish a new steady state (i.e., new water concentration, C_W) in a day or two. However, for other chemicals, there is no observed reduction in C_W during the exposure phase. Thus, to consider variation between chemicals in the water, it would be beneficial to obtain water samples throughout the exposure phase and record a concentration-time profile of chemicals in water for BCF calculations. This may reduce errors associated with BCF's due to concentrations of chemicals in water that may not be constant throughout the exposure phase, other methods of measuring the BCF's may be favored.

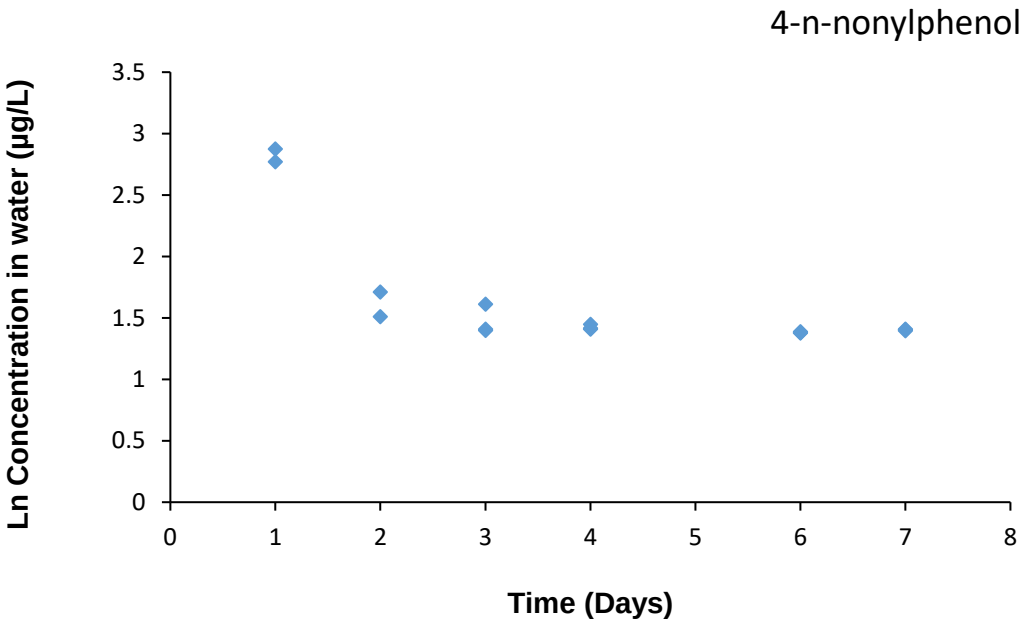


Figure 8. **Natural logarithm of the concentration of 4-n-nonylphenol (log K_{ow} = 5.76) in water (µg/L) throughout exposure phase of the aqueous bioconcentration test with rainbow trout (BCF test #4).**

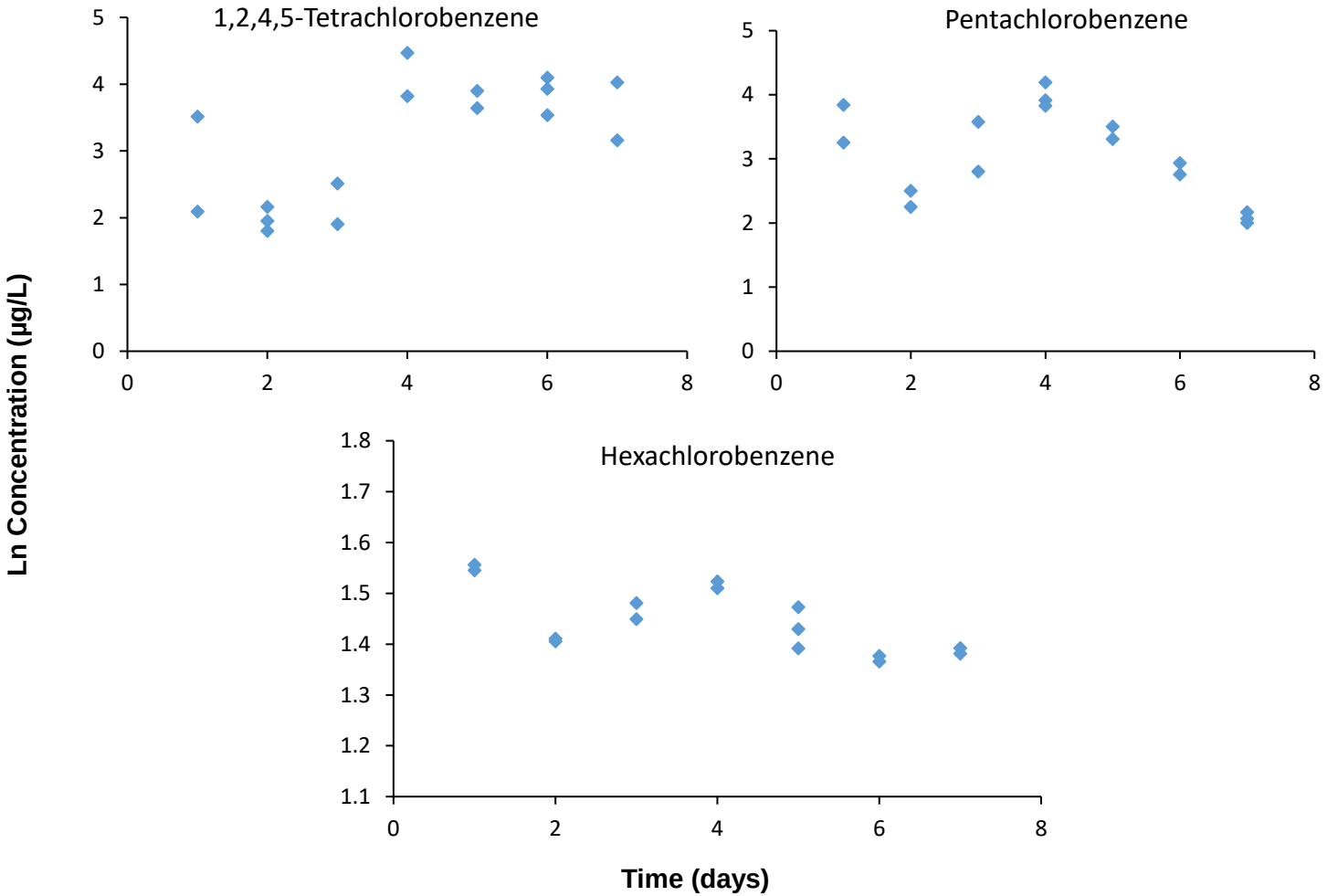


Figure 9. **Natural logarithm of the concentrations of three reference chemicals in water throughout the exposure phase of the aqueous BCF test with rainbow trout (BCF test #4).**

4.2.4. Passive dosing: EVA-water partitioning

Loading the EVA was variable between chemicals due to the solubility of each compound in the solvent (i.e., methanol) used to dissolve the chemicals prior to adding them to the EVA. In general, chemicals with lower log K_{OW}'s dissolved more readily in the methanol. However, chemicals with higher K_{OW} (pentachlorobenzene and hexachlorobenzene) required more manipulation (heat and sonication) to dissolve. For hexachlorobenzene, dosing concentrations had to be reduced for the substance to dissolve in methanol. Second, chemicals with high potential to biotransform required a lot of chemical to be added to the EVA to ensure concentrations of chemicals above detection limits in the fish throughout the depuration phase and in most cases, the concentrations in fish were only above detection limits until the halfway mark (day ~7). Despite some challenges with low solubility chemicals, the chemical loading process was feasible for all test and reference chemicals in the study.

The Log K_{EW} values reported in Table 13 and Table 14 were compared to the Log K_{OW} values found in the peer-reviewed literature to identify the relationship between measured log K_{EW} and empirical octanol-water partition coefficients (Log K_{OW}). The Log K_{EW} and Log K_{OW} exhibit a positive correlation ($R^2 = 0.67$). This is expected as both the Log K_{EW} and Log K_{OW} are measurements of a chemical's partitioning ability between a surrogate for lipids and water (i.e., EVA and octanol). The information in this study may contribute to future studies looking at the potential of K_{EW} as a replacement metric for Log K_{OW} for passive dosing experiments. With this technique, the reliance on a Log K_{OW} that has been determined using various protocols across laboratories could potentially be reduced with the measurement of a Log K_{EW} for aqueous BCF tests.

Table 13. Summary of means of the log C_{EVA} (µg/L), log C_W (µg/L) and Log K_{EW} at the beginning of the exposure phase (t=0) of four BCF tests. Results of test chemicals are separated by A) test only and B) test and reference chemicals. Standard error of the mean is reported for all values (± SE).

BCF Test #	Chemical	Log C_{EVA} (± SE) µg/L	Log C_W (± SE) µg/L	Log K_{EW} = Log C_{EVA}-Log C_W
BCF test 1	Methoxychlor (A)	4.14 (0.02)	0.04 (0.01)	4.11 (0.02)
	Methoxychlor (B)	4.28 (0.03)	0.07 (0.02)	4.20 (0.04)
	1,3,5-trichlorobenzene	4.75 (0.08)	1.53 (0.13)	3.23 (0.15)
	1,2,4,5-tetrachlorobenzene	5.16 (0.02)	0.80 (0.01)	4.36 (0.02)
	Pentachlorobenzene	5.28 (0.01)	0.23 (0.01)	5.05 (0.01)
	Hexachlorobenzene	5.16 (0.01)	0.20 (0.01)	4.97 (0.01)
BCF test 2	Pyrene (A)	5.06 (0.01)	0.87 (0.004)	4.19 (0.01)
	Pyrene (B)	4.73 (0.11)	0.58 (0.01)	4.14 (0.11)
	1,3,5-trichlorobenzene	4.83 (0.07)	1.14 (0.02)	3.69 (0.08)
	1,2,4,5-tetrachlorobenzene	5.15 (0.07)	0.80 (0.003)	4.35 (0.07)
	Pentachlorobenzene	5.26 (0.06)	0.33 (0.01)	4.93 (0.06)
	Hexachlorobenzene	4.64 (0.05)	0.46 (0.01)	5.10 (0.05)
BCF test 3	Cyclohexyl salicylate (A)	7.17 (0.08)	2.67 (0.08)	4.50 (0.11)
	Cyclohexyl salicylate (B)	7.30 (0.01)	2.69 (0.08)	4.61 (0.08)
	1,3,5-trichlorobenzene	5.21 (0.01)	1.59 (0.02)	3.61 (0.02)
	1,2,4,5-tetrachlorobenzene	5.32 (0.01)	0.67 (0.14)	4.65 (0.14)
	Pentachlorobenzene	5.34 (0.008)	0.22 (0.09)	5.12 (0.09)
	Hexachlorobenzene	5.02 (0.009)	0.21 (0.02)	5.23 (0.02)
BCF test 4	4-n-nonylphenol (A)	8.46 (0.006)	1.23 (0.02)	7.23 (0.02)
	4-n-nonylphenol (B)	8.47 (0.03)	1.18 (0.04)	7.28 (0.05)
	1,3,5-trichlorobenzene	5.17 (0.05)	< 0.025 µg/mL	< 0.025 µg/mL
	1,2,4,5-tetrachlorobenzene	5.29 (0.01)	1.56 (0.2)	3.73 (0.2)
	Pentachlorobenzene	5.23 (0.03)	0.9 (0.02)	4.3 (0.03)
	Hexachlorobenzene	4.79 (0.03)	0.68 (0.08)	4.11 (0.08)

Table 14. Mean Log K_{EW} obtained from four BCF tests and Log K_{OW} values obtained from peer-reviewed literature studies. Standard error of the mean is reported (± SE).

Chemical	Log K_{EW} (± SE)	Log K_{OW} (Ref)
Test		
Methoxychlor	4.16 (0.03)	4.68, 5.08, 5.67 (US EPA, 2012; Hansch & Hoekman, 1995; Howard, 2017)
Pyrene	4.17 (0.06)	4.88 , 4.93 (US EPA, 2012; Hansch & Hoekman, 1995)
Cyclohexyl salicylate	4.55 (0.09)	4.7, 4.87 (US EPA, 2012; Laue et al., 2014)
4-n-nonylphenol	7.85 (0.02)	5.76, 5.99, 6.1 (US EPA, 2012; Adolfsson-Erici et al., 2012)
Reference		
1,3,5-trichlorobenzene	3.51 (0.08)	3.93 , 4.19 (US EPA, 2012; Hansch & Hoekman, 1995)
1,2,4,5-tetrachlorobenzene	4.27 (0.11)	4.57, 4.64 (US EPA, 2012; Hansch & Hoekman, 1995)
Pentachlorobenzene	4.86 (0.05)	5.17, 5.22 (US EPA, 2012; Hansch & Hoekman, 1995)
Hexachlorobenzene	4.85 (0.04)	5.73, 5.86 (US EPA, 2012; De Bruijn et al., 1989)

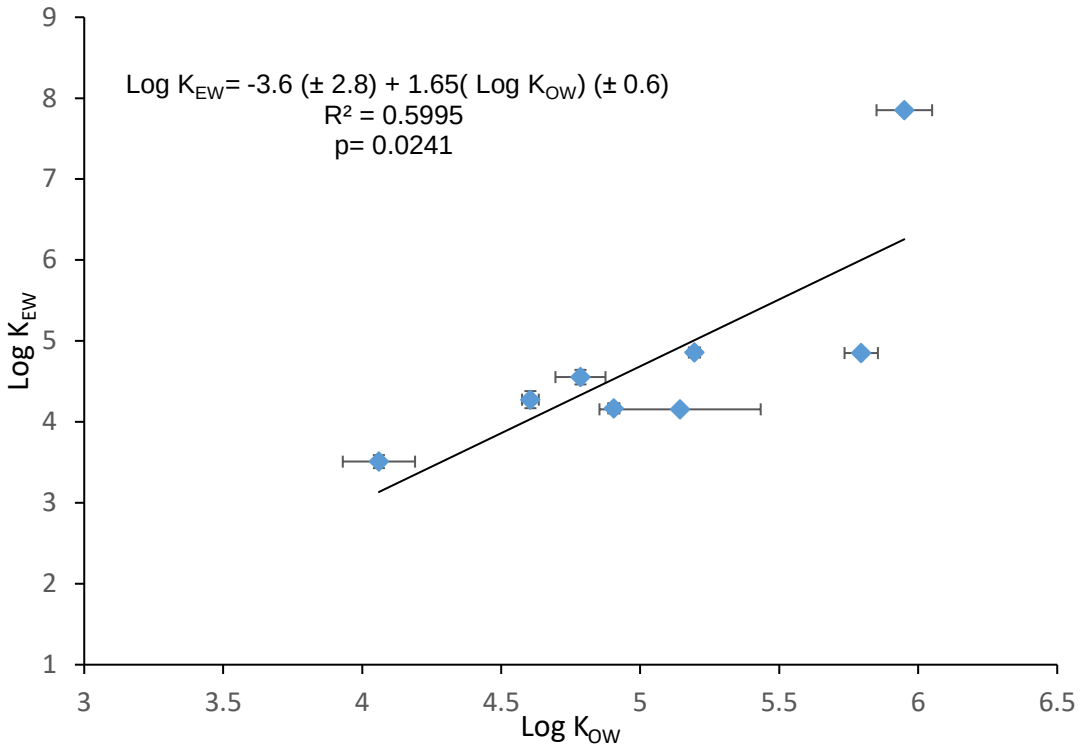


Figure 10. The relationship between the mean Log K_{EW} from four BCF tests (test chemicals N= 2, reference chemicals N=4) and mean Log K_{OW} from literature. The standard error of the means (± SE) is reported for measured Log K_{EW}'s and Log K_{OW}'s obtained from peer-reviewed literature studies.

4.3. Chemical depuration

4.3.1. Concentrations of chemicals in fish tissue

The concentrations of the test and reference chemicals in fish tissue (µg/g) declined over time throughout the depuration phases of the four BCF tests. The depuration rate constants (k_T, day^{-1}) for each chemical were calculated according to the OECD 305 test guideline as the natural logarithm of the concentration in fish over time (Equation 17).

4.3.2. Depuration rate constants (k_T, day^{-1})

The whole-organism depuration rate constants (k_T, d^{-1}), calculated from the natural logarithm of fish concentrations over time (Equation 17), are summarized in Tables 15-18.

The relationship between the depuration rate constants of the non-biotransformed reference chemicals to the reciprocal of the octanol-water partition coefficient ($1/K_{OW}$) showed a strong positive correlation for all BCF tests (Figure 17 and Figure 18).

Depuration rate constants of the reference chemicals (k_T) decreased with increasing hydrophobicity (represented as K_{OW}). The decline can partially be explained by the tendency of respiratory elimination via the gills to decrease with increasing chemical hydrophobicity in fish (Lo et al., 2016). Overall, the total depuration rate constants of the test and reference chemicals decreased with increasing hydrophobicity.

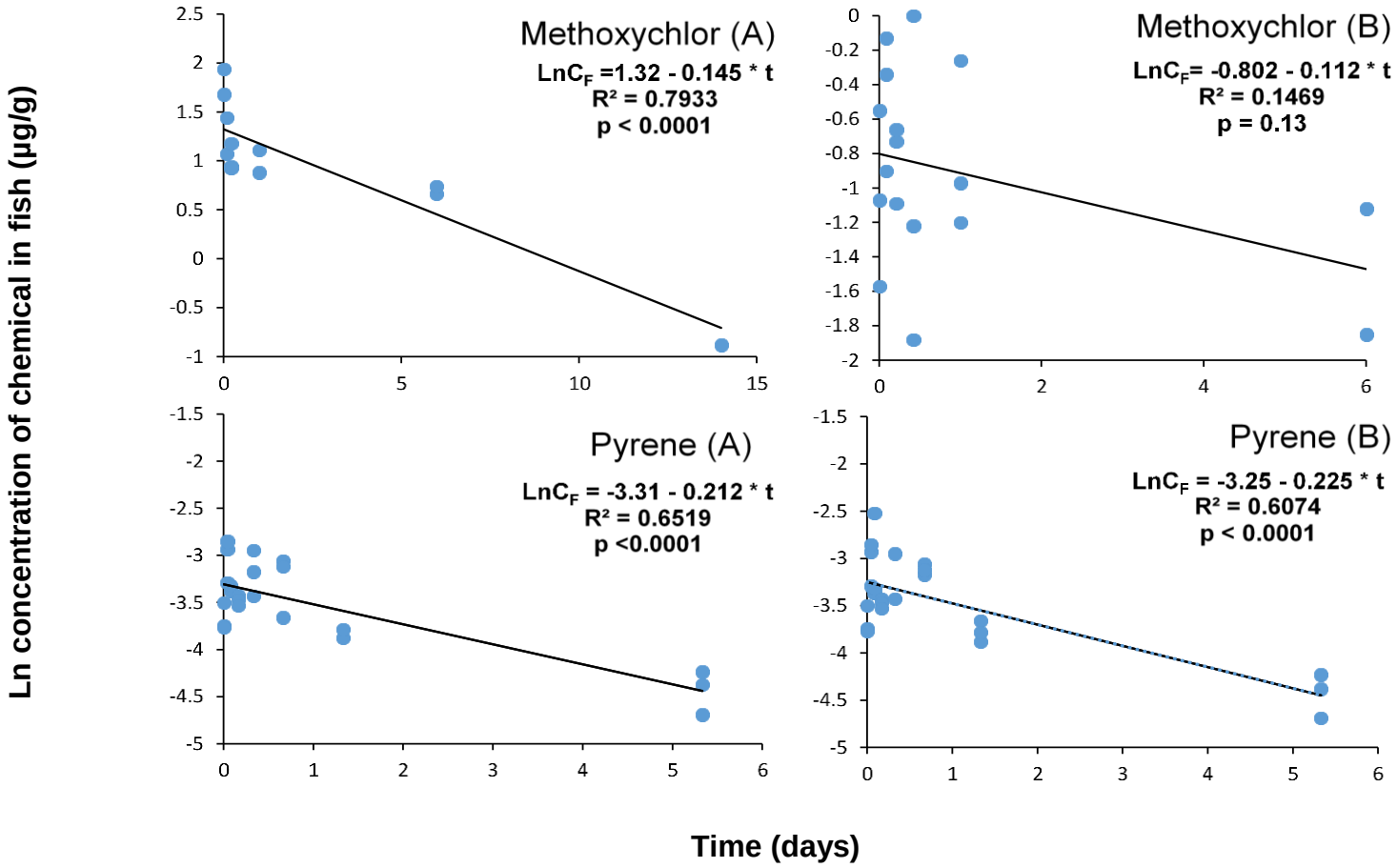


Figure 11. **The natural logarithm of the concentration of reference chemicals as a function of time in fish (µg/g tissue). Slopes of the linear regressions represent the total depuration rate constants (k_T, d^{-1}) for the test chemicals. Solid black line represents best fit line for individual data points. P values and R^2 values for each fitted line are reported.**

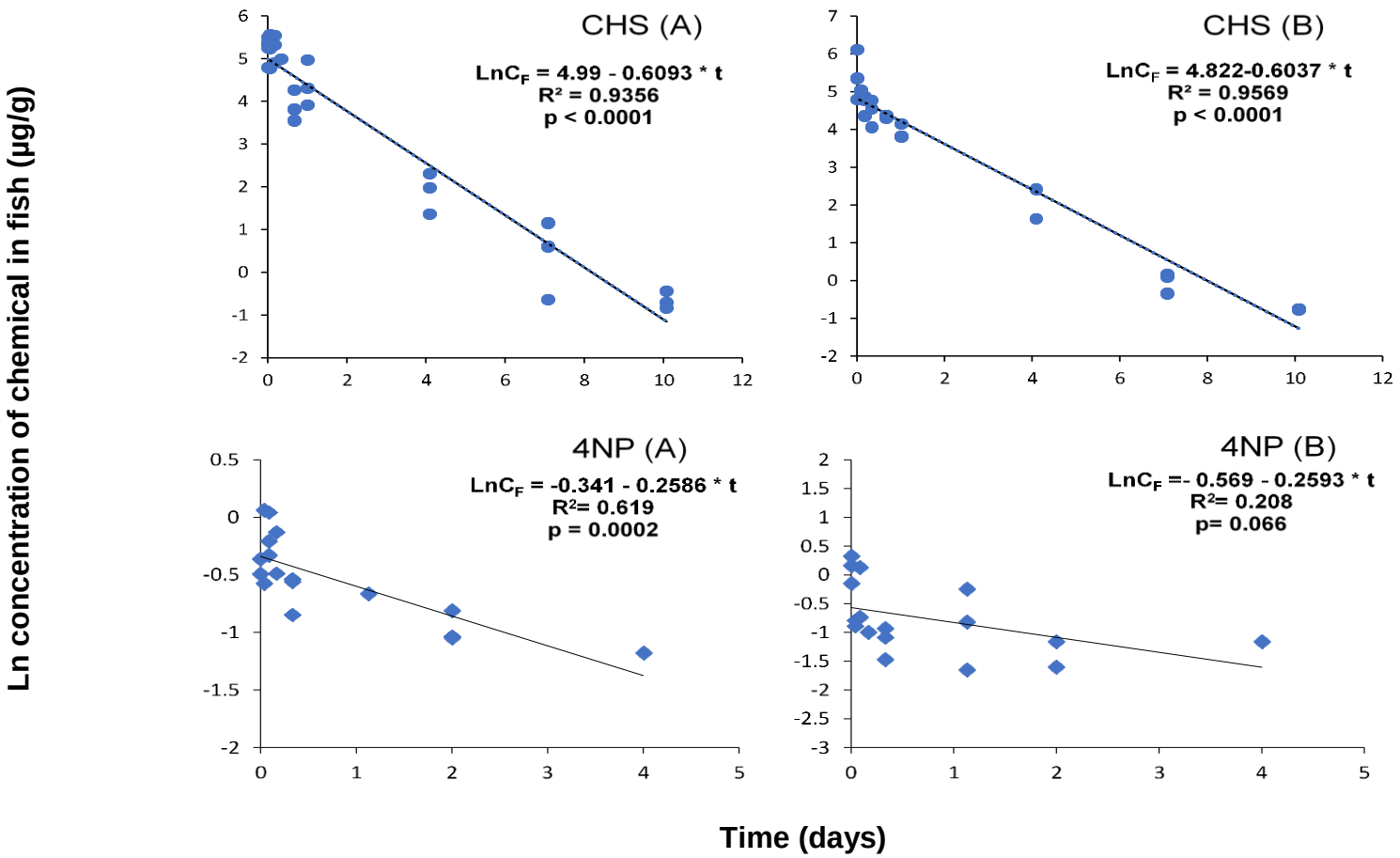


Figure 12. **The natural logarithm of the concentration of reference chemicals as a function of time in fish (µg/g tissue). Slopes of the linear regressions represent the total depuration rate constants (k_T, d^{-1}) for the test chemicals. Solid black line represents best fit line for individual data points. P values and R^2 values for each fitted line are reported.**

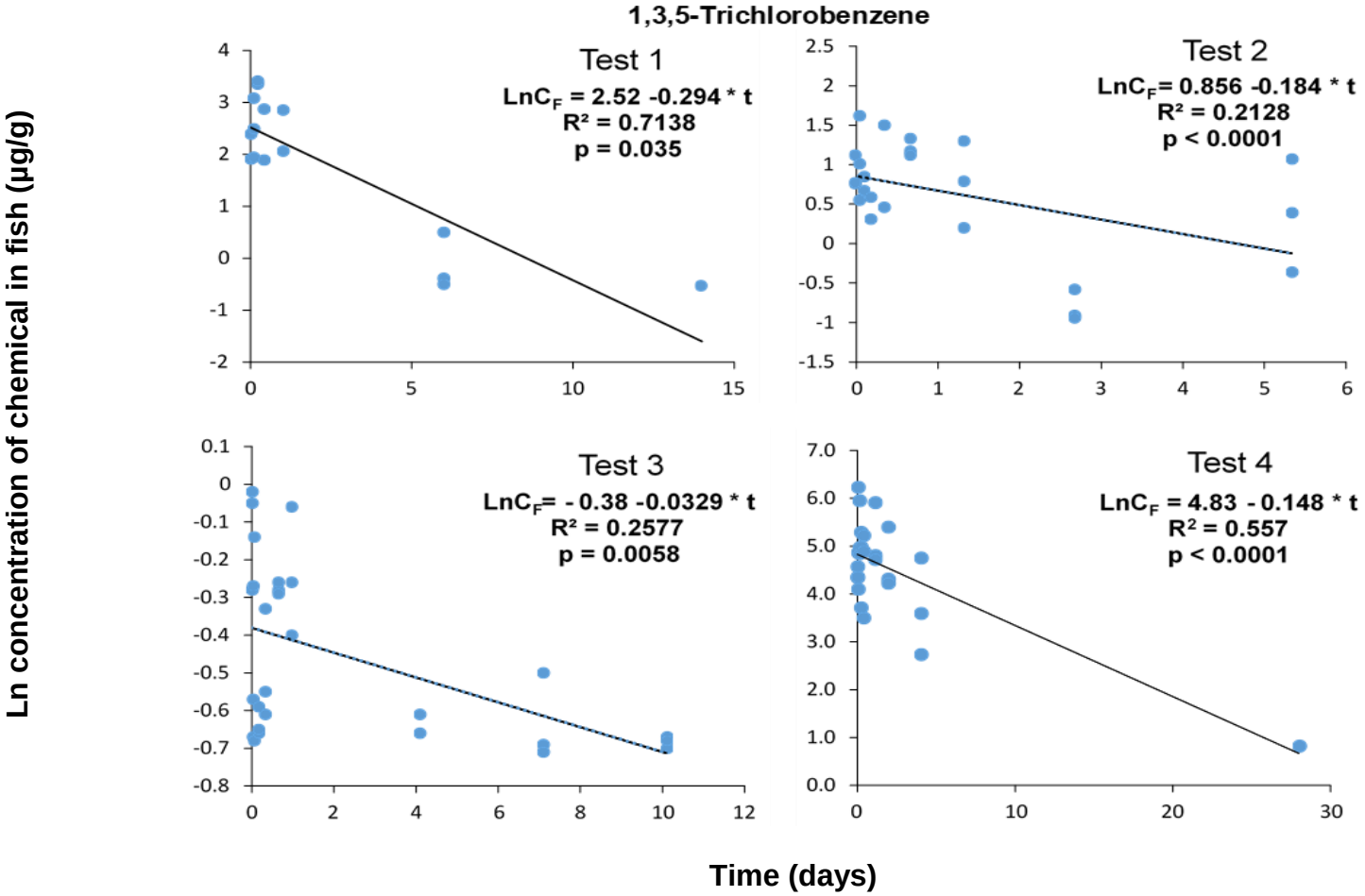


Figure 13. **The natural logarithm of the concentration of reference chemicals as a function of time in fish (µg/g tissue). Slopes of the linear regressions represent the total depuration rate constants (k_T, d^{-1}) for the test chemicals. Solid black line represents best fit line for individual data points. P values and R^2 values for each fitted line are reported.**

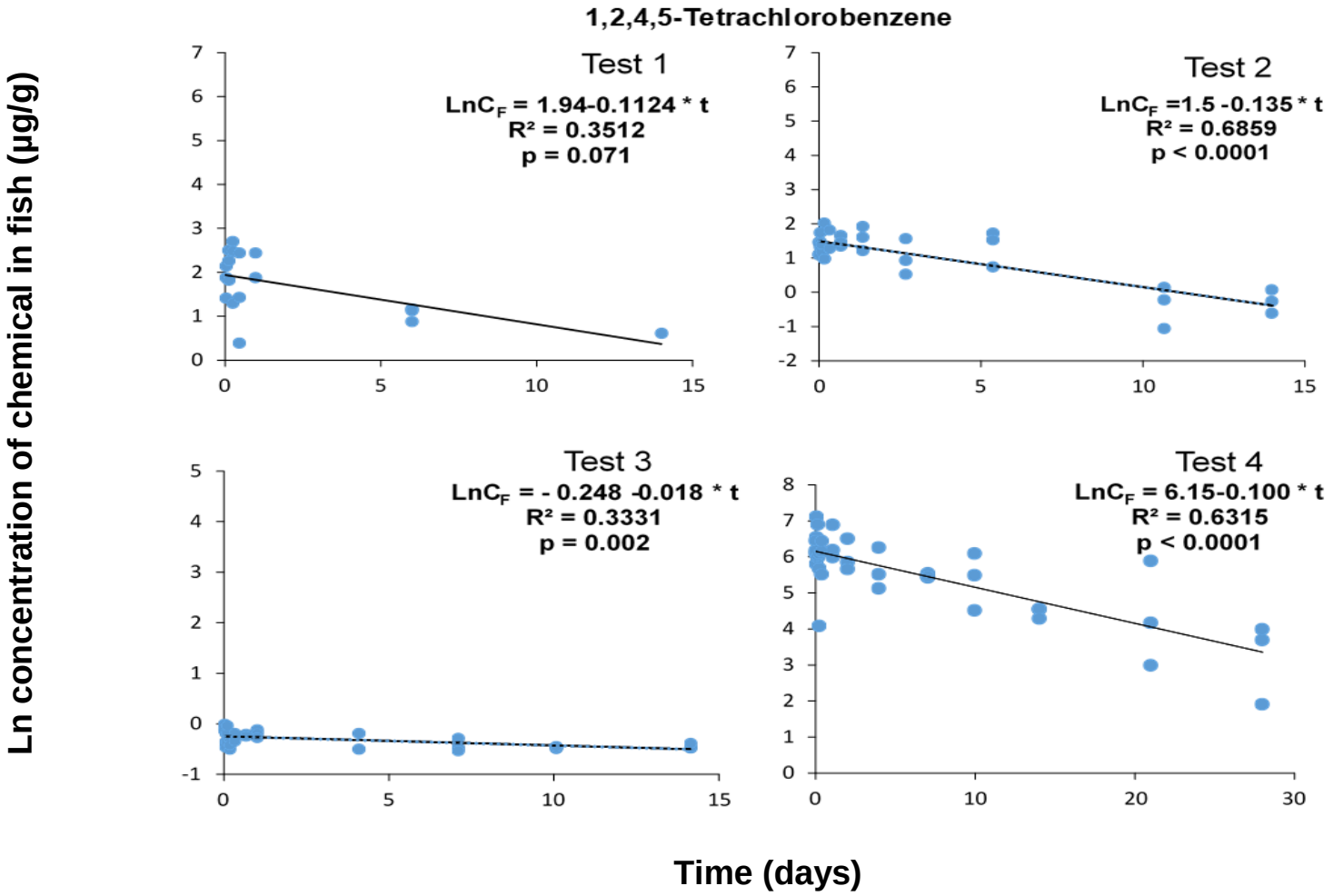


Figure 14. **The natural logarithm of the concentration of reference chemicals as a function of time in fish (µg/g tissue). Slopes of the linear regressions represent the total depuration rate constants (k_T, d^{-1}) for the test chemicals. Solid black line represents best fit line for individual data points. P values and R^2 values for each fitted line are presented.**

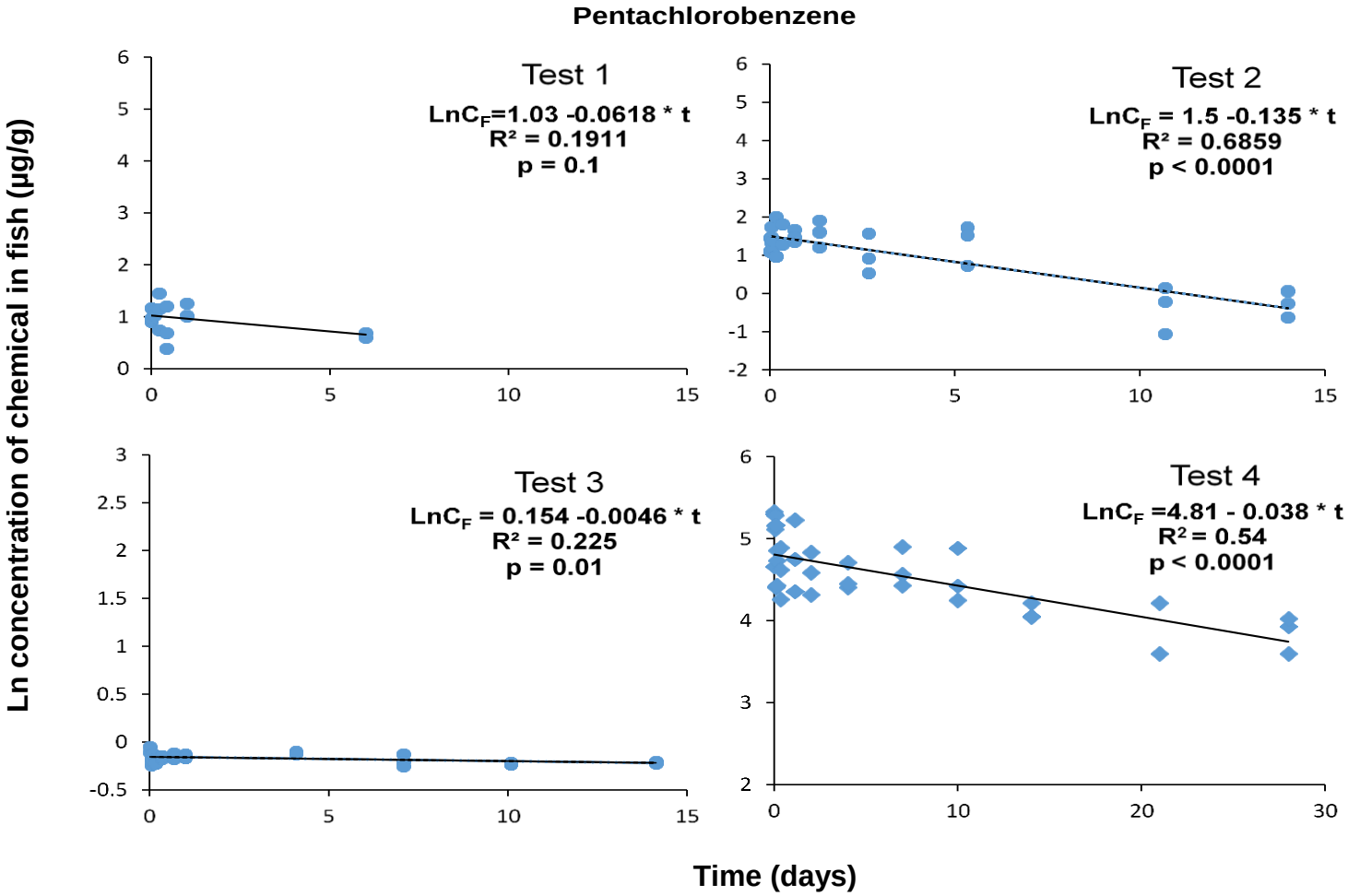


Figure 15. **The natural logarithm of the concentration of reference chemicals as a function of time in fish (µg/g tissue). Slopes of the linear regressions represent the total depuration rate constants (k_T, d^{-1}) for the test chemicals. Solid black line represents best fit line for individual data points. P values and R^2 values for each fitted line are reported.**

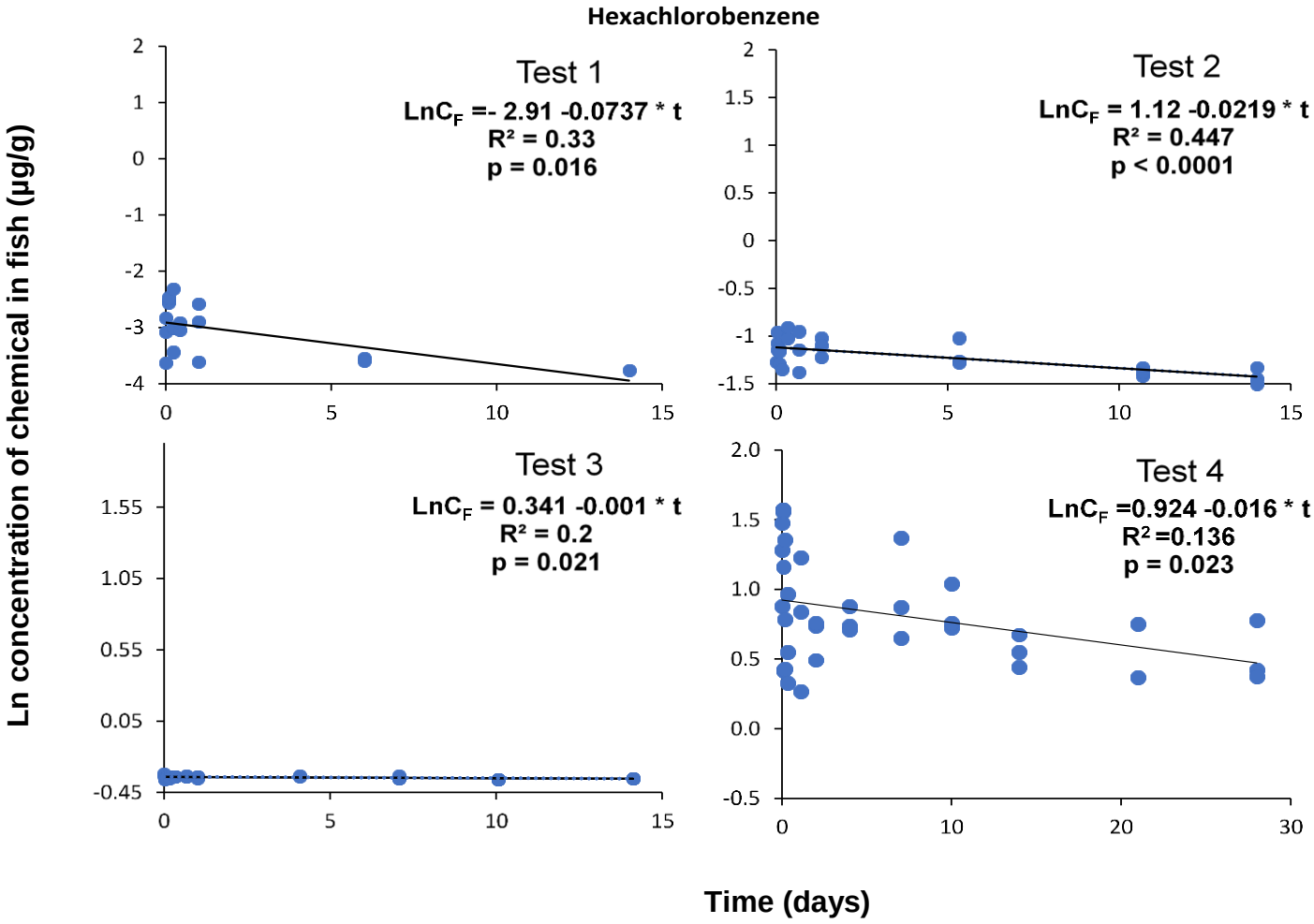


Figure 16. **The natural logarithm of the concentration of reference chemicals (µg/g tissue) as a function of time in fish tissue analyzed using GC/MS with major outliers removed. Slopes of the linear regressions represent the total depuration rate constants (k_T, d^{-1}) for the test chemicals. Solid black line represents best fit line for individual data points. P values and R^2 values for each fitted line are reported.**

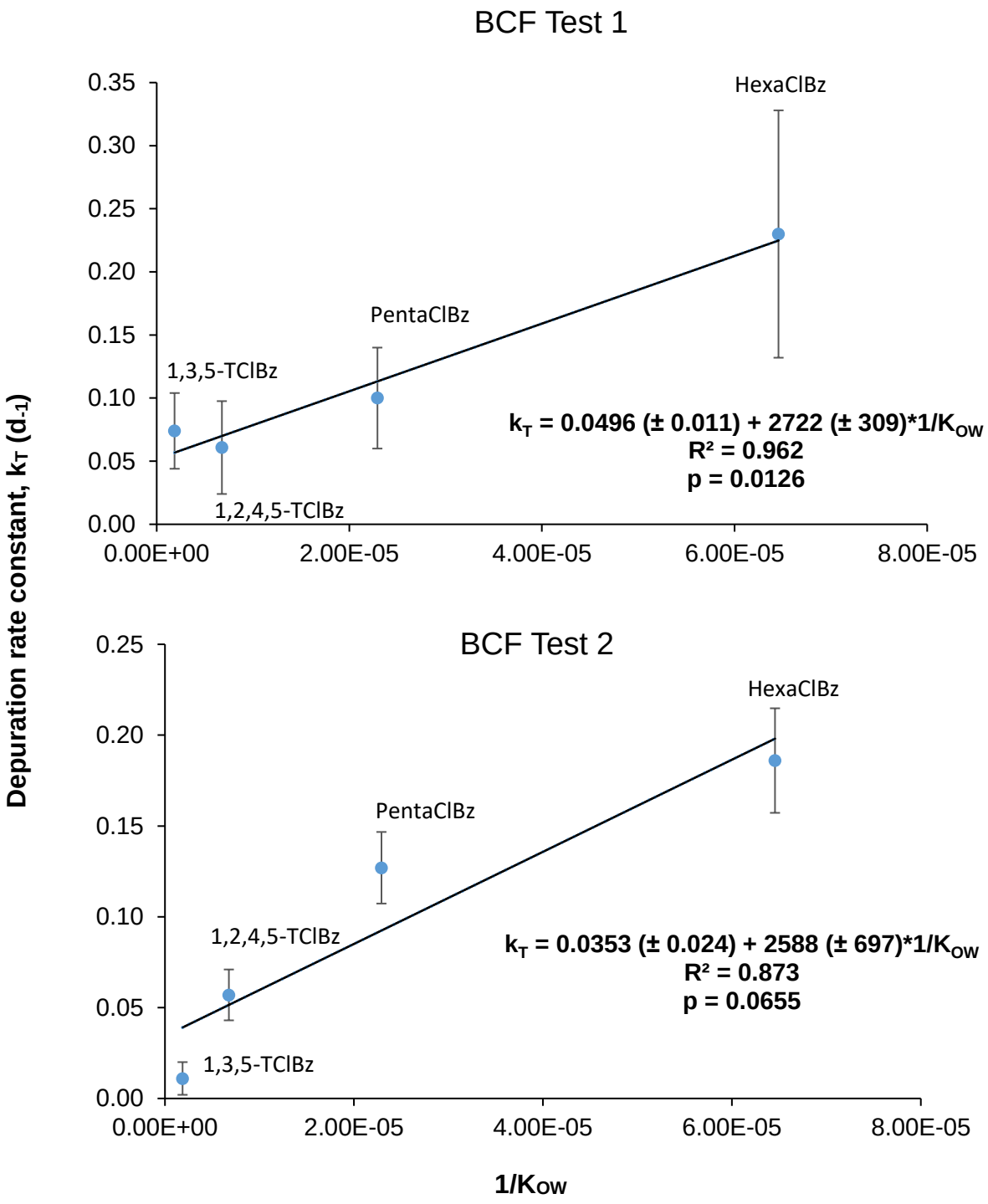


Figure 17. **The total depuration rate constants (k_T) of four reference chemicals as a function of the reciprocal of their respective octanol-water partition coefficients (1/K_{OW}). Error bars represent standard error of the mean depuration rate constants. The line represents a linear regression of k_T and 1/K_{OW} (N = 4).**

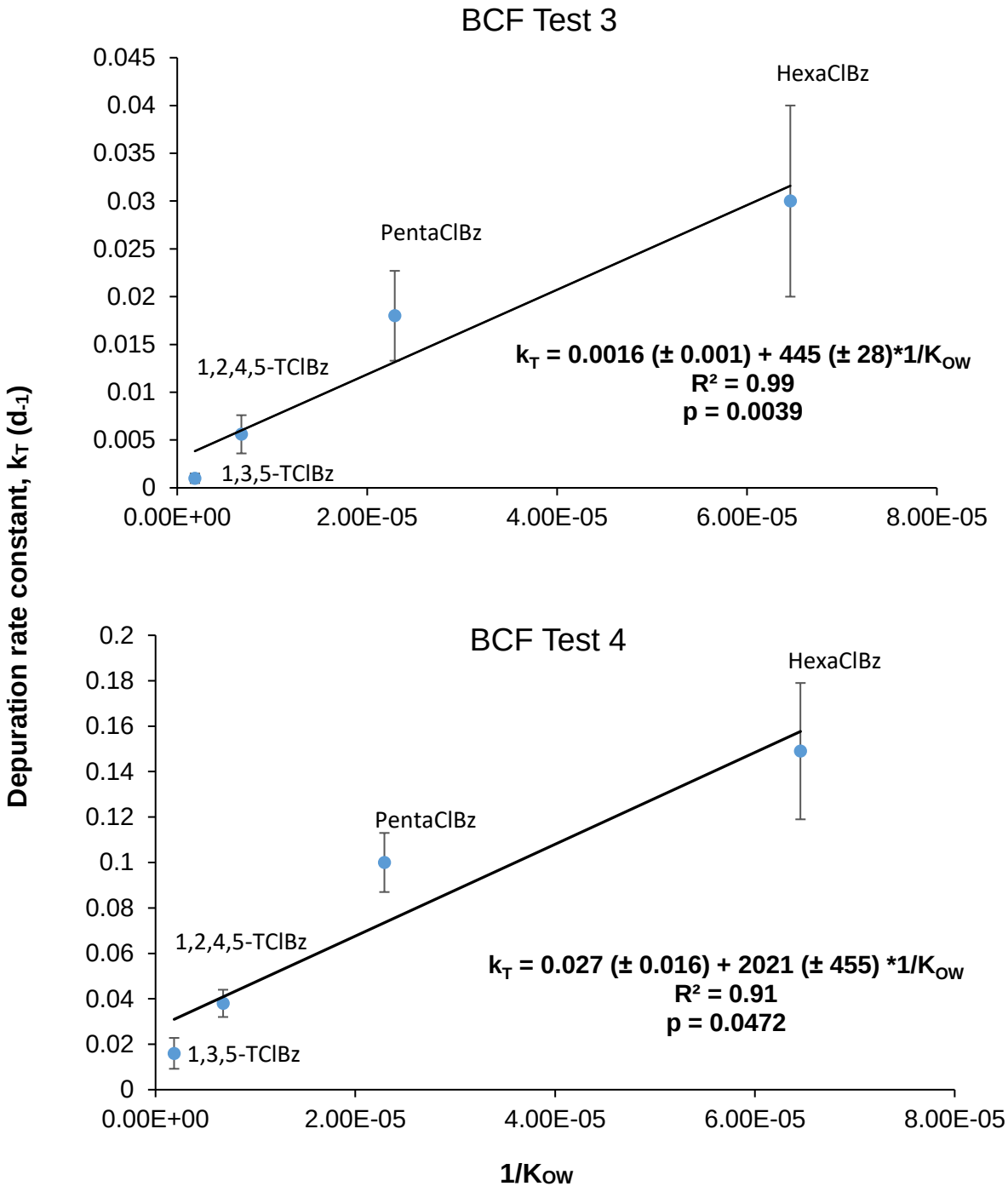


Figure 18. **The total depuration rate constants (k_T) of four reference chemicals as a function of the reciprocal of their respective octanol-water partition coefficients (1/K_{OW}). Error bars represent standard error of the mean depuration rate constants. The line represents a linear regression of k_T and 1/K_{OW} (N = 4).**

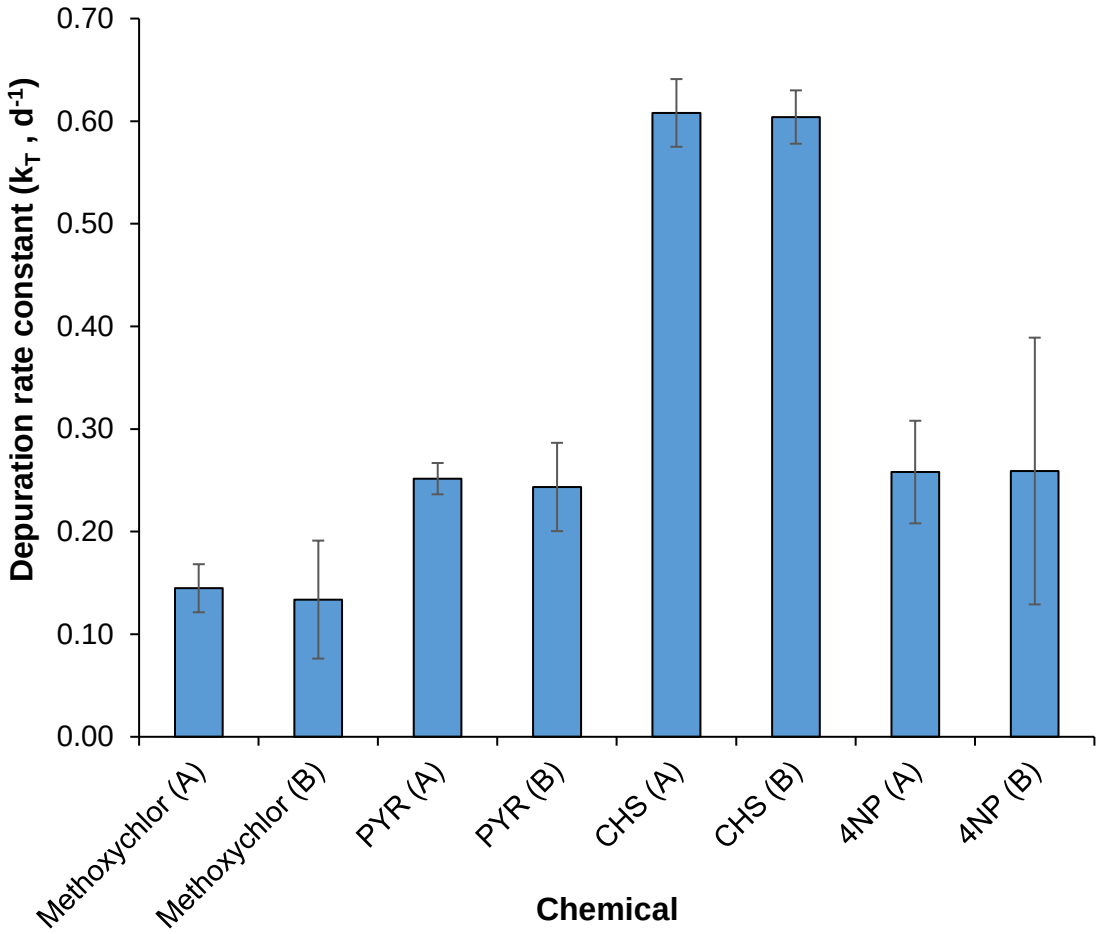


Figure 19. **Measured depuration rate constants (k_T) of the four test chemicals in treatment tanks without reference chemicals and from simultaneous exposure to reference chemicals. (A) refers to treatment tanks with test chemicals alone and (B) the treatment tanks with test and reference chemicals. Error bars represent the standard error of k_T.**

Table 15. Water depletion rate constants (k_W), uptake (k_1) rate constants derived from a) Equation 18 and b) Equation 19 and depuration rate constants (k_T) derived from the natural logarithm of the concentration of chemicals in fish over time (µg/g tissue) during the depuration phase of aqueous BCF test 1. Standard errors of the mean rate constants are reported (±SE).

Chemical	k_W, d^{-1} (±SE)	k_1 (a), d^{-1} (±SE)	k_1 (b), d^{-1} (±SE)	k_T, d^{-1} (±SE)
Test				
Methoxychlor (A)	0.08 (0.03)	1.64E+03 (5.46E+02)	1.77E+03 (3.83E+02)	1.45E-01 (4.50E-02)
Methoxychlor (B)	0.10 (0.09)	2.41E+02 (9.5E+01)	2.61E+02 (1.03E+02)	1.10E-01 (5.75E-02)
Reference				
1,3,5-trichlorobenzene	0.21 (0.54)	8.07E+01 (5.65E+01)	1.14E+02 (2.17E+02)	2.30E-01 (9.80E-02)
1,2,4,5-tetrachlorobenzene	0.19 (0.12)	2.25E+02 (1.59E+02)	2.80E+02 (1.67E+02)	1.00E-01 (4.00E-02)
Pentachlorobenzene	0.17 (0.08)	3.09E+03 (1.68E+03)	3.59E+03 (1.21E+03)	6.08E-02 (3.68E-02)
Hexachlorobenzene	0.04 (0.11)	9.86E+01 (1.76E+01)	1.01E+02 (4.00E+01)	7.40E-02 (3.00E-02)

Table 16. Uptake (k_1) rate constants derived from a) Equation 18 and b) Equation 19 and depuration rate constants (k_T) derived from natural logarithm of the concentration of chemicals in fish over time (µg/g tissue) during the depuration phase of aqueous BCF test 2. Standard errors of the mean depuration rate constants are reported (±SE).

Chemical	k_W, d^{-1} (±SE)	k_1 (a), d^{-1} (±SE)	k_1 (b), d^{-1} (±SE)	k_T, d^{-1} (±SE)
Test				
Pyrene (A)	0.38 (0.15)	2.13E+01 (1.90E+01)	4.42E+01 (2.95E+01)	2.10E-01 (4.00E-02)
Pyrene (B)	0.59 (0.05)	3.69E+00 (3.61E+00)	1.24E+01 (4.06E+00)	2.20E-01 (4.00E-02)
Reference				
1,3,5-trichlorobenzene	0.74 (0.43)	3.44E+01 (3.44E+01)	1.42E+02 (3.30E+02)	1.86E-01 (2.88E-02)
1,2,4,5-tetrachlorobenzene	0.70 (0.10)	2.40E+02 (2.39E+02)	8.06E+02 (4.60E+02)	1.35E-01 (1.97E-02)
Pentachlorobenzene	0.74 (0.11)	1.70E+02 (1.71E+02)	5.10E+02 (3.21E+02)	5.80E-02 (1.40E-02)
Hexachlorobenzene	0.00 (6.25)	2.19E+02 (3.49E+01)	2.19E+02 (4.87E+03)	2.00E-02 (9.00E-03)

Table 17. Uptake (k_1) rate constants derived from a) Equation 18 and b) Equation 19 and depuration rate constants (k_T) derived from natural logarithm of the concentration of chemicals in fish over time (µg/g tissue) during the depuration phase of aqueous BCF test 3. Standard errors of the mean depuration rate constants are reported (±SE).

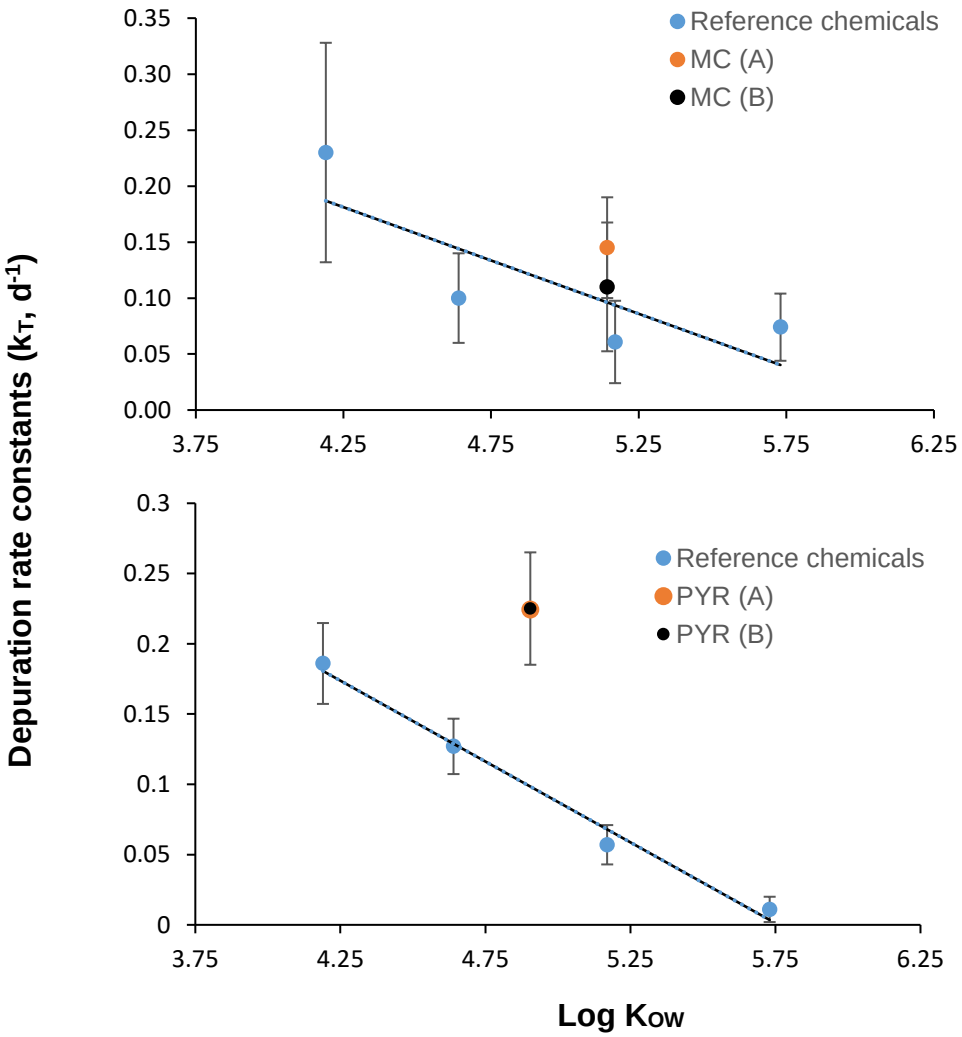
Chemical	k_W, d^{-1} (±SE)	k_1 (a), d^{-1} (±SE)	k_1 (b), d^{-1} (±SE)	k_T, d^{-1} (±SE)
Test				
Cyclohexyl Salicylate (A)	0.39 (0.42)	5.15E+02 (4.53E+02)	1.88E+03 (2.14E+03)	6.10E-01 (3.40E-02)
Cyclohexyl Salicylate (B)	0.39 (0.42)	5.89E+02 (5.64E+02)	2.17E+03 (2.64E+03)	6.00E-01 (2.60E-02)
Reference				
1,3,5-trichlorobenzene	0.33 (0.42)	1.85E+02 (1.54E+02)	2.70E+02 (5.32E+02)	3.29E-02 (1.00E-02)
1,2,4,5-tetrachlorobenzene	0.30 (0.42)	5.96E+02 (4.72E+02)	8.22E+02 (1.68E+03)	1.80E-02 (4.70E-03)
Pentachlorobenzene	0.25 (0.42)	1.06E+03 (7.80E+02)	1.33E+03 (2.59E+03)	4.60E-03 (2.00E-03)
Hexachlorobenzene	0.31 (0.42)	7.93E+02 (6.39E+02)	1.08E+03 (2.15E+03)	1.00E-03 (5.00E-04)

Table 18. Uptake (k_1) rate constants derived from a) Equation 18 and b) Equation 19 and depuration rate constants (k_T) derived from natural logarithm of the concentration of chemicals in fish over time (µg/g tissue) during the depuration phase of aqueous BCF test 4. Standard errors of the mean depuration rate constants are reported (±SE).

Chemical	k_W, d^{-1} (±SE)	k_1 (a), d^{-1} (±SE)	k_1 (b), d^{-1} (±SE)	k_T, d^{-1} (±SE)
Test				
4-n-nonylphenol (A)	0.20 (0.005)	1.94E+01 (1.22E+01)	2.78E+01 (4.91)	2.58E-01 (5.00E-02)
4-n-nonylphenol (B)	0.19 (0.005)	3.63E+01 (2.45E+01)	5.02E+01 (2.24E+01)	2.59E-01 (1.30E-01)
Reference				
1,3,5-trichlorobenzene	NA	NA	NA	1.49E-01 (3.00E-02)
1,2,4,5-tetrachlorobenzene	0.09 (1.0)	4.93E+03 (2.32E+03)	4.93E+03 (1.48E+04)	1.00E-01 (1.30E-02)
Pentachlorobenzene	0.22 (0.41)	1.26E+03 (8.37E+02)	1.54E+03 (2.76E+03)	3.80E-02 (6.00E-03)
Hexachlorobenzene	0.01 (0.33)	1.11E+02 (1.90E+01)	1.11E+02 (1.26E+02)	1.60E-02 (6.80E-03)

4.3.3. *In vivo* biotransformation rate constants (k_M, d^{-1})

The biotransformation rate constants were calculated based on the relationship between the depuration rate constants of test and reference chemicals with their respective Log K_{OW} or $1/K_{OW}$ in all BCF tests and are summarized in Table 19. The k_M for Methoxychlor was the lowest followed by 4-n-nonylphenol, pyrene and then cyclohexyl salicylate with the highest k_M (Table 19). The general rank order of k_M's measured in this study is in agreement with the rank order of k_M's determined *in vitro* using hepatocytes and liver S9 fractions reported in an inter-laboratory ring trial (J. Nichols et al., 2018).The k_M values corresponding to methoxychlor were in agreement with k_M values estimated from the EPI suite QSAR model (US EPA, 2012). However, the k_M values derived for pyrene and cyclohexyl salicylate were much lower than QSAR estimates. The lack of agreement for pyrene and cyclohexyl is likely not due to the presence of non-biotransformed reference chemicals as the measured k_M's were very similar between the treatment tanks with single test chemicals and the treatment tanks containing a mixture of the test and reference chemicals. Past research has indicated that the presence of multiple biotransformed chemicals may result in lower biotransformation rates due to competitive inhibition (Lo et al., 2015). This phenomenon was recorded in *in vitro* biotransformation tests using rainbow trout liver S9 homogenates (Lee et al., 2014; Lo et al., 2015). Due to the reference chemicals ability to resist metabolization, they are unlikely to contribute to competitive inhibition. Thus, an aqueous BCF test in which rainbow trout were exposed to a mixture of the four test chemicals simultaneously would reveal whether the biotransformation rate constants would be affected by the presence of other biotransformed chemicals.



BCF Test 1

k_T = 0.586 -0.095(log K_{OW})

R^2 = 0.667

p = 0.1832

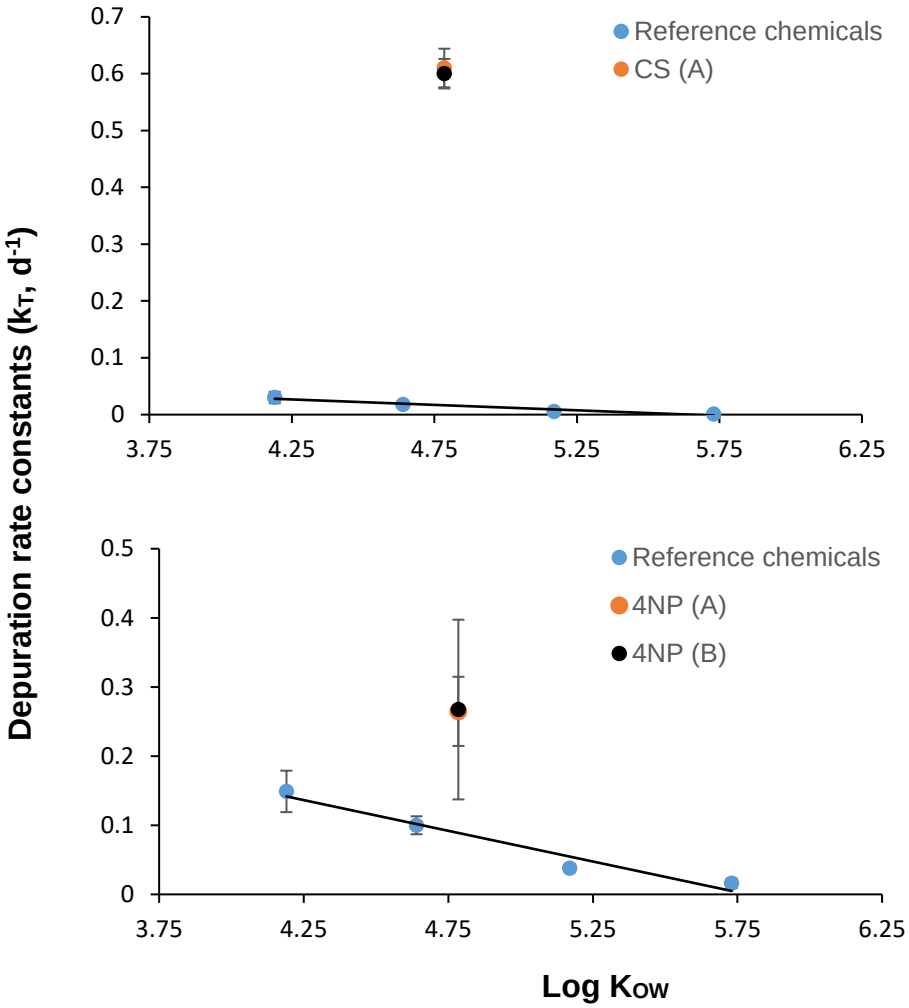
BCF Test 2

k_T = 0.663-0.115(log K_{OW})

R^2 = 0.988

p = 0.0058

Figure 20. Illustrative diagram of the relationship between depuration rate constants (k_T) of test chemicals and four reference chemicals ($k_{T,R}$) and the octanol-water partition coefficient (log K_{OW}) values. Error bars represent the standard error (± SE) of depuration rate constants derived from the linear regression of the Ln concentration of chemical in fish over the depuration phase (µg/L).



BCF Test 3

k_T =0.108 -0.019(log K_{OW})

R^2 = 0.951

p = 0.0250

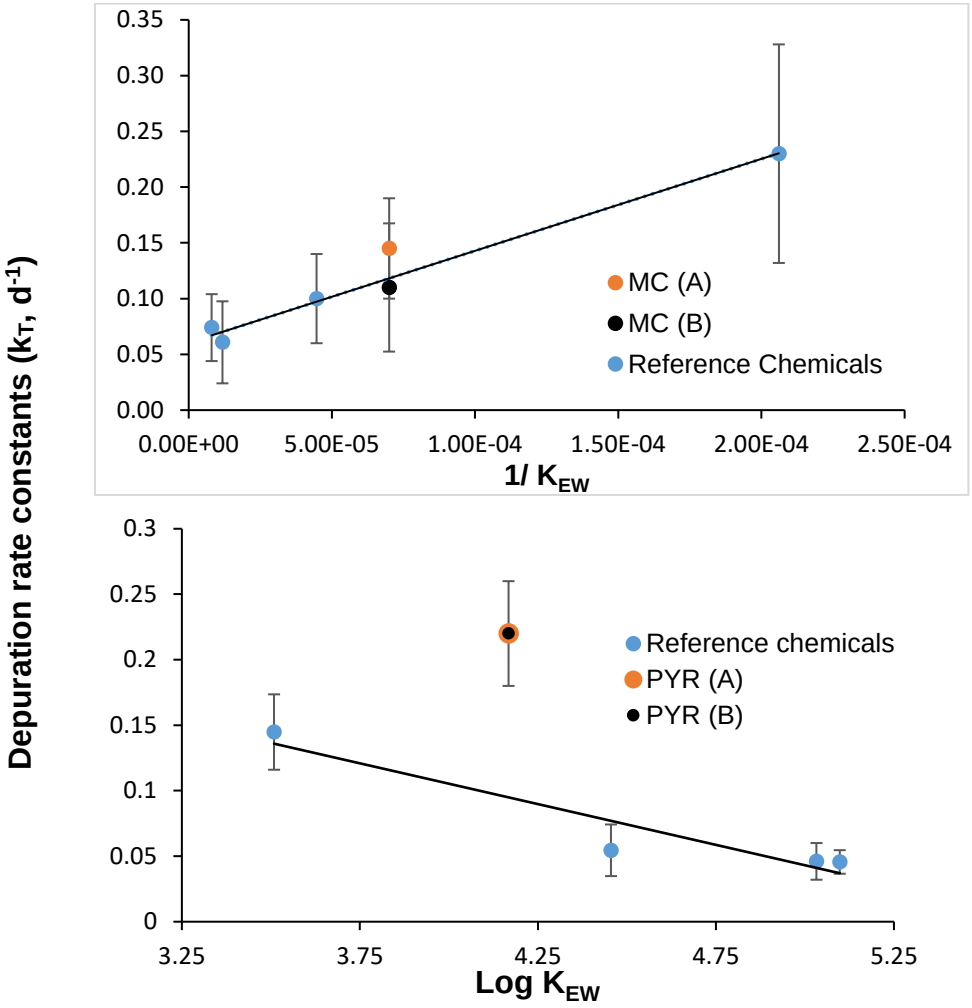
BCF Test 4

k_T =0.514-0.089(log K_{OW})

R^2 = 0.958

p = 0.0211

Figure 21. Illustrative diagram of the relationship between depuration rate constants (k_T) of test chemicals and four reference chemicals ($k_{T,R}$) and the octanol-water partition coefficient (log K_{OW}) values. Error bars represent the standard error (± SE) of depuration rate constants derived from the linear regression of the Ln concentration of chemical in fish over the depuration phase (µg/L).



BCF Test 1

k_T= 0.0585 + 835.1(1/K_{EW})

R^2 = 0.994

p = 0.003

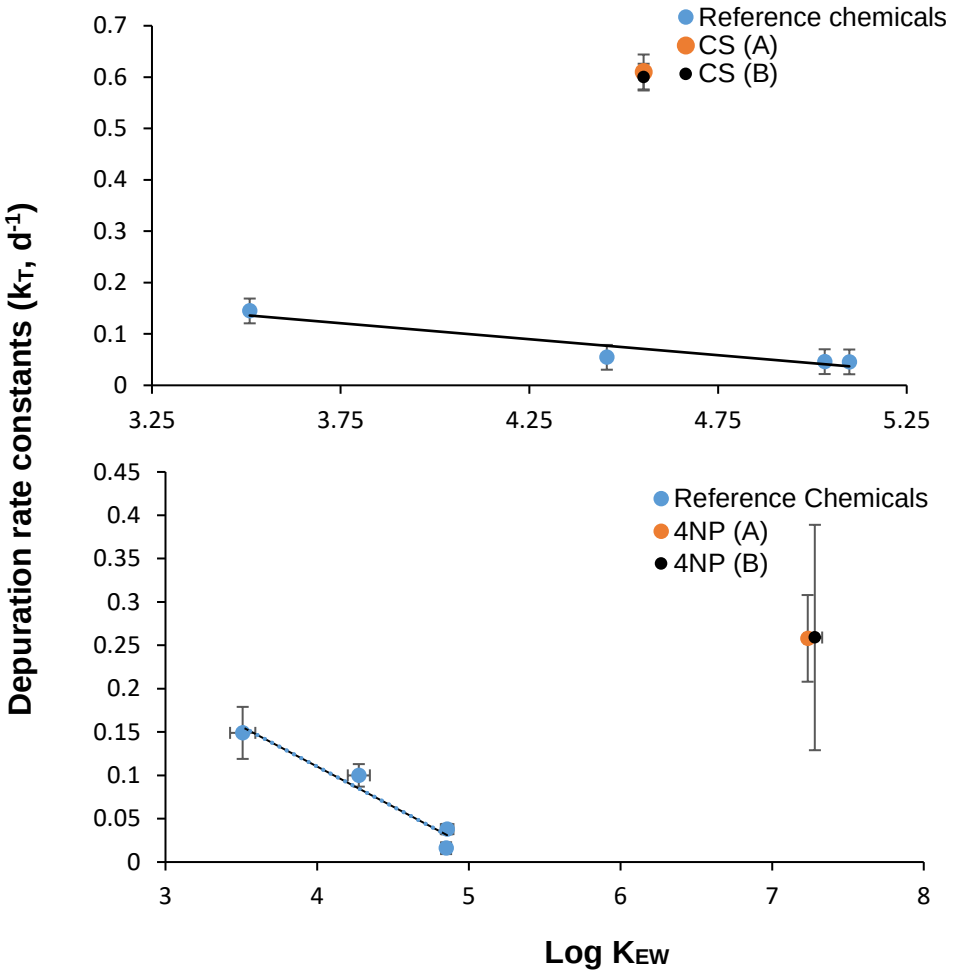
BCF Test 2

k_T = 0.479 – 0.0867*logK_{EW}

R^2 = 0.852

p = 0.0767

Figure 22. Illustrative diagram of the relationship between depuration rate constants (k_T) of test chemicals and four reference chemicals ($k_{T,R}$) and the EVA-water partition coefficient (log K_{EW}) values. Error bars represent the standard error (± SE) of depuration rate constants derived from the linear regression of the Ln concentration of chemical in fish over the depuration phase (µg/L).



BCF Test 3

$k_T = 0.0936 - 0.0172*\log K_{EW}$

$R^2 = 0.944$

$p = 0.0287$

BCF Test 4

$k_T = -0.0923 + 0.479 * \text{Log } K_{EW}$

$R^2 = 0.948$

$P = 0.0263$

Figure 23. Illustrative diagram of the relationship between depuration rate constants (k_T) of test chemicals and four reference chemicals ($k_{T,R}$) and the EVA-water partition coefficient (log K_{EW}) values. Error bars represent the standard error (± SE) of depuration rate constants derived from the linear regression of the Ln concentration of chemical in fish over the depuration phase (µg/L).

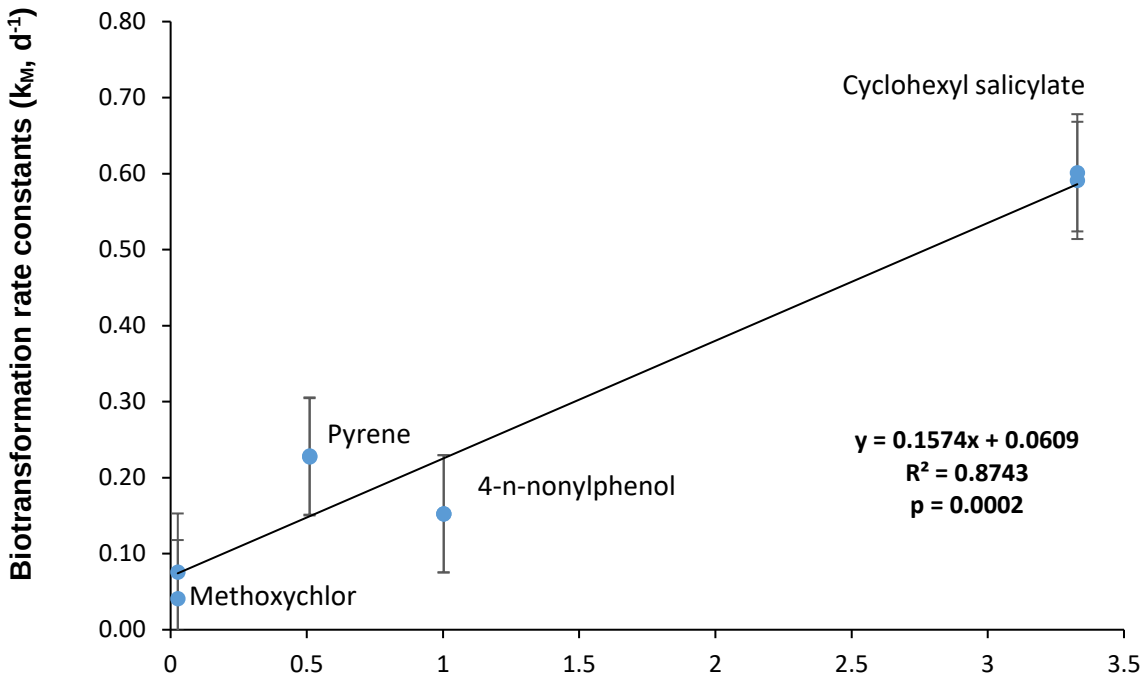


Figure 24. **Observed whole-organism biotransformation rate constants (k_M) from present study (calculated from K_{OW}) as a function of BCFBAF QSAR-predicted (EPI Suite v4.11) biotransformation rate constants corrected for mean fish weights and water temperatures during each BCF test. The solid line represents the regression fit of the biotransformation rate constants in each treatment tank.**

Table 19. **Whole body biotransformation rate constants (k_M) for test chemicals alone (A) and in the presence of reference chemicals (B) determined from depuration rate constants of test and reference chemicals in aqueous BCF tests using K_{OW} in $k_{T,R}$ calculations. k_M estimates from EPI suite using the Arnot-Gobas BCFBAF model for mean fish weights and water temperatures in each BCF tests (US EPA, 2012).**

Test Chemicals	$k_{T,R}$ (using K_{OW}) (±SE), d^{-1}	k_M (± SE) (d^{-1})	$k_{M,\ BCFBAF}$ (d^{-1})
Methoxychlor (A)	6.92E-02 (1.08 E-02)	0.08 (4.63E-02)	0.026
Methoxychlor (B)	6.92E-02 (1.08 E-02)	0.04 (5.85 E-02)	0.026
Pyrene (A)	6.75E-02 (2.55 E-02)	0.15 (4.74 E-02)	1.00
Pyrene (B)	6.75E-02 (2.55 E-02)	0.15 (4.74 E-02)	1.00
Cyclohexyl salicylate (A)	8.90E-03 (1.06 E-02)	0.60 (3.40 E-02)	3.33
Cyclohexyl salicylate (B)	8.90E-03 (1.06 E-02)	0.59 (2.60 E-02)	3.33
4-n-nonylphenol (A)	3.05E-02 (1.57 E-02)	0.23 (5.24 E-02)	0.51
4-n-nonylphenol (B)	3.05E-02 (1.57 E-02)	0.23 (1.31 E-01)	0.51

4.3.4. Bioconcentration factors

Table 20. Mean kinetic bioconcentration factors (BCF_k) of test and reference chemicals for all four BCF tests and lipid-corrected kinetic BCFs (BCF_{kL}) using (a) the mean concentrations of chemical in water during the exposure and (b) the exponential decline in concentrations of chemicals during the exposure phase. Standard error of the mean is reported (± SE).

Chemical	$BCF_{k,\ ww}$ [a] (±SE) L kg^{-1}	$BCF_{k,\ ww}$ [b] (±SE) L kg^{-1}	BCF_{kL} [a] (±SE) L kg^{-1}	BCF_{kL} [b] (±SE) L kg^{-1}
Test				
Methoxychlor	3.96E+03 (3.36E+03)	5.46E+03 (4.61E+03)	1.50E+05 (1.26E+05)	1.17E+05 (9.85E+04)
Pyrene	3.2E+01 (2.3E+01)	1.34E+02 (7.70E+01)	1.37E+04 (2.58E+03)	2.85E+03 (1.65E+03)
Cyclohexyl salicylate	4.86E+02 (3.6E+01)	3.36E+03 (2.67E+02)	1.59E+05 (1.46E+04)	4.77E+04 (2.97E+04)
4-n-nonylphenol	6.8E+01 (2.1E+01)	1.51E+02 (4.30E+01)	1.45E+03 (4.49E+02)	3.22E+03 (9.20E+02)
Reference				
1,3,5-trichlorobenzene	1.45E+03 (8.8E+02)	3.89E+03 (2.23E+03)	3.11E+04 (1.88E+04)	8.31E+04 (4.76E+04)
1,2,4,5-tetrachlorobenzene	2.33E+04 (1.67E+04)	2.62E+04 (1.23E+04)	4.99E+05 (3.56E+05)	5.61E+05 (2.63E+05)
Pentachlorobenzene	4.02E+04 (3.2E+04)	8.65E+04 (6.83E+04)	8.59E+05 (6.83E+05)	1.85E+06 (1.46E+06)
Hexachlorobenzene	1.15E+05 (1.09E+05)	2.75E+05 (2.69E+05)	2.46E+06 (2.33E+06)	5.87E+06 (5.74E+06)

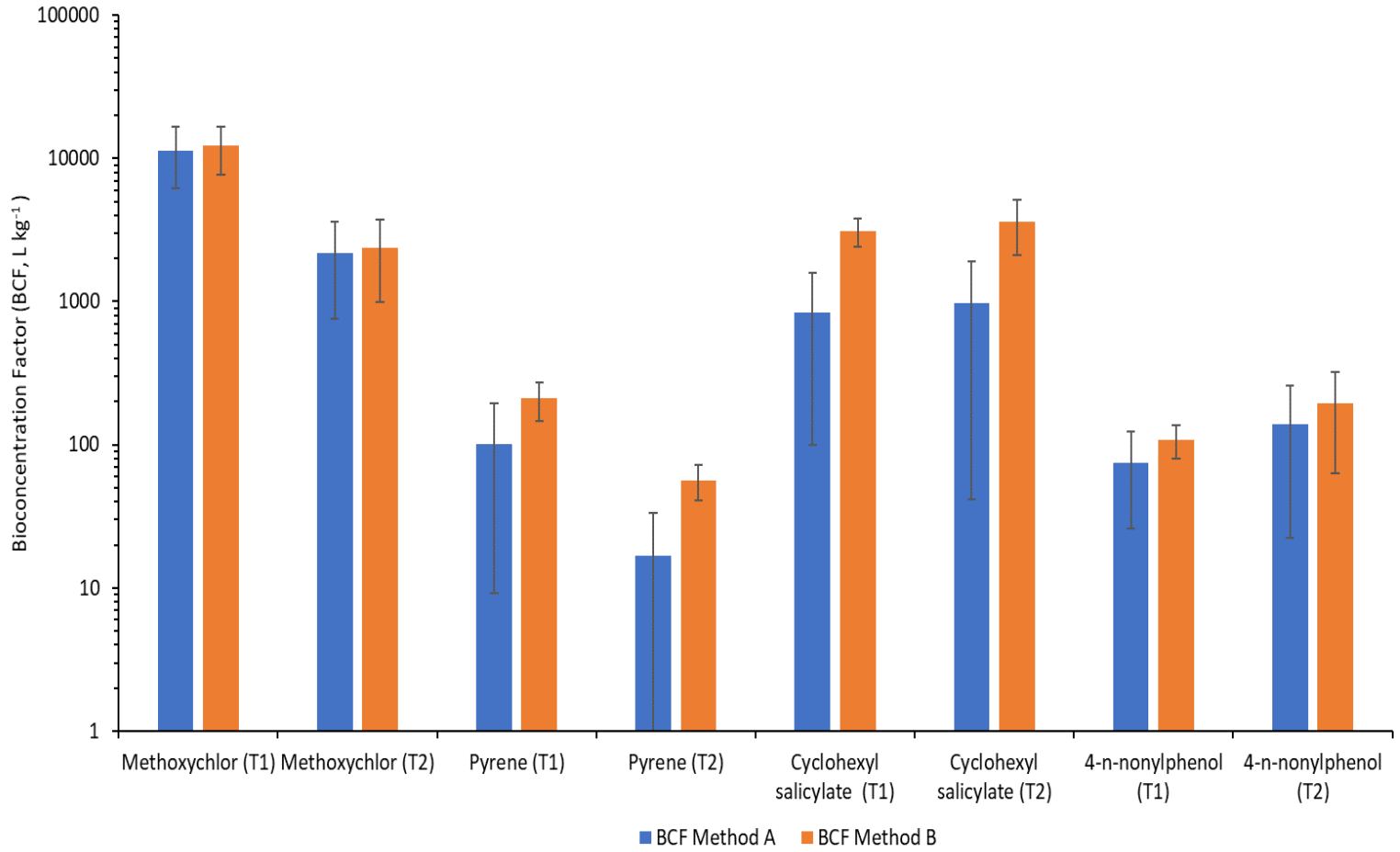


Figure 25. **Mean bioconcentration factors of test chemicals in fish determined from present study in BCF tests with (T1) and without (T2) reference chemicals. The kinetic bioconcentration factor (BCF_k, a) using k_1 derived by Equation 18, the bioconcentration factor using k_1 derived by Equation 19 (BCF_k, b) and lipid normalized BCFs using each method are illustrated. Error bars represent the standard error of the mean BCFs (± SE).**

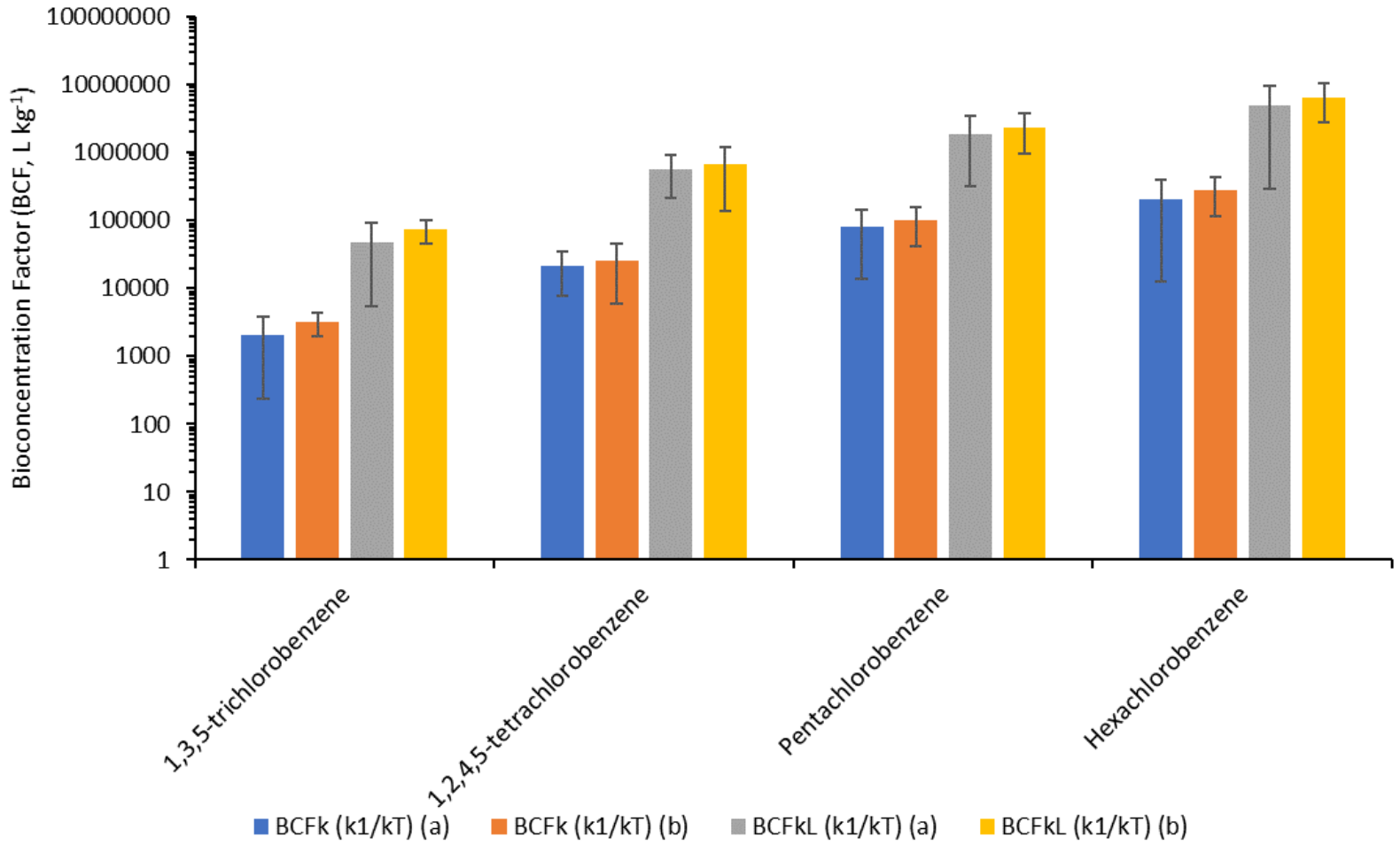


Figure 26. **Mean bioconcentration factors of reference chemicals in fish determined from present study. The kinetic bioconcentration factor (BCF_k, a) using k_1 derived by Equation 18, the bioconcentration factor using k_1 derived by Equation 19 (BCF_k, b) and lipid normalized BCFs using each method are illustrated. Error bars represent the standard error of the mean BCFs (± SE).**

Table 21. Comparison of the mean measured and model estimated [a] chemical bioconcentration factors (BCFs). *In vivo* BCFs from the current study and literature studies. Model estimated BCFs assuming no biotransformation and with estimated biotransformation rate constants.

Chemical	Mean measured i*n vivo* BCF_Ks [b] L kg wet wt^{-1}	Empirical i*n vivo* BCFs L kg^{-1}	Estimated BCFs [a] $k_M = 0$	Estimated BCFs [a] $k_M \neq 0$
Test				
Methoxychlor [72-43-5]	7,292 (3,087)	174	5,229 - 5,763	4,080
Pyrene [129-00-0]	134 (84)	78	3,490 - 3,755	1,008
Cyclohexyl Salicylate [25485-88-5]	3,356 (3,955)	400	2,371 - 2,419	123
4-n-nonylphenol [104-40-5]	151 (43)	290 - 896	16,549 - 21,121	333
Reference				
1,3,5-trichlorobenzene [108-70-3]	3,153 (2,524)	150 - 14,000	1,572	834
1,2,4,5-tetrachlorobenzene [95-94-3]	25,938 (12,477)	5,012 - 12,589	4,046	2,337
Pentachlorobenzene [608-93-5]	99,601 (64,368)	7,600	10,320	6,485
Hexachlorobenzene [118-74-1]	274,926 (268,527)	28,500	18,730	17,090

[a] Model estimates obtained using the BCFBAF program v3.01 in EPI Suite TM v4.1. Fish weights and water temperatures summarized in results.

[b] Mean BCF_Ks calculated with k_1 determined using method B, Equation 19.

BCF interpretation

The bioconcentration factor (BCF) is a function of uptake and depuration processes from water in fish. More specifically, the kinetic BCF (BCF_k) is the ratio of the uptake rate constant (k_1, d^{-1}) and the total elimination or depuration rate constant (k_T, d^{-1}) as described in Equation 3. Therefore, as the depuration rate constant (k_T) of chemical decreases, BCF_k increases. Alternatively, as the uptake rate constant (k_1) increases, the BCF_k increases as well. The BCF_k was obtained using k_1 calculated two different methods, one in which the uptake rate was measured assuming the concentrations of chemical in water would remain constant throughout the exposure phase (Equation 18, method A), the other method assumed an exponential decline in concentration following the addition of fish to the system, as described by a depletion rate constant (Equation 19, method B). For certain chemicals, the latter may be more representative of how hydrophobic chemicals behave in a semi-static system to which fish are added, as demonstrated in Figure 8. Though for others, an average concentration of chemical in water throughout the exposure phase is more representative (Figure 8).

BCFs measured using the uptake rate constants described by an exponential decline in concentrations of chemical in water tended to be higher than BCFs calculated assuming the concentrations in water remained constant at the beginning of the exposure phase (t=0). Though, regardless of the method used, a one-way ANOVA followed by a Tukey-Kramer HSD test ($\alpha = 0.05$) revealed that the measured k_1 values were not statistically different ($p= 0.875$) due to the large experimental error incurred throughout the experimental process, as is the case in most BCF tests for hydrophobic chemicals.

Of the four test chemicals, methoxychlor had the highest measured BCF_ks, followed by cyclohexyl salicylate and then 4-n-nonylphenol. Pyrene exhibited the lowest range of BCF_ks. As with the uptake rate constants (k_1), due to the large amount of error contained in the measurement of the BCF_k, the differences in BCF_ks across all four test chemicals were not significantly different as revealed by an ANOVA ($\alpha = 0.05$) followed by a Tukey-Kramer HSD ($p= 0.195$).

For the reference chemicals, the measured BCF_ks generally increased from 1,3,5-trichlorobenzene, 1,2,4,5-tetrachlorobenzene, pentachlorobenzene with the highest BCF_ks recorded for hexachlorobenzene. Similarly, BCF_ks obtained from the literature for

the reference chemicals followed the same pattern (Table 21). The BCF_ks measured for hexachlorobenzene were high compared to empirical values for three of the BCF tests (pyrene, cyclohexyl salicylate and 4-n-nonylphenol). For the first BCF test (methoxychlor), the measured BCF_ks for hexachlorobenzene were low compared to empirical values. This may be due to the difficulty dissolving hexachlorobenzene in methanol. For example, there may have been a high concentration of hexachlorobenzene in the water in an unavailable form, due to its high hydrophobicity. Hexachlorobenzene and chemicals with low solubility and high hydrophobicity start to become difficult to work with for aqueous BCF tests. This limitation is mentioned in the OECD 305 test guidelines and seems to pose a similar issue in this study (OECD, 2012).

The BCF_ks were normalized according to mean lipid content in fish in each BCF test to get a lipid normalized kinetic BCF (BCF_{kL}). This method is based on the idea that the lipid content plays a significant role in controlling the extent of bioaccumulation of hydrophobic chemicals within an organism, as well as between organisms (Gobas, 2001). In studies where lipid content has little variation over time, the bioconcentration factor is often proportional to the lipid content of the organism (Gobas & MacKay, 1987). Therefore, BCFs tend to be higher in organisms with higher lipid contents (Gobas, 2001; Schlechtriem et al. 2012). Normalizing the BCF to the measured lipid content is used to reduce variability in comparisons across measured BCF values (Schlechtriem et al., 2012). Though this method is generally accepted, particularly for incorporating comparisons between concentration measurements from specific tissues that differ in lipid content (Gobas, 2001), it may contain more error compared to the BCF_k as a result of incorporating the additional error associated with the lipid content measurements.

A challenge of using BCFs to determine the bioaccumulative potential of a chemical is the uncertainty and variability in BCF data (Arnot and Gobas 2006; Arnot and Gobas 2009). Sources of uncertainty include but are not limited to; test concentrations above water solubility, uncertain exposure durations at steady-state, the lack of data to characterize water concentrations and concentrations either near baseline toxicity range or below detection limit (Arnot & Gobas, 2006; Parkerton et al., 2008). Furthermore, due to the wide range of methods for BCF testing and different fish species chosen, empirical BCFs may contain significant error and the quality should be evaluated based on accepted criteria (Arnot & Gobas, 2006).

One main limitation of this bioconcentration factor test is the need to obtain the appropriate values for K_{OW} of test and reference chemicals. Measurements of the log K_{OW} of chemicals can contain significant error due to differences in methodology and experimental error. Measuring the K_{EW} directly in the same study as the BCF was expected to reduce error associated with using a K_{OW} obtained using various techniques across different laboratories, there was no observable difference in error using the measured EVA–water partition coefficient (K_{EW}) of the test and reference chemicals in this study. Therefore, while K_{EW} may not replace the use of K_{OW}s, they can contribute additional information about the test substances.

This study does not address the potential accumulation or toxicity of metabolites derived from biotransformation. Biotransformation reactions can result in the detoxication of parent compounds into more hydrophilic metabolites that are more easily excretable from the organism (Livingstone, 1998; Oost, Beyer, & Vermeulen, 2003; Vives et al., 2005). Alternatively, biotransformation reactions can produce metabolites that are more toxic than the parent compounds, termed bioactivation (Spurgeon et al., 2010; Van der Linde et al., 2001; Weisbrod et al., 2009). For example, methoxychlor undergoes oxidative metabolism by hepatic cytochrome P450 resulting in metabolites with high estrogenic activity (Bulger, Muccitelli, & Kupfer, 1978; Hu & Kupfer, 2002).Therefore, it is necessary to identify potentially harmful metabolites when investigating the bioaccumulative potential of a chemical, to address the potential adverse effects of bioactivation reactions.

Finally, recent literature suggests that biotransformation rates are affected by mixtures of chemicals and therefore, single chemical tests may overestimate biotransformation rates for chemicals that are commonly found as mixtures in the environments (Trowell et al., 2018). In this study, no significant differences were observed between treatments with single test chemicals and treatments with test and reference chemicals. Since this does not reveal whether biotransformation rate constants would be affected by the presence of other biotransformed substances, an additional study in which a BCF test with all four biotransformed test chemicals is necessary to identify the behaviour of biotransformed substances in a mixture with other biotransformative substances. Continuing to investigate the biotransformation potential of hydrophobic substances will contribute to more accurate assessments of new and existing chemicals and improve our ability to protect the environment and human health.

Chapter 5. Conclusion

Efforts are being made to simplify the current BCF testing protocols for HOCs by reducing animal use, costs and time to more efficiently test the increasing number of chemicals used globally. The main objective of the study, to develop and test a streamlined aqueous bioconcentration test for hydrophobic chemicals in fish, was met. Compared to the OECD 305 test guidelines, the present study reduced the required number of fish by approximately 17%, cut the test time in half and reduced costs per chemical by approximately 80%. The reductions in the present study will make BCF testing more efficient, while providing an aqueous BCF test that also allows measuring the *in vivo* biotransformation rate constants (k_M, d^{-1}) of HOCs in fish.

The testing approach includes the use of non-biotransformed reference chemicals, a method that has previously been used successfully for dietary bioconcentration tests (Gobas & Lo, 2016; Lo et al., 2015). A comparison between treatments with the test chemicals on their own, and the test chemicals in the presence of reference chemicals revealed that the presence of conservative reference chemicals does not affect the k_M and BCF of the test chemicals. While maintaining a constant concentration of chemical in water over the uptake phase proved difficult with the passive dosing method used in this study, this limitation can be addressed by adapting the methods for data analysis, as was done by using a concentration depletion rate constant (k_{Water}).

Finally, the test provides an alternative method for estimating the partitioning behavior using a sorbent-phase (i.e., EVA)-water partition coefficient (K_{EW}). Though K_{EW} does not necessarily reduce error associated with K_{OW}, it provides a relatively simple method of calculating the partition coefficient alongside the BCF. It also lends the possibility of estimating the partitioning behavior of a chemical for which there are no available empirical K_{OW} values.

It is important to have empirical data for substances that are subject to a range of *in vivo* k_M's to strengthen and evaluate *in vitro* testing and *in vitro* to *in vivo* extrapolation (IVIVE). Previously, accepted and reliable methods for measuring *in vivo* biotransformation rate constants did not exist (J. W. Nichols et al., 2013; Uchea et al., 2013; Weisbrod et al., 2009). Therefore, this study provides the *in vivo* biotransformation

rate constants required to evaluate alternative methods of obtaining bioaccumulation data.

For future studies, the empirical data generated from this study can be used to evaluate IVIVE and *in vitro* estimates of biotransformation rate constants and BCFs. This research can be expanded to include a wider range of chemicals and different fish species to investigate intra-species variability. Collecting water samples throughout one of the BCF tests provided some insight to how the concentrations of chemicals in water act throughout an exposure using an EVA-chemical reservoir, and the alternative method of measuring k_1 (i.e., using k_{Water}) seemed to return more consistent results. Finally, a mixture study is necessary to identify the behaviour of highly biotransformed substances in a complex mixture with other biotransformative substances to identify potential effects on biotransformation rate constants as chemicals are often found in complex mixtures in the environment. Continuing to investigate the biotransformation potential of hydrophobic substances will contribute to more accurate assessments of new and existing chemicals and improve the ability to protect the environment and human health.

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Appendix A.

GCMS calibration curves

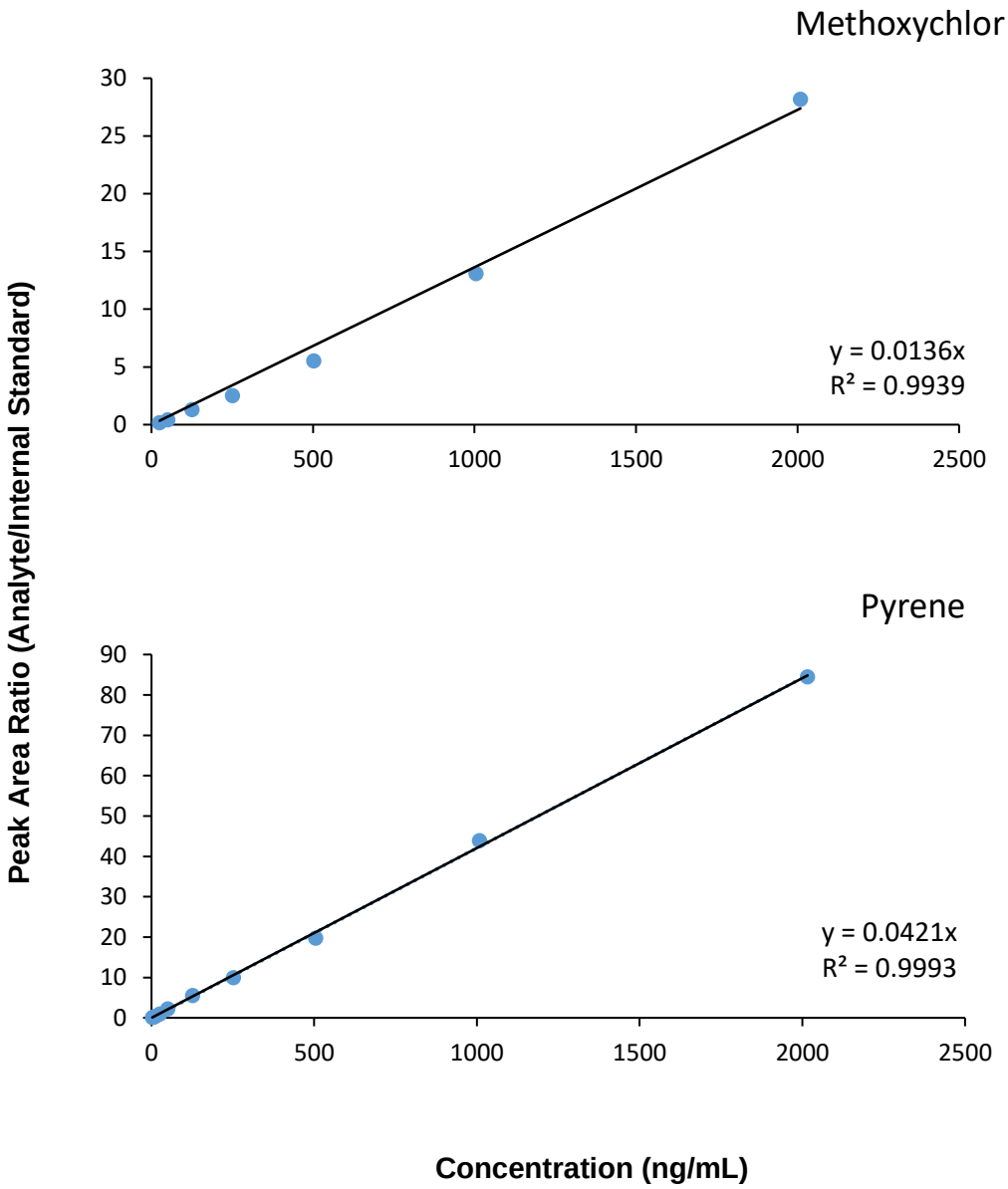

Figure A-1. **Calibration curves for test chemicals methoxychlor and pyrene made up in hexane showing the response, measured in peak area of the test chemical relative to the internal standard (d12-chrysene) as a function of the test concentrations of chemical (ng/mL).**

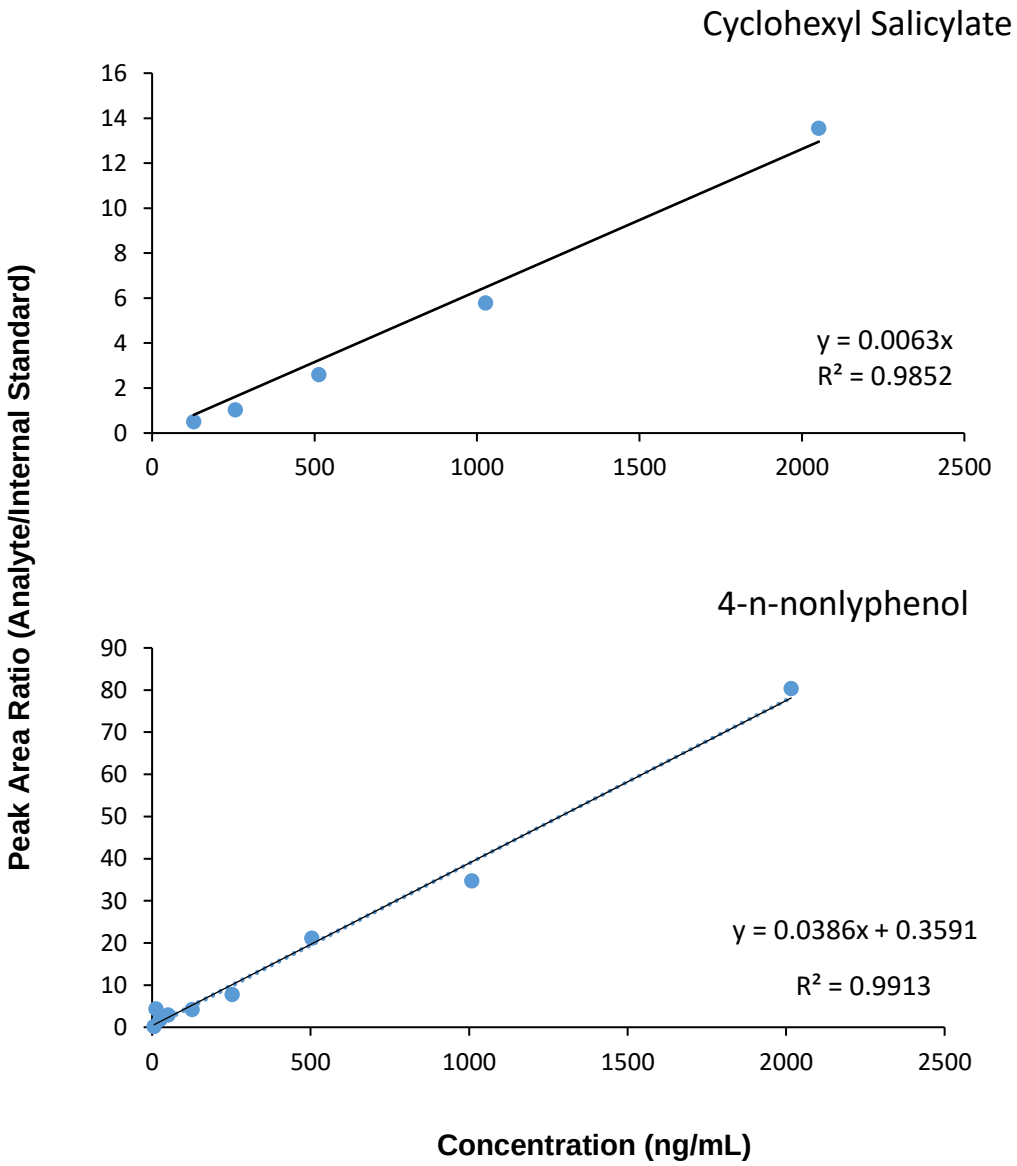

Figure A-2. **Calibration curves for test chemicals cyclohexyl salicylate and 4-n-nonylphenol made up in hexane showing the response, measured in peak area of the test chemical relative to the internal standard (d12-chrysene) as a function of the test concentrations of chemical (ng/mL).**

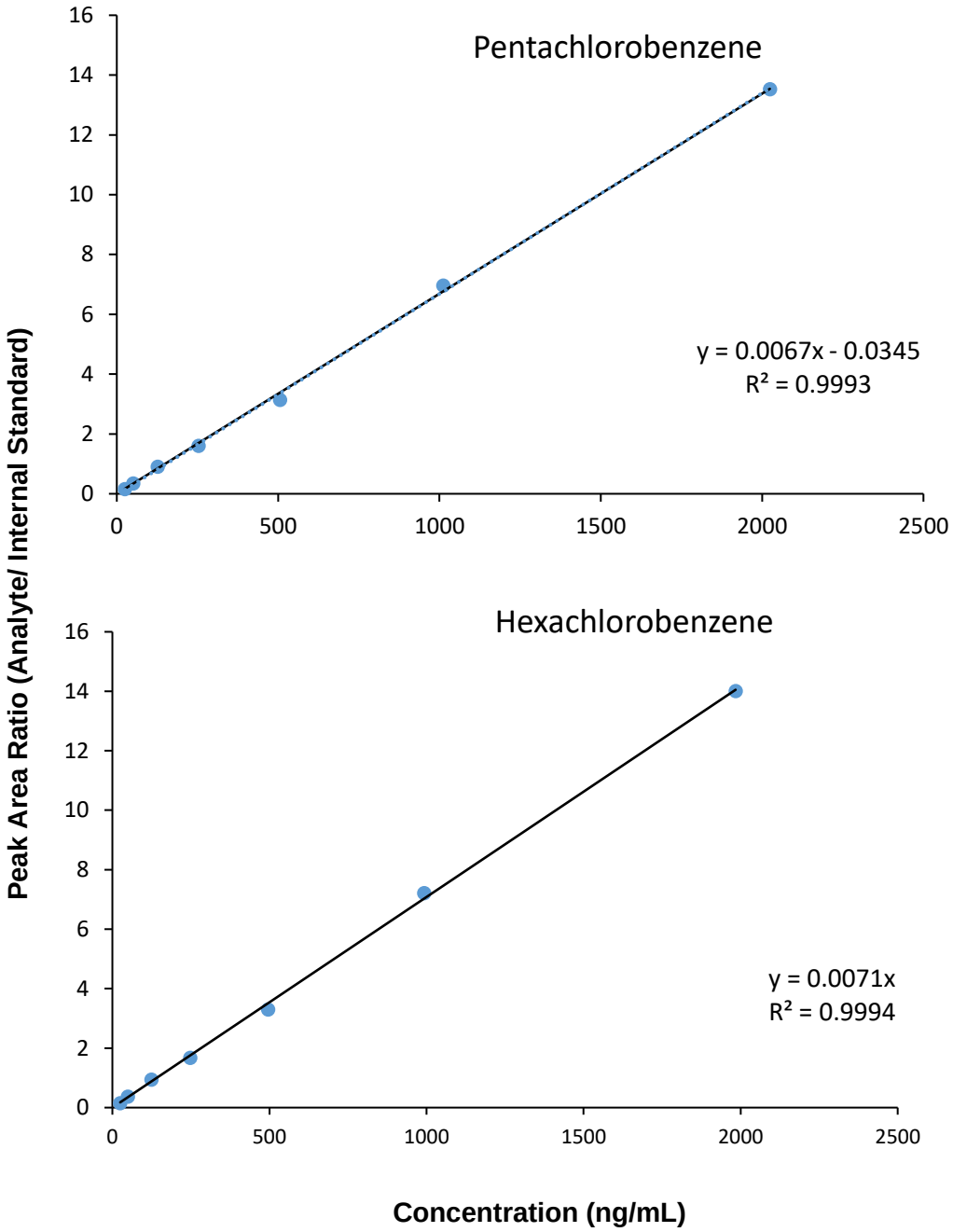

Figure A-3. Calibration curves for reference chemicals pentachlorobenzene and hexachlorobenzene showing the response, measured in peak area of the test chemical relative to the internal standard (d12-chrysene) as a function of the test concentrations of chemical (ng/mL).

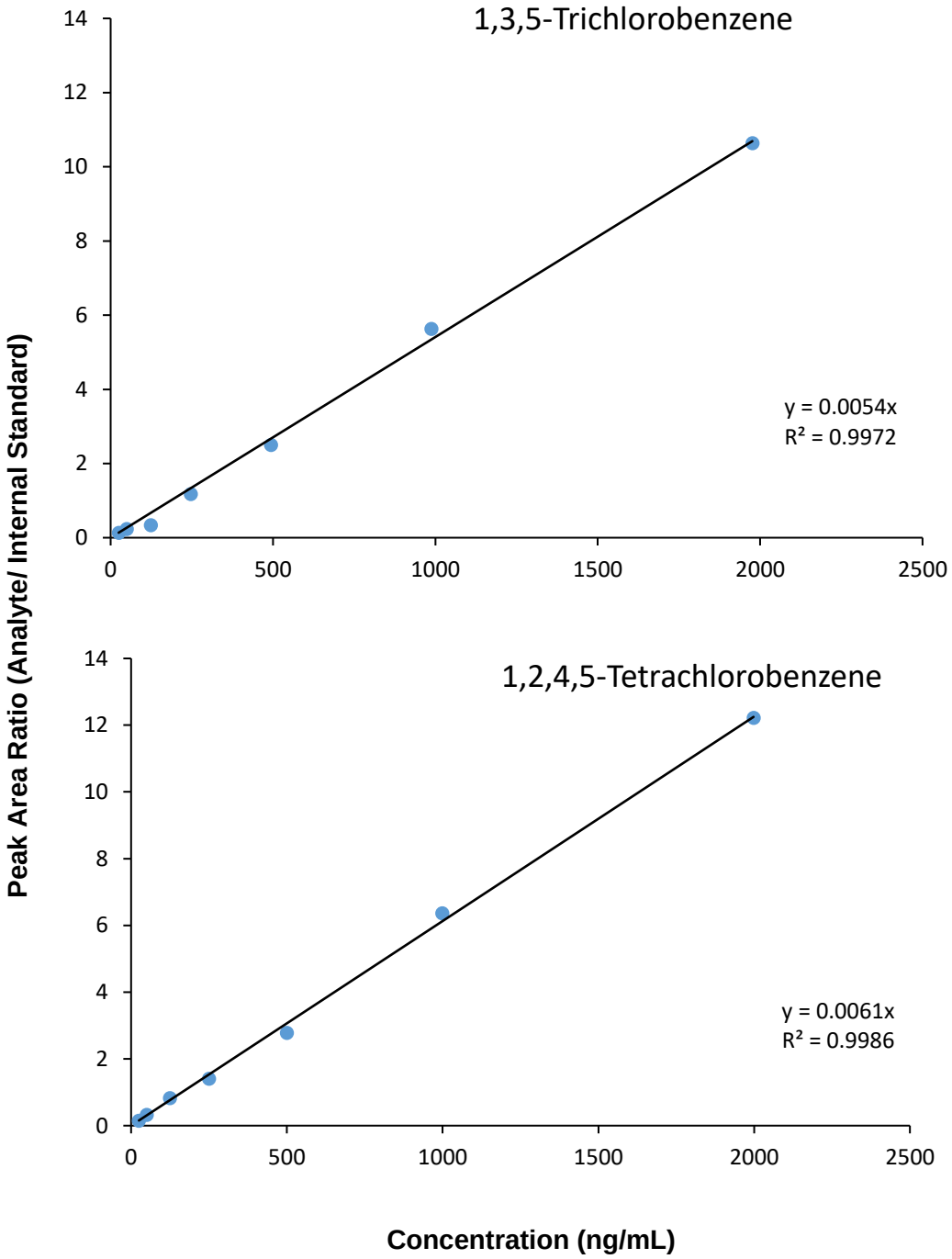

Figure A-4. **Calibration curves for reference chemicals 1,3,5-trichlorobenzene and 1,2,4,5-tetrachlorobenzene showing the response, measured in peak area of the test chemical relative to the internal standard (d12-chrysene) as a function of the test concentrations of chemical (ng/mL).**

Appendix B.

Growth of rainbow trout

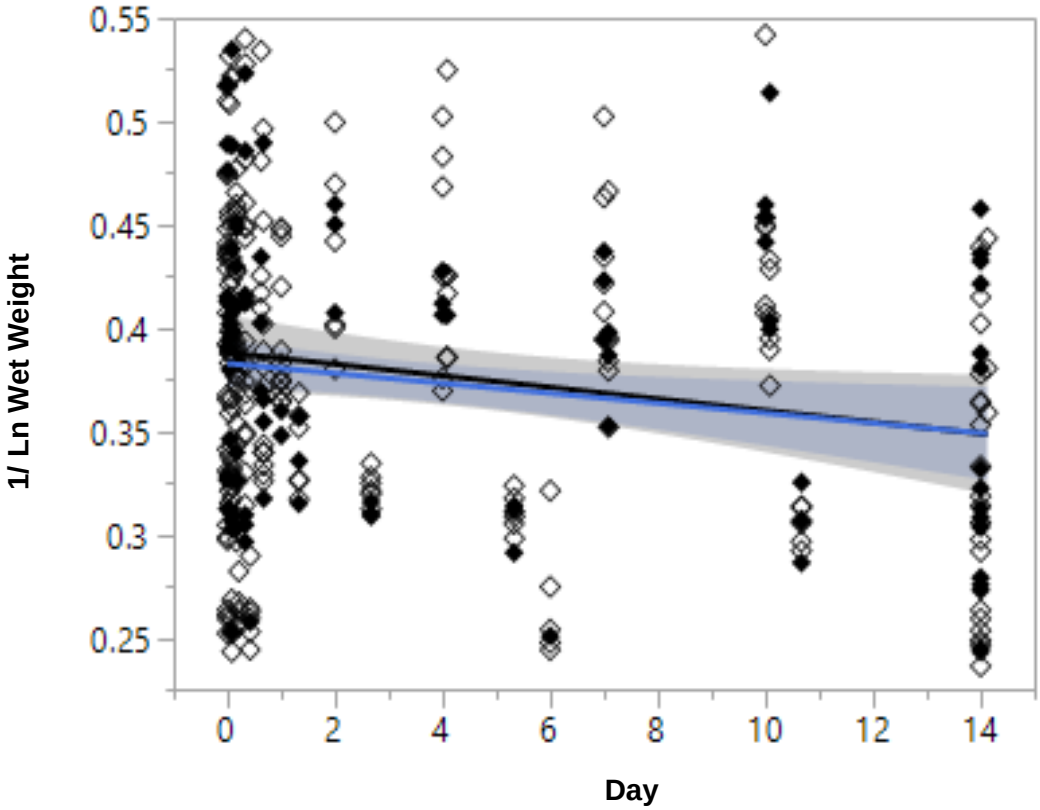

Figure B-1. **Growth of test and control fish measured by the reciprocal natural logarithm of the wet weight (g) versus time (day) with linear regression fit line and 95% confidence intervals.**

Table B-1. **Linear regression fit equation and parameter estimates (R^2, p value and number of individuals (N)) for the growth of control and treatment fish from four BCF tests.**

Treatment (legend)	Linear Regression Fit (±SE)	Statistics
Control (◆)	1/Ln ww = 0.388 ± 0.0092 - 0.0028 ± 0.0013* Day	R^2= 0.0446 p = 0.036 N = 99
Test (◇)	1/Ln ww = 0.388 ±0.0057 – 0.0024 ± 0.001 * Day	R^2= 0.0246 p = 0.0145 N = 242

Appendix C.

Extraction Efficiency and Limits of Detection

Table C-1. Extraction efficiency of test and reference chemicals following the QUEcHERs extraction method from fish tissue.

Chemical	Extraction efficiency %
Test	
Methoxychlor	108
Pyrene	116
Cyclohexyl Salicylate	140
4-n-nonylphenol	NA
Reference	
1,3,5 Trichlorobenzene	108
1,2,4,5 Tetrachlorobenzene	117
Pentachlorobenzene	104
Hexachlorobenzene	94

Note: Extraction efficiencies exceeded 100% due to sample corrections and matrix effects.

Table C-2. Molecular weights and limits of detection (LOD) for each test and reference chemical, determined from calibration curves compiled in solvent (i.e., hexane), water and fish.

Chemicals	MW (g/mol)	LOD (ng/mL)	LOD (ug/mL)	LOD (in mol/kg fish)
Test				
Pyrene	2.02E+02	2.53E+01	2.53E-02	1.25E-05
Methoxychlor	3.46E+02	2.52E+01	2.52E-02	7.30E-06
Cyclohexyl Salicylate	2.20E+02	2.58E+01	2.58E-02	1.17E-05
4-n-nonylphenol	2.20E+02	2.52E+01	2.52E-02	1.14E-05
Reference				
1,3,5-Trichlorobenzene	1.81E+02	2.48E+01	2.48E-02	1.37E-05
1,2,4,5-Tetrachlorobenzene	2.16E+02	2.51E+01	2.51E-02	1.16E-05
Hexachlorobenzene	2.85E+02	2.49E+01	2.49E-02	8.75E-06
Pentachlorobenzene	2.50E+02	2.54E+01	2.54E-02	1.02E-05

Calculating matrix effect (ME):

$$\%ME = \frac{\text{Analyte signal (post-extraction spiked matrix)}}{\text{Analyte Signal (solvent)}} * 100\ \%$$

Combining recovery (R) and matrix effect (ME):

$$\%PE = \frac{\%R * \%ME}{100\%}$$

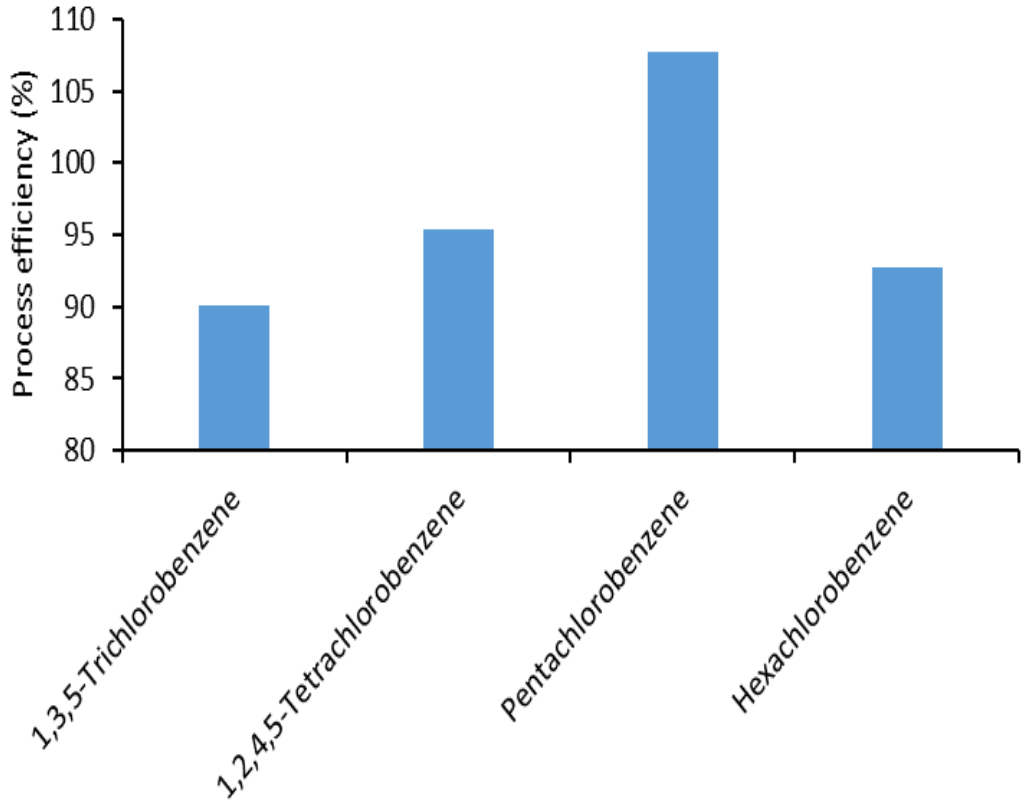

Figure C-1. Process efficiency (%) of four reference chemicals during the QUEChERs extraction of chemicals from fish.

Appendix D.

Acute and chronic toxicity of test and reference chemicals

Table D-1. Acute (LC_{50}) and chronic (NOAEC) toxicity of test and reference chemicals in fish.

Chemicals [CASN]	Acute Toxicity (96-hr LC50) (µg/L) [Ref]	Chronic Toxicity (NOAEC, µg/L) [Ref]	Species
Test			
Pyrene [129-00-0]	>2000 [a]	NA	*Oncorhynchus mykiss*
Methoxychlor [72-43-5]	8.8-132	0.5 [b]	*Oncorhynchus mykiss*
Cyclohexyl salicylate [25485-88-5]	3600	470 (14-days)	*Danio rerio*
4-n-nonylphenol [104-40-5]	140-920 [c,d]	1- 80 [e, f]	*Oncorhynchus mykiss*
Reference			
1,3,5-trichlorobenzene [108-70-3]	5480 [g]	40 [g]	*Oncorhynchus mykiss, Danio rerio*
1,2,4,5-tetrachlorobenzene [95-94-3]	1000	0.34 [i]	*Oncorhynchus mykiss*
Pentachlorobenzene [608-93-5]	34	270	*Oncorhynchus mykiss, Danio rerio*
Hexachlorobenzene [118-74-1]	1000	0.08 [j]	*Oncorhynchus mykiss*

a) (Kennedy, 1990)
b) (US EPA (United States Environmental Protection Agency), 2018)
c) (Dwyer, 1996)
d) (Thaler & Plowright, 1980)
e) (Hébert et al., 2009)
f) (Ward, Duff, & Currie, 2006)
g) (Bosma, vander Meer, Schraa, Tros, & Zehnder, 1988)
h) (ECHA, 2018)
i) NOAEL, 0.34 mg/kg/day (rat, oral), kidney lesions, (Chu, Villeneuvel, Valli, & Secours, 1984)
j) NOAEL, 0.08 mg/kg/day (rat, oral), liver toxicity, (Arnold et al., 1985)

Appendix E.

Model estimates and corrections

Table E-1. Mean water temperatures and wet weights of fish in each bioconcentration test used to correct biotransformation rate constants ($k_{M, BCFBAF}$) estimated in EPI-Suite using the Arnot-Gobas BCFBAF model.

Chemical	$k_{M, BCFBAF}$, N	Temperature, N (°C)	Weight, N (kg)	Temperature, X (°C)	Weight, X (kg)	$k_{M,BCFBAF}$, X
Methoxychlor	3.78E-02	1.50E+01	1.00E-02	1.19E+01	3.92E-02	2.60E-02
Pyrene	1.25E+00	1.50E+01	1.00E-02	1.27E+01	2.18E-02	1.00E+00
Cyclohexyl salicylate	3.52E+00	1.50E+01	1.00E-02	1.31E+01	1.16E-02	3.33E+00
4-n-nonylphenol	5.22E-01	1.50E+01	1.00E-02	1.27E+01	1.00E-02	5.10E-01

The Arnot-Gobas BCFBAF model predicts the biotransformation rate constants ($k_{M,N}$) normalized to a 10 g fish in 15 °C water. Using mean temperatures and fish weights measured in the four BCF tests, the biotransformation rate constants were corrected ($k_{M,X}$).

$$k_{M,X} = k_{M,N}\left(\frac{W_x}{W_N}\right)^{-0.25}\exp(0.01(T_X - T_N))$$

Where, W_X is the mass of the fish (kg), W_N is the normalized mass of the fish (0.01 kg), T_X is the study-specific water temperature (°C), and T_N is the normalized water temperature (15 °C) (US EPA, 2012).