Dietary Biotransformation and Bioaccumulation of Cyclic Siloxanes in Rainbow Trout (*Oncorhynchus mykiss*).

by

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Ethics Statement

The author, whose name appears on the title page of this work, has obtained, for the research described in this work, either:

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Abstract

Cyclic volatile methyl siloxanes (cVMS) are commonly used down-the-drain manufactured substances that were assessed for their bioaccumulation (B) potential in Canada and the European Union, which resulted in different regulations. Discrepancies between regulations resulted in part from the unique intrinsic characteristics of cVMS, their log K_{OW} values, which suggests that cVMS partition out of water and into lipids. Modeled simulations and laboratory-based bioconcentration (BCF) values exceed CEPA 1999 B criteria, while field-based analysis of biomagnification factors (BMFs) can produce values that are below CEPA 1999's B threshold (BCF and/or BMF < 5000) and resulted in biodilution. The current research addressed discrepancies between B values by exploring *in vivo* dietary somatic biotransformation rate constants (k_{BM}) of two cVMS, octamethylcyclotetrasiloxane (D4) and decamethylcyclopentasiloxane (D5), in rainbow trout (*Oncorhynchus mykiss*). The k_{BM}s were derived using a co-exposure of non-metabolized reference chemicals. Plotting the concentration of fish at time (C_F) shows significant but slow somatic depuration rate constants (k_{BT}, d^{-1}) for D4 and D5 (0.045 ± 0.018 (SE); 0.042 ± 0.008 (SE)) and k_{BM} (d^{-1}) (0.039 ± 0.019 (SE); 0.037 ± 0.009 (SE)). The C_F also produced significant, but relatively slow dietary uptake absorption efficiencies (E_D) for D4 and D5 (0.322 ± 0.034 (SE); 0.079 ± 0.008 (SE)). C_F was entered into an Absorption, Distribution, Metabolism, and Excretion Fish Bioaccumulation Calculator (ADME-B) to predict the biotransformation rate constant in the gut (k_{GM},d^{-1}), resulting in relatively fast rate constants of 1.26 ± 0.61 (SE) and 6.46 ± 1.12 (SE) d^{-1} for D4 and D5. Results indicate that the exposure route effects the proportional contribution of somatic (φ_{BM}) and gut (φ_{GM}) biotransformation rate constants on the depuration of D4 and D5. For instance, dietary uptake results in high φ_{GM}s for D4 and D5 (61.34 and 93.02%). Furthermore, the ADME-B calculator indicates that D4 and D5 BCF values are below B criteria (2864.6 ± 1112.1 (SE); 2289.6 ± 532.5 (SE)). Results are consistent with measured environmental concentrations that show biodilution.

Keywords: octamethylcyclotetrasiloxane; decamethylcyclopentasiloxane; down-the-drain chemicals; bioaccumulation; biotransformation; bioconcentration factor; octanol-water partition coefficient; rainbow trout; biomagnification factor; depuration; dietary *in vivo* testing; rate constants

Dedication

This project is dedicated to the type of persistence that we hope to see in the world: love, friendship, and unwavering support. Thank you to my husband, family, and friends, who have persistently shown me all three.

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Completing this project was the ultimate team effort. I would like to start by thanking my senior supervisor, Dr. Frank Gobas, for providing me with the opportunity to take on this project: thank you for your guidance, understanding, and your constant sunny attitude. Your expertise on biological principles and use of analytical concepts to capture the mysteries of bioaccumulation were invaluable to this project and to my understanding of how science can shape policy to better protect our natural environment. I would also like to extend my appreciation to my committee member, Dr. Christopher Kennedy, for taking the time, consideration, and providing the biological expertise to help guide my research and understanding of my project.

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To my friends (old and new): you are truly all an inspiration. You ground me, encourage me, and never cease to amaze me.

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List of Acronyms

ACN	Acetonitrile
B	The bioaccumulation potential of a substance as outlined in s. 64 of CEPA 1999
BAF	Bioaccumulation Factor
BCF	Bioconcentration Factor
BoR	A Board of Review comprised of experts and legal aids established under ss.333(1) of CEPA 1999 that are assigned to assess the PBiT of a substance under s. 64 of CEPA 1999
BMF	Biomagnification Factor
bw	Body Weight (kg)
C	Concentration
C_D	Concentration of Chemical in the Diet (g chemical.kg feed^{-1})
$C_{DILUTED}$	Test Chemical Feed that was Diluted with Reference-Spiked Feed
C_F	Concentration in the Fish Body
CEPA 1999	The Canadian Environmental Protection Act of 1999
C_{OC}	Concentration of organic content in water
C_{STOCK}	Starting Feed Stock Containing 0.2 g Test Chemical.kg feed^{-1}
cVMS	Cyclic Volatile Methyl Siloxane
C-18	Octadecylsilane
D4	Octamethylcyclotetrasiloxane
$D4_{HIGH}$	Tank Fed Spiked Feed containing 2 g D4.kg^{-1} ww fish
$D5_{HIGH}$	Tank Fed Spiked Feed containing 2 g D5.kg^{-1} ww fish
D5	Decamethylcyclopentasiloxane
DSL	The Domestic Substance List is a list of substances imported, manufactured or used in Canada at amounts $\geq$ 100 kg.year^{-1}
d12	Chrysene-d12
EC	Environment and Climate Change Canada
ECHA	The European Chemicals Agency, which manages technical and administrative regulatory action for substances in the European Union
E_D	Uptake Absorption Efficiency

$E_{D,R}$	Relationship between the absorption efficiency for the reference chemicals and their respective log K_{OW}S
ES	External Standard
EU	European Union
GC/MS	Gas Chromatography/Mass Spectrometry
HCBz	Hexachlorobenzene
IS	Internal Standard
iT	The inherent toxicity of a substance as outlined in CEPA 1999
k_T	Depuration Rate Constant (d^{-1})
k_{BT}	Depuration Rate Constant in the body of the fish (d^{-1})
$k_{BT,R}$	Depuration rate constant a reference chemical in the body of the fish (d^{-1})
$k_{T,R}$	Depuration rate constants of the reference chemicals (d^{-1})
k_D	Dietary Uptake Rate Constant (d^{-1})
k_E	Elimination Rate Constant via Fecal Egestion Rate
k_G	Elimination Rate Constant via Growth Dilution (d^{-1})
k_M	Elimination Rate Constant via Biotransformation (d^{-1})
K_{OC}	Octanol-Organic Carbon Partition Coefficient
K_{OW}	Octanol-Water Partition Coefficient
k_1	Gill respiration uptake rate constant (d^{-1})
LOEC	Lowest observed effect concentration ($g.L^{-1}$)
M_B	Mass (g) of chemical in the body
M_G	Mass (g) of chemical in the gut
$MgSO_4$	Anhydrous Magnesium Sulfate
MS-222	Tricaine Methanesulfonate for Euthanizing Fish
MSC	The Member State Committee
M4Q	Tetrakis(trimethylsiloxy)siloxane
NaCl	Sodium Chloride
NOEC	No observed effect concentration
P	The persistence ($t_{1/2}$) of a substance as outlined in s. 64 of CEPA 1999
PA	Peak Area
PAR	Peak Area Ratio
$PAR_{PRE\ XT,C}$	Peak Area Ratio of the Pre Extracted Standard Curve at a known

	concentration
$PAR_{POST\ XT,C}$	Peak Area Ratio of the Post Extracted Standard Curve at a known Concentration
PBiT	Persistence (P), bioaccumulation (B) and inherent toxicity (iT) criteria as outlined in s. 64 of CEPA 1999.
PBT	Persistent, Bioaccumulative and Toxic substance classification under REACH
PCBs	Polychlorinated Biphenyls
PCBz	Pentachlorobenzene
PCB 52	2,2',5,5' - Polychlorinated Biphenyl
PCB 153	2,2',4,4',5,5' - Polychlorinated Biphenyl
PCB 209	2,2',3,3',4,4',5,5',6,6' - Polychlorinated Biphenyls
PNOEC	Predicted no effect concentration
POST XT	Standard Curves Created by Spiking Tissue/Feed after Extraction
PSA	Primary Secondary Amine
PRE XT	Standard Curves Created by Spiking Tissue/Feed before Extraction
QuEChERS	Quick, Easy, Cheap, Effective, Rugged Safe Extraction Method
RBF	Round Bottom Flask
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals is the substance regulatory authority in the European Union
rpm	Rotations Per Minute
SCCS	The Scientific Committee on Consumer Safety
SD	Standard Deviation of the Mean
SE	Standard Error of the Mean
SEHSC	Silicones Environmental, Health and Safety Council
SIL	Tetrakis(trimethylsilyl)siloxane
TCBz	Tetrachlorobenzene
vP	Ver Persistent as classified under REACH Annex XIII
vPvB	Very Persistent, Very Bioaccumulative substances as classified under REACH
ww	Wet Weight (kg)
WWTP	Wastewater Treatment Plants

φ_{BM}	The proportional contribution of the biotransformation rate constants for the somatic tissue (%)
φ_{GM}	The proportional contribution of the biotransformation rate constants for the gut (%)

Glossary

Bioaccumulation	A process resulting in the accumulation of chemical substances within an organism that is exposed to chemical sources (i.e., dietary, respiration, environmental media, etc.) and where accumulation occurs at a faster rate than the organism's ability to eliminate the substance
Bioaccumulation Factor (BAF)	A numerical value representing the degree of bioaccumulation of a substance as measured under field or lab-derived conditions, which considers the dietary uptake of a chemical substance
Bioconcentration	The process of chemical uptake by an organism from its environmental media via respiration and/or dermal application and does not include dietary uptake
Bioconcentration Factor (BCF)	A laboratory-based assessment that derives a numerical value of the degree of bioaccumulation of a chemical substance from respiration and/or dermal application and does not include dietary uptake
Biomagnification	The process of increasing chemical accumulation from prey to predator species and is often assessed using dietary-based laboratory or in the field experiments and measurements
Biomagnification Factor (BMF)	The numerical degree to which biomagnification occurs from predator to prey species
Biotransformation	The process of converting a chemical to its metabolites via enzymatic activity within an organism
Biotransformation rate constant (k_M)	The rate constant describing the rate of conversion from a chemical to its metabolites which reduced the concentration of the parent substance within an organism

Depuration	The reduction of chemical concentration within an organism, which considers all modes of elimination (i.e., fecal, urination, biotransformation, somatic growth, etc.)
Depuration rate constant (k_T)	The numerical value expressing the rate of chemical elimination in an organism over time, which occurs after the chemical spiked diet has been replaced with a control diet
Dietary absorption efficiency (E_D)	The numerical value expressing an organisms ability to incorporate chemicals from their diets into the organism's cells
Dietary uptake rate constant (k_D)	The numerical value expressing an organism's rate of chemical uptake from the diet
Octanol-water partition coefficient (K_{OW})	The numerical value expressing the ratio between a chemical in 1-octanol and water. It is used to determine the hydrophilic or hydrophobic tendencies of a chemical
Polychlorinated biphenyls (PCBs)	A class of organic chemicals consisting of chlorinated double benzene rings and known for their bioaccumulation tendancies
Somatic biotransformation rate constant (k_{BM})	A numeric value expressing the relationship of chemical elimination from somatic tissues in an organism (e.g., does not include the stomach or intestines)
Somatic depuration rate constant (k_{BT})	A numeric value expressing the chemical elimination from somatic tissues within an organism after the spiked died has been replaced with a control diet

Chapter 1.
Introduction

Octamethylcyclotetrasiloxane (D4) and decamethylcyclopentasiloxane (D5) are man-made chemicals commonly used in personal care products that have the potential to enter aquatic environments after in-shower-use as down-the-drain chemicals (EC and HC 2008b/a). Given their extensive use in Canada, D4 and D5 were screened and evaluated under s. 64 of the Canadian Environmental Protection Act (CEPA 1999) to determine if they exceed criteria for persistence (P), bioaccumulation (B), and inherent toxicity (iT) in the Canadian environment. The *Screening Assessment for the Challenge of D4* (2008b) predicted that it exceeds s. 64 CEPA 1999 criteria for P (sediment and air) and could exceed iT criteria, thus producing lethal health effects in early life-stage and sensitive species at environmental concentrations within its solubility limit. The *Screening Assessment for the Challenge of D5* (2008a) predicted that D5 exceeds criteria for P (air, water and sediment). The *Screening Assessment* (2008a) also stated that D5 could exceed iT criteria and could cause adverse effects to pelagic organisms via non-polar narcosis and cancerous modes at its water solubility limit.

Results from the *Screening Assessments* (EC and HC 2008b/a) provoked a proposal to add D4 and D5 to the Domestic Substance List (DSL), the creation of a risk management plan enacted under the Chemicals Management Plan, and the listing of D4 and D5 as Schedule 1 toxic substances under CEPA 1999 (EC and HC 2009). The *Screening Assessments* (EC and HC 2008b/a) and the resulting Risk Management Approach (EC and HC 2009) also asserted that D4 and D5 did not conclusively meet s. 64 CEPA 1999 B criteria based on the available information that produced inconsistent results concerning risk. However, since relevant information was not available, it was determined that the Precautionary Principle should be applied to reduce the risk of uncertainty associated with the iT potential outlined in the literature.

Stakeholders filed an opposition to the proposed listing of D4 and D5 as toxic substances under Schedule 1. Stakeholders provided new evidence concerning D5 and a Board of Review (BoR) comprised of experts was assembled under s. 333(1) of CEPA 1999 to assess D5's PBiT potential (BoR 2011, Giesy et al. 2016). The D5 BoR (2011) determined that D5 does not exceed s. 64 CEPA 1999 PBiT criteria and does not pose a risk to the environment. The BoR made its conclusions based on measured biodilution, which was assumed to be from biotransformation (i.e., metabolic breakdown via

enzymatic activity within the fish), and measured environmental concentrations that are below iT or B potential.

A BoR was not established for D4 because new evidence was not presented. D4 has since been added to the Schedule 1 toxic substances list based on P and iT assessments from the *Screening Assessment* (EC and HC 2008b). D4 is currently regulated in industrial effluents at concentrations ≤ 17.3×10^{-5} $g.L^{-1}$ and/or total quantities of ≤ 4000 $g.year^{-1}$ (GC 2017).

Furthermore, the EU assessed D4 and D5 and regulated concentrations to < 0.1% in personal care products. D4 and D5 were regulated because they met criteria for very persistent and very bioaccumulative (vPvB) substances under Article 14(2)(f) of the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulations (ECHA 2018).

The findings of the D5 BoR (2011), the regulation of D4 in Canada, the precautionary-based regulation of D4 and D5 in the EU, and inconsistencies within the literature concerning the bioaccumulation potential of D5 prompted additional research into understanding the depuration kinetics of cVMS in aquatic organisms (Woodburn et al. 2013). It was proposed that biotransformation rates are responsible for measured biodilution occurring in the environment (Powell et al. 2017, EC and HC 2009) and the effect of biotransformation on concentration had not been properly captured by predictive BCF models (EC and HC 2009). Though overall elimination rates have been studied, isolating the body compartments (i.e., body tissue vs. digesta) responsible for metabolising cVMS had not been evaluated. As such, isolating biotransformation rate constants could help explain the inconsistences between predicted and observed cVMS concentrations and help settle regulatory differences between Canada and the EU.

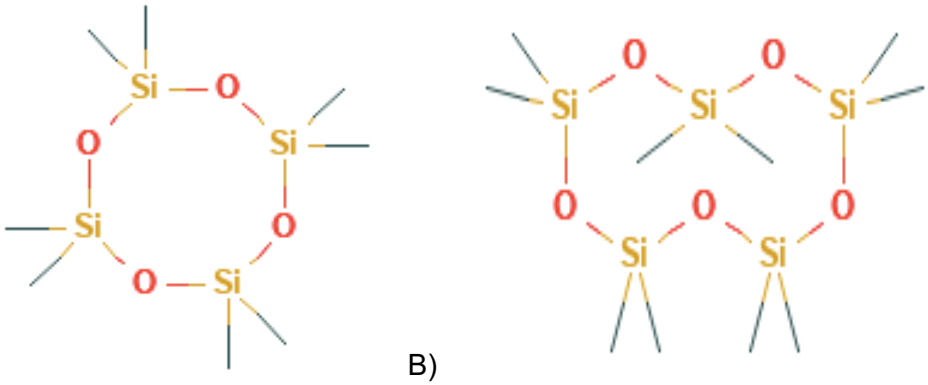
The main objectives of the current study include: 1) to determine the biotransformation rate constants (k_M) of D4 and D5 in rainbow trout and 2) to determine the contribution of biotransformation to the depuration rate and BCF of D4 and D5 in fish.

Chapter 2.

Background

2.1. Cyclic Volatile Methyl Siloxanes (cVMS)

Cyclic volatile methyl siloxanes (cVMS) are non-naturally occurring synthetic compounds that are comprised of a characteristic methylated silicon ring. Dow Chemical ®^{TM} is one of the primary producers of cVMS in North America. Of the varieties of cVMS manufactured by Dow Chemical ®^{TM}, octamethylcyclotetrasiloxane (D4) and decamethylcyclopentasiloxane (D5), are the focus of this study. D4 and D5 are relatively small organosilicon molecules with molecular masses of 296.62 and 370.78 g.mol^{-1}, respectively (EC and HC 2009). D4 and D5 are comprised of carbon, hydrogen, oxygen and silicon (D4: $C_8H_{24}O_4Si_4$; D5: $C_{10}H_{30}O_5Di_5$) (PubMed 2019) and have four and five-methyl groups extending from their silicon rings (Figure 2.1A/B).



A) B)

Figure 2.1. Chemical structure of A) octamethylcyclotetrasiloxane (D4) and B) decamethylcyclopentasiloxane (D5) as retrieved from PubMed (2019a/b).

2.2. Canadian Use and Exposure Routes

Large quantities of D4 and D5 are imported into and used in Canada. For instance, in 2006 an estimated 1-10 million kg.year^{-1} of both D4 and D5 were imported into Canada (EC and HC 2008). It is estimated that ≥ 80% of imported D4 is used to produce silicone polymers or copolymers that become chemically transformed via processing (EC and HC 2009). Estimates suggest that the remaining amount of D4 enters the air (~12%) and wastewater (~5%) via personal or industrial product use and processing (EC and HC 2009).

In 2010, it was estimated that 3.3 million kg of D5 is used each year in Canada, with an estimated primary use (99.9%) in consumer-care products (BoR 2011), primarily

due to their emulsification, lubricating, surfactant, and defoaming properties. The amount of D5 in personal care products ranges in concentration (0.001-100%) with the majority of products comprised of ≤ 5% (EC and HC 2009). The remaining D5 is used as raw or intermediate materials to create silicone polymers and in commercial dry cleaners (EC and HC 2009). It is estimated that > 80% of D5 is released into air and ~10% is released to wastewater systems via down-the-drain product use, with some release associated with processing (EC and HC 2009, Gobas et al. 2015b). It is also estimated that ~40% of the relative concentration of D5 in conditioners and other rinse-off after in-shower use personal care products enter wastewater treatment plants (WWTPs) from drains (Mackay et al. 2015). Thus, rinse-off after in-shower-use personal care products result in the greatest source of potential exposure to aquatic organisms.

D4 and D5 have large socio-economic benefits in Canada. D4 and D5 are associated with the preparation and manufacturing sub-sector that produces shampoos, conditioners, face and body creams, and makeup, etc. In 2005, this industry produced $1.3 billion dollars in revenue, was associated with 196 working establishments, and employed 5608 people in Canada (EC and HC 2009).

D4 and D5 are also relatively cheap substances, costing $10 kg^{-1} in 2005 (EC and HC 2009). When alternatives are available, they tend to cost slightly more than D4 and D5 ($11-13 kg^{-1}). Furthermore, the Government of Canada has not evaluated the effectiveness or the persistence, bioaccumulative and inherent toxicity (PBiT) capabilities of alternatives for D4 and D5 under s. 64 of CEPA 1999 (EC and HC 2009).

2.3. Biological Exposure

D4 and D5 can enter human and aquatic organisms through multiple exposure routes including lung or gill respiration, uptake via the skin from direct application, and dietary uptake. Aquatic organisms are primarily exposed to cVMS due to their extensive use in personal care products as down-the-drain or wash-off chemicals. Down-the-drain or wash-off chemicals are commonly associated with consumer care products that enter the environment via wastewater systems.

WWTPs filter cVMS from wastewater. WWTPs remove ~92% of cVMS from entering the environment (Mackay et al. 2015, Wang et al. 2015). It is hypothesized that the majority of cVMS become bound to the organic components of aerosols and wastewater sludge (Mackay et al. 2015). The remaining quantities of free cVMS can enter aquatic biota via gill respiration or through the diet. The widespread use of cVMS

has resulted in ubiquitous concentrations throughout various environmental compartments (air: Genualdi et al. 2011; landfill biogas/air: McBean 2008, Steer et al. 2008; soil, influent, effluent, receiving water and wastewater sediment: Wang et al. 2013b) and organisms including invertebrates, fish and marine mammals (D4: Sanchis et al. 2016, Kaj et al. 2005a/b, Brooke et al. 2009a/b/c, Kierkegaard et al. 2010, Kierkegaard et al. 2011, Kierkegaard et al. 2012a/b; D5: Kaj et al. 2005b, Brooke et al. 2009a/b/c, Warner et al. 2010, Kierkegaard et al. 2010). Furthermore, cVMS presents the greatest exposure potential to biota in aquatic environments compared to those that respire air because cVMS are oxidized via hydroxyl radicals in the atmosphere and are no longer bioavailable (Mackay et al. 2015).

2.4. The Canadian Environmental Protection Act, 1999

The Government of Canada aims to protect the Canadian environment and human health from harmful substances through the Canadian Environmental Protection Act, 1999 (CEPA 1999). CEPA 1999 evaluates the risk of chemical substances if amounts ≥ 100 kg are manufactured, imported and/or used in Canada. Substances that meet the previous criteria are added to the domestic substances list (DSL). The DSL is assessed under CEPA 1999 by Health Canada and Environment and Climate Change Canada (EC). Health Canada assesses DSL substances for their potential for human exposure and the EC focuses on assessing environmental health. Both Health Canada and the EC assess if substances on the DSL are persistent (P), bioaccumulative (B) and/or inherently toxic (iT) as outlined in CEPA 1999. If a DSL substance is determined to be P and/or B and iT to humans or non-human organisms, they undergo a screening level risk assessment. If PBiT potential meets CEPA 1999 criteria, the substance is determined to be 'CEPA toxic' and is added to the list of toxic substances with possible regulatory responses. If more information is required to determine P and/or B causing iT, the substance is placed on a list of priority substances for further assessment. If the substance does pose risk as outlined by CEPA 1999, no further action is taken.

Persistence is defined as any substance that has a half-life ($t_{1/2}$) that exceeds the time outlined in the *Persistence and Bioaccumulation Regulations* (The *Regulations*) (Table 2.1).

Table 2.1. List of media and metrics for persistence (P) and bioaccumulation (B) criteria as listed in the *Persistence and Bioaccumulation Regulations* associated with CEPA 1999.

Medium/Parameter	Value
Air	$t_{½} \geq 2$ days
Water	$t_{½} \geq 6$ months
Sediment	$t_{½} \geq 1$ year
Soil	$t_{½} \geq 6$ months
BAF	≥ 5000
BCF	≥ 5000
log K_{OW}	≥ 5

CEPA 1999 classifies B substances using the octanol-water partitioning coefficient (K_{OW}), the bioconcentration factor (BCF), and the bioaccumulation factor (BAF). According to The *Regulations*, if a substance has a log $K_{OW} > 5$ it meets B criteria under s. 64 of CEPA 1999. See Table 2.1 for B classification criteria values from The *Regulations*.

The BCF and BAF are two metrics used by CEPA 1999 to estimate if a substance will bioaccumulate into an organism passively from water and/or via the diet. The *Regulations* define the BCF as the "ratio of the concentration of a substance in an organism to the concentration in water, based only on the uptake from the surrounding medium," where primary uptake is via the gills. The BAF is defined as "the ratio of the concentration of a substance in an organism to the concentration in water, based on uptake from the surrounding medium and food," where uptake is passive via the gills and active through the diet. The BCF can only be derived within a lab, whereas the BAF can be determined in the lab or in the field. If the BCF or BAF > 5000, then the substance meets B criteria under s. 64 of CEPA 1999 (Table 2.1)

The *Regulations* further prioritize the BAF over the BCF, and the BCF over the K_{OW}. Prioritizing BAF is useful because field BAF measurements represent a real-world measure of the concentration of a substance within a fish compared to its surrounding environments from both respiration and dietary uptake routes. The BAF also captures the ability of a substance to accumulate in an organism via the substance's bioavailability and the organism's ability to metabolize that substance. In contrast, the BCF is derived using lab tests, and the K_{OW} is a lab-derived or model predicted value. If BAF, BCF or K_{OW} exceed CEPA 1999 B criteria then concentrations could produce toxic effects and the substance is investigated to determine its hazard potential.

Other real-world data such as the biomagnification factor (BMF), the trophic magnification factor (TMF) and the biota-sediment accumulation factor (BSAF) are not criteria under CEPA 1999 but can be used as supporting evidence to assess the risk posed by a substance. A BMF, TMF and/or BSAF value greater than 1 indicates that biomagnification or trophic magnification is occurring (CEPA 1999).

CEPA 1999 classifies a substance as a B substance if it meets the following criteria: 1) if continuous dietary or gill respiration exposure of the substance over time results in the accumulation of the substance in cells, 2) if the rate of uptake is greater than rate of elimination, and 3) causes harm to organisms. A substance's potential to cause 'harm to organisms' is assessed when determining it's inherent toxicity (iT). The iT of a substance is based on the "hazard a substance presents to the environment" (CEPA 1999). The iT hazard is categorical and is based on the environmental impacts associated with the potency of a test substance from either predicted models or empirical studies. The *Regulations* propose that a substance is iT in non-human organisms if one or several of the following conditions or criteria exist: 1) If acute concentrations of ≤ 0.001 $g.L^{-1}$ produce lethal concentrations (LC_{50}) or effect concentrations (EC_{50}) in 50% of the test population, or chronic exposure at concentrations ≤ 0.0001 $g.L^{-1}$ produces no observed effect concentration (NOEC) in the test population, 2) If concentrations > 0.001 $g.L^{-1}$ could produce hazard based on other substance-related considerations such as metabolism or high molecular weights, and 3) If a substance has a log K_{OW} > 6 then the substance could have effects to wildlife based on its intrinsic nature to partition from water into lipids.

2.5. cVMS and B Criteria

The above-mentioned CEPA 1999 criteria recognizes the potential for cVMS to be classified as B substances based on their intrinsic chemical properties. For instance, D4 and D5 have log K_{OW}s > 5 (D4 = 6.49; D5 = 8.03), which exceeds B criteria (EC and HC 2008). According to The *Regulations,* the log K_{OW} values of D4 and D5 are high enough (> 6) to garner special consideration concerning their potential effects to wildlife based on their ability to partition out of surrounding water and into lipids.

Measured and modeled BCF and BAF values provide conflicting predictions concerning cVMS bioaccumulation potential. The measured and modeled bioaccumulation potential values for D4 range well below and above CEPA 1999 B criteria for BCF and BAF (Table 2.2). A study measuring BCF by Opperhuizen et al.

(1987) on goldfish produced below B criteria values. Since then, various studies measuring BCF have produced values that are greater than 5000 (Fackler et al. 1995, Drottar et al. 2005). Predicting the BCF of D4 using the Arnot and Gobas (2003) model produced an even more variable range of values above and below CEPA 1999 B criteria. Using the Arnot and Gobas (2003) model to predict bioaccumulation potential also produced above B criteria for BAF (Table 2.2). Studies assessing field-based and dietary *in vivo* concentrations of D4 including the BSAF, TMF, BMF, kinetic BMF (BMF_k), lipid-corrected BMF (BMF_L) and kinetic/lipid corrected BMF ($BMF_k(L)$) also produced conflicting evidence for and against whether D4 met B substance classification (Table 2.2).

Table 2.2. Representative values of the bioaccumulation potential of octamethylcyclotetrasiloxane (D4) using various field-based and modeled metrics. All metrics were obtained via empirical studies unless otherwise stated. Values obtained from studies prior to 2008 have been summarized from EC and HC (2008b). Post-2008 values were added from their respective original sources.

Test Organism	Metric	Value	Unit	Reference	Notes
---	Log K_{OW}	6.49		EC and HC 2008	
P. reticulata	BCF	1,090	L. kg^{-1} ww	Opperhuizen et al. 1987	
P. promelas	BCF	8,700	L. kg^{-1} ww	Fackler et al. 1995	
P. promelas	BCF	12,600	L. kg^{-1} ww	Drottar et al. 2005	
Mid. trophic level fish	BCF	1,413 - 12,589	L. kg^{-1} ww	Arnot and Gobas 2003	Modeled
Mid. trophic level fish	BAF	12,022 - 467,735		Arnot and Gobas, 2003	Modeled
O. mykiss	BMF	0.28	kg feed.kg^{-1} ww	Woodburn et al. 2013	
O. mykiss	BMF_L	0.62 - 0.75	kg lipid.kg $lipid^{-1}$	Drottar et al. 2007, Domoradzki 2008a, SEHSC 2008b	
O. mykiss	BMF_L	0.66	kg lipid.kg $lipid^{-1}$	Woodburn et al. 2013	
O. mykiss	BMF_k	1.7	kg lipid.kg $lipid^{-1}$	Woodburn et al. 2013	
O. mykiss	$BMF_k(L)$	4.8	kg lipid.kg $lipid^{-1}$	Woodburn et al. 2013	
Midge	BSAF	0.70 - 2.2	kg dry w.kg^{-1} ww	Kent et al. 1994	
Aquatic Environ.	TMF	0.5 - 0.6		Powell et al. 2017	

Measured and modeled BCF and BAF values produced conflicting bioaccumulation potential for D5. Measured and modeled BCF values varied from below to above B criteria (1200 – 13 700) (Table 2.3). BMF, BMF_k, BMF_L and BMF_k(L) values range below and above B criteria (Table 2.3). Other field-based measurements including BSAF and TMF also range below and above B criteria (Table 2.3).

Table 2.3. Representative values of the bioaccumulation potential of decamethylcyclopentasiloxane (D5) using various field-based and modeled metrics. All metrics were obtained via empirical studies unless otherwise stated. Values obtained from studies prior to 2008 have been summarized from EC and HC (2008a). Post-2008 values were added from their respective original sources.

Test Organism	Metric	Value	Original Study	Unit	Reference	Notes
---	$logK_{OW}$	8.03			EC 2008	
Various Aquatic	BCF	1120 - 13 300		$L.kg^{-1}$ ww	Gobas el al. 2015a	Reference
Various Aquatic	BCF	5900 - 137000		$L.kg^{-1}$ ww	Gobas et al. 2015b	Reference
P. reticulata	BCF	1040		$L.kg^{-1}$ ww	Opperhuizen 1987	
P. reticulata	BCF	1120	Opperhuizen 1987	$L.kg^{-1}$ ww	Gobas et al. 2015b	Modeled
P. reticulata	BCF_L	800	Opperhuizen 1987	$L.kg^{-1}$	Gobas et al. 2015b	Modeled
P. promelas	BCF	3800		$L.kg^{-1}$ ww	Annelin and Frye 1989	
P. promelas	BCF	2100	Annelin and Frye 1989	$L.kg^{-1}$ ww	Gobas et al. 2015b	Modeled
P. promelas	BCF	4450 - 4920		$L.kg^{-1}$ ww	Parrot et al. 2010	
P. promelas	BCF	4500	Parrot et al. 2010	$L.kg^{-1}$ ww	Gobas et al. 2015b	Modeled
P. promelas	BCF_k	13300		$L.kg^{-1}$ ww	Drottar 2005	
P. promelas	BCF_k	13700	Drottar 2005	$L.kg^{-1}$ ww	Gobas et al. 2015b	
P. promelas	BCF_k	5300		$L.kg^{-1}$ ww	Drottar 2005	Applied k_T
P. promelas	BCF_k	5900	Drottar 2005	$L.kg^{-1}$ ww	Gobas et al. 2015b	Applied k_T
O. mykiss	BCF_k	3.4 ± 1.4		$kg\ lipid.kg^{-1}lipid$	Drottar 2006	
O. mykiss	BCF_k	0.27	Drottar 2006	$kg\ feed.kg^{-1}ww$	Gobas et al. 2015b	Modeled
P. reticulate	BMF_L	0.08		$kg\ lipid.kg\ lipid^{-1}$	Opperhuizen 1987	
P. reticulate	BMF	0.05	Opperhuizen 1987	$kg\ feed.kg^{-1}ww$	Gobas et al. 2015b	Modeled
O. mykiss	BMF	0.32		$kg\ feed.kg^{-1}ww$	Woodburn et al. 2012	
O. mykiss	BMF_L	0.85		$kg\ lipid.kg\ lipid^{-1}$	Woodburn et al. 2012	
O. mykiss	BMF_k	1.3		$kg\ lipid.kg\ lipid^{-1}$	Woodburn et al. 2012	
Aquatic environ.	BMF_k (L)	0.83		$kg\ lipid.kg\ lipid^{-1}$	Gobas et al. 2015b	
O. mykiss	BMF_k (L)	3.4		$kg\ lipid.kg\ lipid^{-1}$	Woodburn et al. 2012	
L. variegates	BSAF	4.29	Krueger et al. 2010	$kg\ dry\ w.kg^{-1}ww$	Gobas et al. 2015b	
L. variegates	BSAF	4.4		$kg\ dry\ w.kg^{-1}ww$	Gobas et al. 2015b	
Aquatic environ.	TMF	0.2 - 3.2			Gobas et al. 2015a	
Aquatic environ.	TMF	1.62 - 5.58			Borga et al. 2012	
Aquatic environ.	TMF	< 1			Gobas et al. 2015b	

Models that consider the solubility limitations of D4 and D5 indicate that they are not toxic or bioaccumulative to biota that are exposed to various types of cVMS-containing environmental media. Such models used the principles of fugacity and activity to apply the intrinsic limitations, including the solubility, of cVMS in multiple types of environmental media. These models allow assessors to create risk profiles based on the chemical's ability to accumulate within a given environmental media (i.e., air, water, sediment, biota, etc.) at concentrations that could cause toxic effects by comparing measured environmental concentrations to concentrations applied in toxicity tests on various species. The activity and/or fugacity models have been applied to measured D4 and D5 environmental concentrations, NOECs, and adverse effect concentrations from toxicity tests (D4: unpublished assessment using the Gobas et al. (2015) Thermodynamic Activity Calculator for Solid and Liquid Hydrophobic Organic Chemicals in Environmental Media: Excel Model ver. 1.1; D5: Gobas et al. 2015b). Examining D4 and D5 using the fugacity and/or activity models allowed assessors to remove any toxicity test results that are obtained from unrealistic concentrations that cannot be achieved in a given environmental media. Removing unrealistic toxicity data allowed activity and/or fugacity models to predict that cVMS are not bioaccumulative or toxic in multiple organisms that are exposed to cVMS from various environmental media.

Overall, metrics derived from the field and models that consider metabolism (e.g., BMF and TMF) and solubility limitations (e.g., fugacity and activity) generally indicate that D4 and D5 are not bioaccumulating in the environment. Metrics from predictive models, including the BCF and K_{OW}, which do not consider metabolism or measured field environmental concentrations, indicate that D4 and D5 may meet B criteria according to s.64 of CEPA 1999 (D4: Table 2.2; D5: Table 2.3).

2.6. Canadian Regulatory Response to cVMS as B Substances

The regulation of cVMS as B substances in Canada has a complicated history. D4 and D5 were identified under the Chemicals Management Plan as high priority substances that required assessment for their high risk to human and ecological health. The Ministers of Health Canada (HC) and Environment and Climate Change Canada (EC) published the *Challenge* for D4 to industry that enacted 'information-gathering-provisions' under s. 71 of CEPA 1999 in February 2007. Given its structural similarity to D4 and thus potential to meet PBiT criteria, the *Challenge* for D5 was published three months later. Both substances prompted human and ecological health assessments.

In November 2008, EC and HC conducted a *Screening Assessment for the Challenge* of D4 (EC and HC 2008b) and D5 (EC and HC 2008a) to assess whether they met PBiT criteria under s. 64 of CEPA 1999. The *Screening Assessment for the Challenge* of D4 predicted that D4 exceeds P criteria in sediment ($t_{1/2}$ = 49 – 588 d) and air ($t_{½}$ = 5.5 – 22.8 d) (EC and HC 2008b).

The *Screening Assessment for the Challenge of D5* found that D5 met criteria for P and iT. D5 exceeded P criteria in air ($t_{½}$ > 3 d), water ($t_{1/2}$ = 1 – 733 d) and sediment ($t_{1/2}$ = 49 – 588 d). Furthermore, it was suggested that the persistence of D5 increases under neutral pH environments and in the cooler temperatures (≤ 5°C) associated with the average Canadian climate (EC and EC 2008a).

The *Screening Assessments* (EC and HC 2008b/a) also stated it was unable to determine if D4 and D5 met B criteria under s. 64 of CEPA 1999 because of high uncertainty associated with conflicting results from the primary literature. For instance, the BMF_L for D4 in aquatic environments was determined to be between 0.62 - 0.75, and thus not bioaccumulative under CEPA 1999 (Drottar 2007, Domoradzki 2008a/b, SEHSC 2008b). However, studies assessing other B criteria values using different models produced variable results. For instance, the Arnot and Gobas model (2003) accounts for variations in metabolic rates and was used to predict the BCF and BAF for D4. The Arnot and Gobas (2003) model produced values that were below and above CEPA 1999 B criteria (BCF = 1413 – 12 589, average = 4365; BAF = 12 022 – 46735, average = 89 125) (EC and HC 2008b). The above-mentioned examples combined with the studies presented in Table 2.2 produced conflicting data. EC and HC Canada (2008b) concluded that the data was insufficient to conclude if D4 met B criteria.

The *Screening Assessment* (HC and EC 2008a) stated it could not determine if D5 meets CEPA 1999 B criteria. The calculated BMF_L values were below B criteria (0.82 - 0.91). The Arnot and Gobas (2003) model was applied using *in vivo* values of D5 metabolism in rainbow trout to calculate a BCF and BAF for D5 (Drottar 2006, Springer 2007). The Arnot and Gobas (2003) model yielded a BCF of 759 – 4265 (average = 1905) and a BAF of 269 153 – 2 630 268 (average = 1 023 293) (EC and HC 2008a), which exceed B criteria for BAF but not for BCF.

The *Screening Assessment for the Challenge of D5* (EC and HC 2008a) also provided statements concerning the possible bioaccumulative nature of D5 including: 1) predicted BCF > 5000, suggesting that fish and lower-trophic level aquatic organisms might be subject to D5 bioaccumulation, 2) D5 might be metabolized differently in

various aquatic species, and 3) BMF and other field-based studies indicate that the potential for D5 to bioaccumulate in fish and invertebrates is low, possibly due to metabolism or reduced bioavailability due to partitioning coefficients that are associated with adhering to organic carbon (i.e., high octanol-carbon partitioning coefficients (K_{OC})) and rapid degradation via hydrolysis.

The *Screening Assessment*s also stated that D4 and D5 have the potential to meet CEPA 1999 iT criteria in aquatic organisms (EC and HC 2008b/a). D4 met iT criteria because of its potential to cause developmental and lethal health effects to early life-stage and sensitive species. Concentrations of D4 that produce hazard have been measured below the solubility limit (5.6×10^{-5} g.L^{-1}). Furthermore, a lowest observed effect concentration (LOEC) of 6.9×10^{-6} g.L^{-1} and a no observed effect concentration (NOEC) of 4.4×10^{-6} g.L^{-1} was determined for early life-stage rainbow trout under 0.001 kg (Sousa et al. 1995). A NOEC for survival and reproduction (8.0×10^{-6} g.L^{-1}) and a LOEC for survival (1.5×10^{-5} g.L^{-1}) were also measured in the water flea (Sousa et al. 1995; EC and HC 2008b).

D5 met iT criteria based on its potential to cause adverse effects via non-polar narcosis and cancerous modes to pelagic organisms at a predicted no effect concentration (PNOEC) of 1.5×10^{-5} g.L^{-1} (EC and HC 2008a). D5's PNOEC coincides with its solubility in water. The *Screening Assessment* identified that the majority of real-world concentrations of D5 in water were below the PNOEC. However, an estimated 6.8% of evaluated WWTP sites (n = 68) had concentrations greater than the PNOEC, thus D5 could meet iT criteria in aquatic organisms (EC and HC 2008a).

The *Screening Assessment for the Challenge of D4 and D5* (EC and HC 2008b/a) found that D4 met two and D5 met three criteria for P, and D4 and D5 were possibly iT under s. 64 of CEPA 1999. Given the above-mentioned results and D4 and D5's potential threat to environmental health, it was proposed that D4 and D5 be added to the DSL inventory uptake initiative. Adding D4 and D5 to the DSL prompted a request for stakeholders to submit any new research for consideration during the risk management phase and that field-level monitoring is enacted under the Chemicals Management Plan in order to verify previous assumptions concerning environmental health. The *Screening Assessments* (EC and HC 2008b/a) also determined that D4 and D5 posed no threat to human health.

On January 31st, 2009 the Ministers published a *Proposed Risk Management Approach* for D4 and D5 under s. 77(6) of CEPA 1999 to list D4 and D5 as Schedule 1

toxic substances starting in February 2011. The report concluded that D4 and D5 are "entering or may enter the environment in a quantity or concentration or under conditions that have or may have an immediate or long-term harmful effect on the environment or its biological diversity (EC and HC 2009)." The *Risk Management Approach* reached their conclusions because D4 and D5 were potentially iT, met P criteria in air and sediment (D4 and D5) and met P criteria in water (D5). Furthermore, the *Proposed Risk Management Approach* outlined that management was necessary because D4 and D5 are man-made substances and their persistence in the environment was primarily from human activity via wash-off down-the-drain personal care products (EC and HC 2009). The management plan also identified that it was not possible to conclude if D4 and D5 met B criteria under s. 64 CEPA 1999. The *Proposed Risk Management Approach* outlined by the Ministers used a weight-of-evidence methodology and applied the Precautionary Principle to suggest that a life-cycle management approach could minimize and/or prevent D4 and D5 release into the environment.

On July 10th, 2009 an allied group of stakeholders including the Silicones Environmental, and Health and Safety Council of North America (SEHSC), filed a *Notice of Objection* under ss. 333(2) of CEPA 1999 to dispute The *Proposed Risk Management Approach*'s recommendation to list D4 and D5 as Schedule 1 substances. SEHSC requested the assembly of a Board of Review (BoR) comprised of scientific experts that would conduct a new assessment to examine the nature and extent of the danger posed by D4 and D5. SEHSC's claim asserted that new scientific information was available which showed that D4 and D5 does not meet CEPA 1999 iT criteria, and that this new information addressed errors made in the screening process. For instance, The *Screening Assessments* of D4 and D5 (EC and HC 2008b/a) relied on the Precautionary Principle when creating the risk management strategies because information was conflicting and lacking at the time. The Precautionary Principle was applied as a conservative measure to protect the environment given the high amount of uncertainty.

Establishing a BoR for D4 was rejected under s. 333(1) because industry failed to provide new evidence. On February 16th, 2011 a Final Order listing D4 as a toxic substance under Schedule 1 of CEPA 1999 was published in the *Canadian Gazette* (GC 2014). The listing was based on the conclusions of the *Screening Assessment for the Challenge of D4* (EC and HC 2008b) "that D4 may be entering the environment in a quantity of concentration or under conditions that have or may have an immediate or long-term harmful effect on the environment or its biological diversity." D4 met iT in acute

and chronic studies on sensitive aquatic organisms at LC_{50}s < 0.001 $g.L^{-1}$, which is below its solubility limit. An acute test on ≤ 0.001 kg rainbow trout produced an LC_{50} of 1.0×10^{-5} $g.L^{-1}$ and a NOEC of 4.4×10^{-6} $g.L^{-1}$. A chronic (e.g., 21 day) test on the water flea produced significant mortality at 1.5×10^{-5} $g.L^{-1}$ with a NOEC of 8.0×10^{-6} $g.L^{-1}$ (Sousa et al. 1995, EC and HC 2008b). As such, proposed *Pollution Prevention Plans* were published in January 2011 to restrict industrial releases to the environment. The *Pollution Protection Plan* outlined reduction targets where D4 effluent concentrations must be ≤ 17.3×10^{-5} $g.L^{-1}$ and/or total quantities must be ≤ 4000 $g.year^{-1}$ (GC 2017). Furthermore, the use of D4 in pesticides is being reassessed by the Pest Management Regulatory Agency and ongoing environmental monitoring is in effect (GC 2014).

On August 21st, 2011 a BoR was established under s. 333(1) of CEPA 1999 in response to SEHSC's *Notice of Objection* for D5 (BoR 2011, Giesy et al. 2016). The BoR was comprised of an expert scientific panel accompanied by legal aids. The legal aids were appointed to help the scientific experts interpret the application of CEPA 1999 PBiT criteria when determining the hazard posed by D5 to the Canadian environment and to clarify the conditions that result in a Schedule 1 listing (BoR 2011, Giesy et al. 2016). The new and existing evidence was presented to the scientific BoR to determine if D5 posed a threat to the environment.

On October 20th, 2011 the Report of the BoR concluded that D5 does not pose a risk to the environment due to apparent biodilution associated with biotransformation and environmental concentrations that were below iT or B potential. Thus, D5 does not meet PBiT criteria according to s. 64 of CEPA 1999 and cannot cause "harmful effects" or "danger" to the environment (BoR 2011, Giesy et al 2016). The BoR submitted their assessment of the environmental "harm" and "danger" posed by D5 to the Minister of the Environment. The Minister of the Environment determined that D5 does not pose "harm" or "danger" to the environment and does not require regulatory action or a Schedule 1 listing. Since D5 is not regulated or listed under Schedule 1 substances, Canadian consumer care products contain variable concentrations of D5.

2.7. European Regulation of cVMS

In 2010, the Scientific Committee on Consumer Safety (SCCS) assessed D4 and D5 to determine possible threats to human and environmental health. The SCCS (2010) assessment classified D4 and D5 as meeting criteria set out in Article 57 of REACH for having a "possible risk of impairing fertility" from toxicity findings in the non-polar

narcosis range and that they could be bioaccumulative to aquatic organisms based on their high K_{OW} values. The results prompted a review in the UK and ultimately the restriction of D4 and D5 in wash-off personal care products in June of 2016. As such, D4 and D5 were added to the REACH Annex XVIII Restricted Substances List on January 10, 2018 (Commission Regulation (EU) 2018/35)). The listing surmised that D4 is persistent, bioaccumulative and toxic (PBT) and that both D4 and D5 are very persistent, very bioaccumulative (vPvB) substances. The listing enforced that "D4 and D5 shall not be placed on the market in wash-off cosmetic products in a concentration equal or greater than 0.1% by weight of either substances, after 31 January 2020."

The European Chemicals Agency (ECHA 2018)'s risk assessment and the Socio-Economic Assessment Committee supported the listing. Both assessments proposed to add D4 and D5 as candidates under inspection to the list of substances with very high concern under Article 57 of REACH's Regulation on June 27th, 2018 with the eventual inclusion of D4 and D5 as authorized substances under Annex XIV of REACH (ECHA 2018).

2.8. Canadian vs. EU Regulation of cVMS

The Canadian and EU regulatory response to cVMS diverged due to inconsistences within the literature that produced varying PBiT estimates, evaluating the risk from contaminated sediment, incorporating risk from impurities associated with mixtures, and varied reliance on the Precautionary Principle. The variation in PBiT estimates are associated with the use of traditional models. As previously mentioned, modeling the fate and PBiT potential of D4 and D5 has produced inconsistent results that range above and below PBiT criteria. Traditional models use high K_{OW}s of non-metabolized hydrophobic chemicals to estimate BCF accurately (Gobas 1993, Arnot and Gobas 2004). These traditional models do not appropriately estimate the BCF for metabolized chemicals (Nichols et al. 2013). The inconsistences associated with cVMS and predictive model outputs suggest that cVMS are being metabolized or biotransformed, where the biotransformation rate is directly related to how fast an organism removes a substance from its cells via chemical alterations.

Canada and the EU arrived at different regulatory responses in part because of the EU's evaluation of risk from cVMS-contaminated sediment. The Member State Committee (MSC) established to assess D4 and D5 PBT found that both D4 and D5 meet vP criteria outlined in REACH Annex XIII (ECHA 2015). Their results were based

on experimental observations, monitoring studies, and simulations that exceed P thresholds. For instance, the MSC evaluated the efficiency of cVMS non-degradation processes in sediment. The MSC determined that such processes did not improve the sediment removal half-life. Given that the MSC proclaims that sediment half-life remains high (D5 $t_{1/2}$ = 800 – 3100 d) this compartment should be prioritized in the assessment of persistence (ECHA 2015, ECHA 2018). In contrast, the Canadian assessment did not focus on exposure to sediment-dwelling organisms and prioritized risk based on exposure rates for pelagic aquatic organisms based on overall exposure potential.

The ECHA (2018) assessment also applied the potential of environmental contamination on behalf of impurities of D4 in products containing D5 while the Canadian BoR (2011) did not consider the risk from mixtures. According to ECHA (2018), “D5 fulfills PBT criteria with impurity [of] D4 in concentration ≥ 0.1 % [ww],” which exceeds the conventional threshold of PBT and vPvB substances under Article 14(2)(f) of REACH (ECHA 2018). Given that D5 is comprised of concentrations of D4 that exceed the threshold, a release of D5 would result in a release of D4. Exposures from impure mixtures that meet PBT and vPvB criteria require that the mixture itself be regulated (ECHA 2018).

Furthermore, the EU regulations rely heavily on the Precautionary Principle to protect the environment while Canada relies on the Precautionary Principle if uncertainty is high due to lack of real-world data. For instance, D4 remains regulated in both Canada and the EU because new information to combat D4’s PBiT potential was not presented so additional review was not required (BoR 2011). D5 is not regulated in Canada because new evidence was made available, a BoR was established, and the BoR concluded that D5 does not meet CEPA 1999 B or iT criteria at measured environmental concentrations (BoR 2011). The ECHA (2018) assessment made its recommendations to regulate D4 and D5 based on a weight-of-evidence based approach that is consistent with applying the Precautionary Principle.

Chapter 3.

Methods

3.1. Chemicals

Octamethylcyclotetrasiloxane (D4, CAS no. 556-67-2) and decamethylcyclopentasiloxane (D5, CAS no. 541-02-6) were test chemicals provided by Dow Corning, Inc. Reference chemicals were chosen because of their high log K_{OW} values associated with lipophilic tendencies and low biotransformation rates in fish. The K_{OW} values for the reference chemicals encompass the K_{OW} values of the test chemicals. Furthermore, previous research has successfully used these reference chemicals to determine the biotransformation rate constants (k_M) of test chemicals (Lo et al. 2015). See Table 3.1 for a list of reference chemicals and their K_{OW}.

Table 3.1. List of reference chemicals, their CAS number (CAS no.), Log K_{OW} and the chemical supplier. Reference chemicals include: 1,2,4,5-tetrachlorobenzene (TCBz), pentachlorobenzene (PCBz), hexachlorobenzene (HCBz), 2,2',5,5'-PCB (PCB-52), 2,2',4,4',5,5'-PCB (PCB-153) and 2,2',3,3',4,4',5,5',6,6'-PCB (PCB-209).

Reference Chemicals	CAS no.	Log K_{OW}	Supplier
TCBz	95-94-3	4.64 [Lo et al 2015]	Aldrich Chemistry
PCBz	608-93-5	5.17 [Lo et al 2015]	Aldrich Chemistry
HCBz	118-74-1	5.73 [Lo et al 2015]	Sigma Aldrich
PCB-52	35693-99-3	6.09 [Lo et al 2015]	AccuStandard
PCB-153	35065-27-1	7.75 [Lo et al 2015]	AccuStandard
PCB-209	2051-24-3	8.27 [Lo et al 2015]	Sigma Aldrich

Tetrakis(trimethylsilyl)siloxane (SIL, CAS no. 4098-98-0) and tetrakis(trimethylsiloxy)silane (M4Q, CAS no. 3555-47-3) were used as internal standards (IS) and sourced from Gelest, Inc. Chrysene-d12 (d12, CAS no.1719-03-5) was used as an external standard (ES) and sourced from Isotec ™.

3.2. Fish

252 juvenile rainbow trout (*Oncorhynchus mykiss*) were used as test subjects to determine the biotransformation rate constant (k_M) of the test chemicals. Fish were housed at Simon Fraser University in 75-gallon fiberglass tanks (n = 36 fish.tank^{-1}). Fish received filtered oxygen directly via gas lines sourced from the Biology Department Facilities at Simon Fraser University. Fish were acclimatized up to a minimum of two weeks in their respective test tanks. Tanks (n = 8) received the following dietary

treatments: control tank (no test chemicals; no reference chemicals), reference tank (no test chemicals; 0.02 $g.kg^{-1}$ feed of reference chemicals), $D4_{HIGH}$ tank (2 $g.kg^{-1}$ feed of D4; 0.02 $g.kg^{-1}$ feed of reference chemical), and $D5_{HIGH}$ tank (2 $g.kg^{-1}$ feed of D5; $g.kg^{-1}$ feed reference chemical). Four other tanks received feed spiked with two lower concentrations of D4 and D5, which will not be discussed further during this study. See Appendix A for a visual of the experimental tank set up.

Tanks were equipped with a flow-through system that sourced de-chlorinated municipal water and was fitted with custom Plexiglas lids to prevent escape. Lids were partially covered to provide the fish a shaded refuge. The flow-through system maintained water temperature at 13.4°C ± 0.5 (SD) and fish were kept under a 12:12 hour light:dark photoperiod.

3.3. Feeding Rates and Spiking Feed

The feeding rate was held at 1% $bw.d^{-1}$ and equal to the growth rate for the entire experiment. Starting weight (kg) was calculated from a sample of extra fish representing 20% of the total population (n = 378) received from the hatchery (w = 0.00824 ± 0.00022 (SE) kg ww). Body weight was re-calculated at each sampling point. The feeding rate was adjusted accordingly to account for body weight and the number of fish removed per tank after each sampling point because fish were sampled before feeding.

Fish feed (Alcan EWOS Pacific for salmonids consisted of 1.5 mm diameter pellets containing 50% protein, 18% lipid, 2% fiber, 2.1% calcium, 1.1% potassium and 0.5% sodium) was spiked with D4 or D5 and the aforementioned reference chemicals. Reference chemicals (0.015 g) were dissolved in hexanes (100 mL) and added to 0.3 kg of feed to create a reference chemical stock. See Appendix B for predicted masses (g) added for each reference chemical. The reference chemicals and feed were mixed by hand in a 100 mL round bottom flask (RBF) for 30 s. The mixed contents were transferred into a 1000 mL RBF containing 0.3 kg of clean feed (total 0.6 kg feed). The 100 mL RBF was washed three times with hexanes and the liquid content was added to a 1000 mL RBF to maximize the transfer of the reference chemicals to the feed. The 1000 mL RBF was capped and shaken by hand (30 s) to ensure equal mixing between the clean and spiked feed. The 1000 mL RBF was then placed on the rotor-evaporator (Yamato - RE47) with the evaporation mode turned off overnight to ensure that the feed was homogenous.

The following day the remaining hexanes were further evaporated in the rotor-evaporator (30°C, 30 min) until visible signs of hexanes were no longer observed. All free-moving feed (i.e., feed that was not adhered to the RBF with saturated lipids) was transferred into a clean 1000 mL RBF. A 0.06 kg feed aliquot was then placed in a 100 mL RBF and set aside as the reference stock. The final estimated concentration of reference chemicals was ~0.02 $g.kg^{-1}$ feed after accounting for a 20% loss during the spiking procedure. See Appendix B for predicted concentrations per chemical in feed.

The fish feed containing the test-chemicals, D4 or D5, was created as two separate concentrated stocks (C_{STOCK}) that were serially diluted with the reference chemical-spiked feed to achieve the desired concentration for each test chemical. The C_{STOCK} was composed of 5 g of D4 or D5 in 0.02 kg of feed that included the reference chemicals. Mixtures were hand shaken (30 s) in a 100 mL RBF to ensure homogeneous dispersion of the test chemical in feed. The mixture was placed on the rotor-rack over night to further homogenize the feed.

$$C_{STOCK} = 5 \text{ g test chemical} / 0.02 \text{ kg feed} = 250 \text{ g test chemical.kg feed}^{-1}. \quad (1)$$

See Appendix B for predicted concentrations of D4 and D5 at each step of the serial dilution.

Each C_{STOCK} was diluted into their respective stocks ($C_{DILUTED}$) by transferring 0.006 kg of feed from C_{STOCK} into 0.054 kg of reference chemical-spiked feed in a 100 mL RBF. The mixture was shaken by hand (0.03 kg feed), capped and placed on the rotor rack (40 rpm, 4 hr). The high concentration-test feed (C_{HIGH} = 2 $g.kg\ feed^{-1}$) was created by transferring 0.006 kg of feed from $C_{DILUTED}$ into 0.054 kg of reference chemical-spiked feed in a 100 mL RBF. The mixture was hand-shaken (~30 s) and then placed on the rotor-rack (40 rpm, 4 hr).

3.4. Sampling Procedure

The uptake period consisted of feeding the fish spiked feed for 10 consecutive days. Fish were sampled in triplicate from each tank on days 2, 3 and 5 of the uptake period. The fish were then fed a clean diet for a depuration phase of 28 days (38 days total). Fish were sampled in triplicate from each tank on days 0, 1, 2, 3, 4, 5, 8, 14 and 28 of the depuration period. Fish were monitored daily and mortalities were recorded. Fish were euthanized on the above-mentioned sample dates using a 1:1 MS – Finquel 222 (MS-222, Argent Laboratories) and bicarbonate buffer in 10 L of water (~5 min).

Buckets were covered to reduce stress and mortality was confirmed by a lack of gill movement.

3.5. Dissection

After fish were euthanized, they were weighed (kg ww) and weights were recorded. The contents of the intestinal tracts were emptied, and both the intestines and stomach were discarded in order to determine the concentration of test and reference chemicals in the fish carcass. Removing the intestine, intestinal contents and stomach prevents the overestimation of test and reference chemicals from undigested feed that might remain in the digestive tract and allows analysis of the rate constants associated with processes in the somatic tissue. The intestinal contents were deposited in 2 mL amber vials and stored at -80°C for a future metabolite analysis that is outside of the scope of this project. See Appendix C for a detailed protocol of the fish dissection.

Carcasses were homogenized in triplicate using an Oster 18-speed blender/blade (Sunbeam Products) with the glass portion replaced with a screw-top mason jar. All equipment was washed (3x) with hexanes, dichloromethane and toluene before use. Homogenized fish samples were then transferred to scintillation vials (15 mL) to reduce headspace. Reducing headspace prevents cVMS from partitioning out of tissue and into air. Samples were frozen (-20°C) until they were thawed for extraction.

3.6. Extraction and Recovery

In order to determine the concentration of the dose given to the fish from spiked feed and concentrations recovered from the tissue of the fish carcasses, extractions were run in triplicate and averaged for treatment tank at time (d). Extractions followed a Quick, Easy, Cheap, Effective, Rugged, Safe (QuEChERS) method modified from Wang et al.'s (2017) cVMS extraction from plasma. Modifications to Wang el al.'s (2017) method were necessary to counteract the high amount of lipids found in tissue versus plasma, to prevent co-elution of chemicals during GC/MS analysis, and to limit the amount of lipids in the samples in order to reduce GC/MS column maintenance. Details of the extraction procedure are recorded below.

3.6.1. Extraction Procedure and Rationale

Triplicate samples of feed or tissue (0.001 kg.sample^{-1}) were weighed into 15 mL conical centrifuge tubes (Falcon - 352096). Triplicate samples were analyzed to account

for variability within the method. Aliquots of the IS, SIL and M4Q, from a 10 mL stock in toluene (CAS no. 108-88-3; Fisher Chemical) solution were added to all tubes. The concentrations of M4Q and SIL ($C = 1.0 \times 10^{-7}$ g.mL^{-1}) remained constant between control and test samples. Acetonitrile (ACN, CAS no. 75-05-8; Fisher Chemical) (1 mL) was added to all centrifuge tubes and then shaken by hand (1 min) to aid in a single-phase extraction (Anastassiades et al. 2003). Centrifuge tubes were placed on the shaker (high, 30 min) to extract chemicals from the sample and capture them in the ACN. ACN has mildly polar properties that draw other mildly polar chemicals out of organic material and into the ACN.

Salts, sodium chloride (NaCl, CAS no. 7647-14-5; Fisher Chemical) (0.2 g) and anhydrous magnesium sulfate ($MgSO_4$, CAS no. 7487-89-9; Fisher Chemical) (0.25 g), were added to centrifuge tubes to aid in liquid-liquid partitioning. Salts force ACN into organic content when mixtures are shaken by hand (1 min) and are allowed to settle (5 min) (Anastassiades et al. 2003). Salts also help control the polarity of the mixture and varying the amount of salts helps to target chemicals of a given polarity (Anastassiades et al. 2003). Pentane (CAS no. 109-66-0; Caledon Laboratories, Ltd.) (4 mL) was added to all centrifuge tubes, placed on a shaker (high, 30 min) to ensure a homogeneous mixture and then centrifuged at 5000 rpm for 10 minutes at 21°C (Beckman Allegro 64R) to further separate the liquid-liquid layer (Wang et al. 2017). Pentane was chosen because it is a nonpolar volatile solvent that is immiscible with ACN and it extracts nonpolar test chemicals when combined with specific amounts of NaCl (Anastassiades et al. 2003).

Pentane (3 mL) per sample was pipetted into a clean centrifuge tube with corresponding labels. All new tubes labeled ‘pooled’ contained the sorbents octadecylsilane (C-18, Agilent Technologies Part No. 5982-5752) (0.18 g), silica-bonded primary secondary amine (PSA, Agilent Technologies Part No. 5982-8382) (0.18 g) and $MgSO_4$ (0.25 g) to aid in lipid-chemical separation. All samples were centrifuged (10 min, 5000 rpm, at 21°C) to separate liquids from sorbents and to complete the first extraction in a series of three. See Appendix D for the rationale for the use of PSA, C-18 and $MgSO_4$ as sorbents.

Fresh pentane (3 mL) was added to the original working tube containing the sample, shaken by hand (5 min) and centrifuged (10 min, 5000 rpm, at 21°C). The top 3 mL of pentane was transferred from the working tube to the pooled 12 mL centrifuge tube containing sorbents and centrifuged (10 min, 5000 rpm, at 21°C) to complete the

second extraction in the series. This process was repeated once more for a total of three extractions that were pooled (9 mL). The pooled tubes were shaken by hand (5 min) to ensure that sorbents and lipids bind together and to dehydrate water. Dehydrating water is important because previous research shows that cVMS concentrations can be overestimated if the internal components of the gas chromatography (GC) machine are made of polydimethylsiloxanes. GC parts composed of polydimethylsiloxanes can react with water to create D4 and D5 (Varaprath et al. 2000). See Appendix D for further detail on water-induced overestimation of cVMS. The pooled tubes were then centrifuged (10 min, 5000 rpm, at 21°C) to separate the liquid pentane layer from the solid sorbents. An aliquot of pentane (6 mL) was removed and stored in an 8 mL vial (VWR – Borosilicate Glass with Phenolic Screw Cap). The samples were then diluted 1000x in pentane to determine the extraction recovery. Each sample also received an aliquot (5.0×10^{-5} $g.mL^{-1}$) of the ES, chrysene-d12 (d12), dissolved in toluene. Samples were kept at -20°C until they were prepped and analyzed using a GC and mass spectrometer (MS).

3.6.2. GC/MS and Ion Selection

An Agilent 6890 GC connected to an Agilent 5973N MS was used to analyze test and reference chemicals from all extracts. The MS contains a cool-on column auto-injector port, a 5% phenyl methyl siloxane-coated column (30 m x 250 µm x 0.25 µm HP – 5MS; Agilent) and deactivated guard column comprised of fused silica (5 m x 530 µm x 0.25 µm; Agilent). The oven was programmed at an initial temperature of 30°C (2 min), increased by 10°C min^{-1} to 160°C (15 min), and then finally increased by 30°C min^{-1} to 300°C. The GC/MS uses helium as a carrier gas under a flow rate of 1 mL min^{-1} with an initial injection of 1.0 µL from a 10-µL gas-tight glass syringe (Agilent).

MS data for the test chemicals, reference chemicals, ES and IS was collected using the selected ion mode. Ions ($m.z^{-1}$) are as follows: D4 = 281, D5 = 355, TCBz = 216, PCBz = 250, HCBz = 284, PCB-52 = 292, PCB-153 = 360, PCB-209 = 498, M4Q = 281, SIL = 232 and d12 = 240. Ion selection for D4, D5, M4Q, d12 and all reference chemicals were in accordance with previous research that had high signal recovery with reduced interference (D4/D5/M4Q: Varaprath et al. 2000, Woodburn et al. 2013; d12/reference chemicals: Lo et al. 2015). Ion selection for SIL was determined by evaluating a diluted amount of SIL in solvent under scan mode within a retention time of 10 minutes to determine which ions have high intensity and few interference peaks. All

MS data was integrated and quantified using the Chemstation software (Hewlett Packard) and transcribed to Microsoft Excel for analysis.

3.6.3. Method Validation – Feed and Fish

Standard curves were used to validate the chemical extraction recovery from fish feed and tissue. A standard curve for test and reference chemicals in feed was created by extracting clean feed and then spiking the matrix-matched pentane with known concentrations (2.0×10^{-9} – 5.0×10^{-7} $g.mL^{-1}$) of test and reference chemicals (POST XT).

Concentrations were plotted against the signal strength for the absolute peak area to create a linear regression for test and reference chemicals in feed. POST XT standard curves were blank corrected using pure pentane.

The method recovery efficiency for tissue was validated by comparing standard curves created by spiking tissue samples before the extraction took place (PRE XT) to standard curves created by spiking tissue samples after the extraction occurred (POST XT). PRE XT curves were constructed by spiking 0.001 kg of clean fish tissue with a known range of test and reference chemicals (0.00025 – 0.015 $g.kg^{-1}$ ww fish) and 0.006 $g.kg^{-1}$ ww fish of IS. POST XT curves were constructed by spiking 0.001 kg samples with a known range of test and reference chemicals (5.0×10^{-9} – 2.5×10^{-7} $g.mL^{-1}$) and $1.0 \times 10^{-7} g.mL^{-1}$ of IS. $5.0 \times 10^{-5} g.mL^{-1}$ of d12, the ES, was added last in both PRE XT and POST XT. The extraction process produces a dilution factor of 9 so the PRE XT samples were spiked with 9x the amount of test, IS, and reference chemical to properly correlate the PRE XT to the POST XT standard curves. A concept error resulted in an underestimation of the dilution factor in the extraction process for the PRE XT, which resulted in PRE XT samples being under-spiked by one third. PRE XT signal strength was corrected x3 to account for the above-mentioned error.

The PRE XT and POST XT standard curves in tissue were corrected using samples from control fish to account for laboratory contamination and overestimates generated within the GC. Samples were then run on the GC/MS to determine linearity in the PRE XT and the POST XT. Linearity was determined by using the concentration-dependent signal strength of the peak area produced by the GC/MS. PRE XT and POST XT standard curves for tissue samples were generated using the peak area ratio (PAR). For instance, peak areas were generated by known concentrations of a test or reference chemical that was divided by the peak areas of the IS to create a PAR. The two IS, M4Q

and SIL, have comparable peak areas to D4 and D5, respectively. Thus, the IS were used to calculate test chemical PAR in tissue from PRE XT and POST XT. The ES, d12, was used to calculate PAR for reference chemicals in the PRE XT and POST XT standard curves.

The absolute peak area (feed) or PAR (tissue) was plotted against the known concentrations of test or reference chemical to create a linear regression. The linear regression determined the goodness of fit via the coefficient of determination and solved for the slope and intercept of a line following the equation y = mx + b, where x is the C (feed: $g.mL^{-1}$; tissue: $g.kg^{-1}$ ww fish) of the test or reference chemical, m is the slope of the linear regression of PAR/C, y is the signal strength (feed: peak area; tissue: PAR) produced at a given concentration, and b is the y-intercept created by the regression.

The linear regressions of the PRE XT and the POST XT were then compared to determine the recovery efficiency of the method for tissue. Recovery was calculated by dividing a PAR generated at a given concentration in the PRE XT ($PAR_{PRE\ XT,C}$) by the equivalent concentration that generated a PAR in the POST XT ($PAR_{POST\ XT,C}$).

$$\%Recovery = (PAR_{PRE\ XT,C} / PAR_{POST\ XT,C}) * 100\%. \quad (2)$$

All percent recoveries calculated were then averaged to determine the mean percent recovery for a given test or reference chemical.

3.7. Determining Concentration from Feed and Fish

3.7.1. Experimental Feed

Actual values of test concentrations in experimental feed were determined using the absolute peak area from a post-extracted curve (POST XT). The blank corrected POST XT standard curve was constructed by extracting clean feed samples that were then spiked at known concentrations (2.0×10^{-9} – 5×10^{-7} $g.mL^{-1}$). Absolute peak area was chosen because PAR resulted in a large range for the y-axis (0.5 – 117 PAR), which could produce inaccurate results since the ratio between test and IS was large. The recovered concentrations of test, reference and IS chemicals were determined using a 1000x dilution, an assumed 90% recovery based on preliminary research, and an assumed 20% loss during spiking.

3.7.2. Experimental Fish

The PRE XT was used to determine unknown concentrations from experimental fish tissue. Concentrations ($g.kg^{-1}$ ww fish) of D4, D5 and reference chemicals in fish

were determined by solving for x by rearranging the equations from the linear regression of the PAR to equal C = (PAR * m) – b. PARs generated by M4Q and SIL were used to determine the C of D4 and D5, respectively. The ES, d12, was used to calculate the PAR and to determine the C of reference chemicals. The ES was used because 1) reference chemicals were in low concentrations that produced relatively smaller peak areas compared to either IS, and 2) the peak areas of d12 were in close range to the peak areas of the reference chemicals. In this case, using d12 to determine the PAR of the reference chemicals theoretically produces less over or underestimation of C compared to IS. All experimental samples were corrected for background contamination using control fish.

3.8. Growth Rate (k_g, kg ww.d^{-1})

The fish growth rate was calculated to determine if the growth over time significantly reduced the concentrations of test and reference chemicals in the tissue. If growth is determined to be non-significant, then the growth dilution factor associated with substance removal is negligible and does not need to be factored into the final analysis. Fish growth rate (k_g, kg ww.d^{-1}) was determined by plotting the weight (kg ww) of fish at time (d) and plotting standard error (n = 3) from each sampling point.

3.9. Lipid Normalization

Concentrations of chemicals in fish (g.kg^{-1} ww fish) were normalized by applying the percent of lipids present in a given tissue sample. Lipid normalization reduces over or underestimation of lipid-soluble chemical concentrations due to the variation of lipids between two tissue samples (Lo et al. 2015).

3.10. Lipid Analysis

Lipid analysis followed modifications to Bligh and Dyer's (1959) lipid extraction method and was conducted on fish sampled from all tanks at three different sampling dates (uptake day 2, depuration day 14 and depuration day 28). These sampling dates represented the onset, middle, and offset of the experiment and were chosen to encompass fish growth over time. The percent of lipid determined per treatment was then multiplied by the concentration of chemical in experimental fish at time. Incorporating the percent lipid into the chemical concentration reduces variation between samples with differing amounts of lipids by making chemical concentration relative to the

percent of lipids (Lo et al. 2015). See Appendix E for a detailed explanation of the lipid analysis procedure and the associated results.

3.11. Rational for Determining *in vivo* Rate Constants in Fish

Determining the whole-body biotransformation rate constant (k_M, d^{-1}) of test and reference chemicals requires the determination of their respective depuration rate constants (k_T, d^{-1}) from an *in vivo* experiment on fish fed a spiked diet. The analysis required measuring the somatic depuration rate constant (k_{BT}, d^{-1}) and the dietary absorption efficiencies (E_D) from actual feeding rates (f, kg feed.kg $fish^{-1}.d^{-1}$) and concentration (g.kg $feed^{-1}$) of test and reference chemicals in the diets since the intestines were excluded from the samples. A relationship between the k_{BT} in reference chemicals was then used to derive the somatic biotransformation rate constant (k_{BM}, d^{-1}) for test chemicals. The whole-body k_M and k_T can then be modeled by using the measured values for k_{BT}, k_{BM}, E_D and C_D as inputs to solve for intestinal biotransformation (k_{GM}) rate and depuration rate constants (k_{GT}) using a two-compartment fish model (body and intestines).

3.12. Uptake Rate Constants (k_D, d^{-1}) and Dietary Absorption Efficiencies (E_D)

The uptake rate constant (k_D, d^{-1}) was determined using a non-linear regression following this equation:

$$C_F = (k_D / k_T) C_D (1 - ^{(-k_T * t)}) \quad (3)$$

Where the concentration of chemical in the fish (C_F, $g.kg^{-1}$ ww fish) equals the ratio of the k_D of the whole-body depuration rate constant (k_T) multiplied by the concentration of chemical in the diet (g.kg $feed^{-1}$) as it is affected by the k_T at time (t, d^{-1}). C_D was also held constant during the uptake period (Lo PhD Thesis 2018).

The dietary absorption efficiency (E_D) was derived to determine k_D, where:

$$k_D = E_D * f \quad (4)$$

The feeding rate (f, kg feed.kg $fish^{-1}.d^{-1}$) was held constant at 1% body weight during the uptake period. The actual f was confirmed by plotting the linear regression of the weight of fish sampled per treatment at time (d) (Gobas and Lo 2016, OECD 2012).

$$E_D = dC_F / d_t = k_D.C_D \quad (5)$$

3.13. Determination of Depuration (k_T) and Biotransformation Rate Constants (k_M)

Determining the whole-fish biotransformation rate constant (k_M, d^{-1}) of test and reference chemicals required deriving their respective depuration rate constants (k_T, d^{-1}) from an *in vivo* experiment on fish fed spiked diets. The current method discarded the intestines and analyzed the tissue from the fish carcass, thus measured values of k_T represent the somatic depuration rate constants (k_{BT}, d^{-1}). The somatic depuration rate constants for test (k_{BT}, d^{-1}) and reference chemicals ($k_{BT,R}$, d^{-1}) were determined by plotting the natural logarithm of C_F ($g.kg^{-1}$ ww fish) over time (t, d) during the 28 days of the depuration phase and assumed that the test and reference chemicals followed first-order kinetics. The slope of the linear regression describing the relationship between C_F and time produced the k_{BT} and $k_{BT,R}$ (Gobas and Lo 2016, OECD 2012).

The somatic biotransformation rate constant (k_{BM}, d^{-1}) for test chemicals were determined by plotting the linear relationship between the $k_{BT,R}$ and K_{OW}^{-1} assuming that the somatic biotransformation rate constants of the reference chemical k_{BM} = 0 (Gobas and Lo 2016). The coefficients (A and B) of the linear regression were then applied in the following equation to solve for $k_{BT,R}$:

$$k_{T,R} = A * (K_{OW}^{-1}) + B \quad (5)$$

The $k_{BT,R}$ and k_{BT}s were plotted against their respective log K_{OW} values to determine relative k_{BM}s for test and reference chemicals. The k_M for the test chemicals were then derived using the $k_{BT,R}$s, when the test chemical k_{BM} and k_G are assumed to equal 0. The equation is as follows:

$$k_{BM} = k_{BT} - k_{BT,R} \quad (6)$$

Finally, the whole-body k_M and k_T was modeled using the measured values for k_{BT}, k_{BM}, E_D and C_D as inputs to solve for intestinal biotransformation rate (k_{GM}, d^{-1}) and depuration rate constants (k_{GT}) using a two-compartment fish model (body and digesta). The whole-fish biotransformation rate constant (k_M, d^{-1}) was generated by inputting *in vivo* C_F ($g.kg^{-1}$ ww fish) data sampled from the fish carcass (k_{BT}, d^{-1}) at time (d) into the Absorption, Distribution, Metabolism, and Excretion Fish Bioaccumulation Calculator (ADME-B, beta ver. 1.14) (Gobas et al. 2018) in order to derive the k_{GM}.

The ADME-B model was created to interpret the OECD 305 bioaccumulation tests in fish for non-ionic, organic chemicals with a log K_{OW} > 3 (Gobas et al. 2019). Determining biotransformation rate constants (k_T) within the compartments required a series of inputs including the substances log K_{OW}, water solubility, mean fish weight, C_D,

proportional feeding rate, uptake duration, lipid contents (fish body and diet), mean water temperature, growth rate, and C_F at time (d).

3.14. Biomagnification Factor

The dietary bioaccumulation factor for test and reference chemicals were determined using the following biomagnification factor (BMF, kg feed.kg^{-1} ww fish) equation:

$$BMF = k_D / k_T \quad (7)$$

And then the biomagnification factor was lipid normalized (BMF_L) using the following equation:

$$BMF_L = BMF / \Phi_L \quad (8)$$

Where Φ_L refers to the lipid content of the fish. BMF_Ls ≥ 1 are expected to bioaccumulate, while those < 1 are unlikely to bioaccumulate.

3.15. Bioconcentration Factor

The bioconcentration factor (BCF, ww) is modeled from dietary tests by solving for the relative contribution of chemical depuration rate constant (k_{BT}, d^{-1}) from the body from gill respiration uptake rate constant (k_1, d^{-1})) based on the estimated values of the concentration of organic content (C_{OC}, kg.L^{-1}) in water and the carbon-water partitioning coefficient (K_{OC}) using the following equation:

$$BCF_{ww} = k_1 / (k_{BT} (1 + C_{OC} * K_{OC})) \quad (9)$$

3.16. Proportional Contribution of the Biotransformation Constants

The proportional contribution of the biotransformation rate constants for the gut (φ_{GM}, %) and the somatic tissue (φ_{BM}, %) are used to determine the relative contribution (i.e., the gut vs. the fish body) of chemical removal based on the exposure route (i.e., via gill respiration or the diet). The equations to solve for the proportional contribution for the biotransformation rate constants are as follows:

$$\varphi_{GM} = k_{GM} * M_G / (k_{GM} * M_G + k_{BM} * M_B) \quad (10)$$

$$\varphi_{BM} = k_{BM} * M_B / (k_{BM} * M_B + k_{GM} * M_G) \quad (11)$$

Where the M_G and M_B refers to the mass (g) of chemical in the gut and body.

3.17. Statistical Analyses

All statistical analyses were determined with Microsoft Excel ver. 14.7.7 and XLStatPlus build 6.7.1.0/Core v6.7.02. Standard curves were analyzed using linear regressions. ANOVAs were used to find significant differences ($p = 0.05$) between the biotransformation rates of test and reference chemicals.

Chapter 4.

Results and Discussion

4.1. Determining Concentration of D4 and D5 in Feed

The concentration in the experimental feed was determined from POST XT standard curves that were spiked with D4, D5 and reference chemicals after the extraction process took place. See Appendix F for the method development for determining test chemical concentrations in feed for D4 and D5 standard curves in feed. The recovery efficiency was previously measured to be 90% for cVMS based on a sequential analysis of D4 extracted from feed. The recovery efficiency was based on a previous extraction method using corn oil as a carrier to spike feed with cVMS, which was not used to create the experimental feed fed to fish. See Supplementary Material 1 for an overview of the recovery efficiency from the corn oil carrier method.

The concentrations for $D4_{HIGH}$ and $D5_{HIGH}$ were 0.84 ± 0.07 (SE) $g.kg^{-1}$ feed and 1.95 ± 0.25 (SE) $g.kg^{-1}$ feed with recoveries of 42 and 98%, respectively. Results indicate that the feed-spiking and extraction method was adequate for D5 but low for D4. The recovery efficiency may be reduced in D4 compared to D5 because D4 has a somewhat higher log K_{OA} value (D4 = 4.29; D5 = 4.94) (Xu and Kropscott 2013, Wang et al. 2013a) and a higher vapor pressure than D5 (D4 = 1.05 mm Hg; D5 = 0.3 mm Hg) (Flaningam 1986, ECHA 2015). A relatively higher log K_{OA} and a higher vapor pressure causes D4 to partition out of octanol (a surrogate for lipids) and into the air more than D5. Thus, given D4's highly volatile nature, it is likely that the majority of losses occur via vaporization during the extraction process. Future research should reduce any exposure to air during the extraction and spiking process by reducing the triple pentane extraction to a single extraction. Performing one extraction compared to triple extractions reduces the amount of air exposure that occurs when adding more solvent (i.e., pentane) to the test tube, thus keeping D4 from partitioning out of feed and into air. Comparing a triple extraction and a single extraction recovery could provide information on the average loss between the two methods.

Future research could also reduce cVMS loses by using a less-volatile solvent, such as hexane. Replacing pentane with a less volatile solvent could improve recovery because less evaporative solvents could keep D4 in solution longer when exposed to air during portions of the extraction method. Using a less volatile solvent could also improve consistencies between samples because the concentration would be less affected by

evaporation. Comparing extractions in pentane and hexane could help decipher which solvent has a greater capability of holding D4 in solution.

The low recovery of D4 from experimental feed might also be associated with using the POST XT standard curve to measure concentrations. The POST XT standard curve may not be as representative of the actual concentrations within the feed as the PRE XT standard curve. POST XT standard curves are unable to capture chemical losses from reactions and the impact of matrix effects incurred during the extraction process since the extracted pentane is spiked after the extraction has taken place ("LC-MS Method Validation" n.d.). In turn, POST XT standard curves may overestimate the amount of test and reference chemical in a given sample compared to PRE XT standard curve. Using PRE XT standard curves could better represent the actual amount of test chemical in a sample because samples are spiked and then extracted. Thus, PRE XT standard curves factor in the extraction process, potential chemical reactions, and any matrix effects that affect the final test concentration ("LC-MS Method Validation" n.d.).

4.2. Fish Growth Rate by Treatment

Fish growth rate constant (k_G, kg ww.d^{-1}) was assessed to ensure that fish growth did not significantly reduce test and reference concentrations in fish as they grew over time. Fish fed control, reference-spiked, and D4-spiked diets showed non-significant increases in weight (kg ww) over time (d) (Figure 4.1A, C and D). Fish fed reference-chemical spiked diets showed significant increases in growth over time, though significance was low ($1.5 \times 10^{-5} \pm 5.4 \times 10^{-6}$ (SE) kg ww.d^{-1}, $p = 0.01$) (Figure 4.1B). Results are consistent with a low dietary f of 0.01 kg feed.kg fish^{-1}.d^{-1} and indicates that treatment did not significantly affect fish growth rate.

Non-significant growth also indicates that somatic growth did not reduce the concentration of test and reference chemicals from dilution. Thus, it was unnecessary to incorporate the somatic growth dilution factor when determining the concentration of test and reference chemicals in tissues. See Table 4.1 for growth rate, mean, standard error, coefficient of determination, and p-values for all treatments.

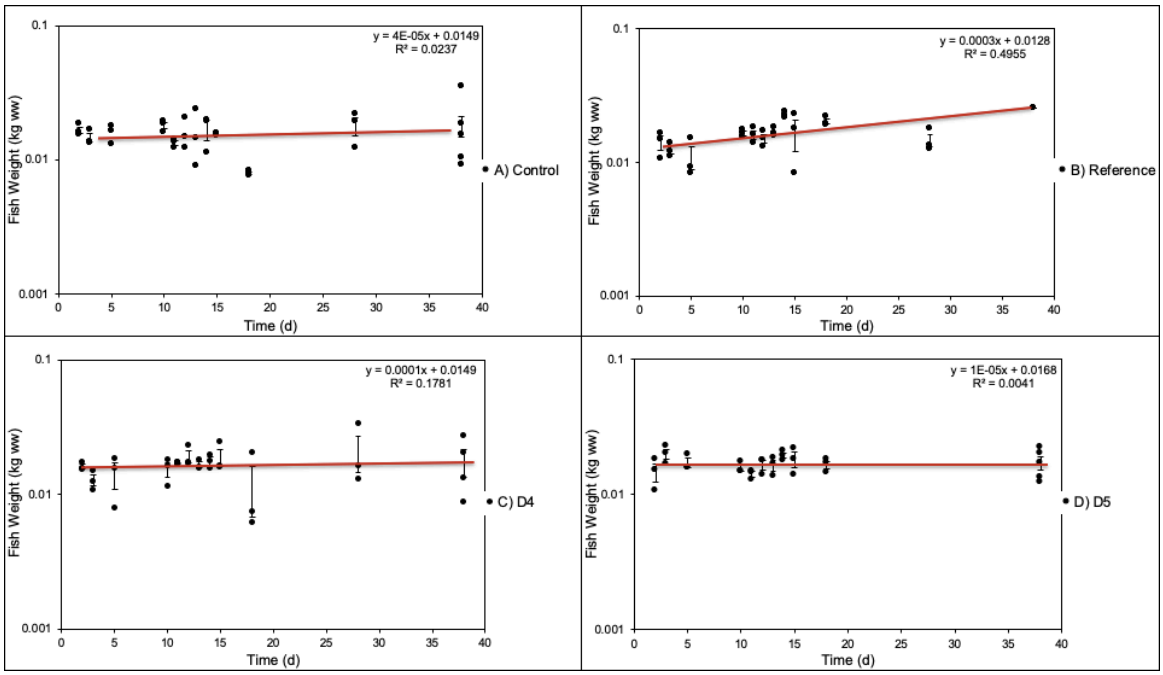


Figure 4.1. **Fish weight (kg ww) over time (d) for fish fed the following treatment diets: A) control, B) reference-spiked feed, C) D4-spiked feed, and D) D5-spiked feed. The red line represents the linear regression of the means and error bars represent the standard errors of the mean.**

Table 4.1. **Coefficients of variation (R^2), means, standard error (SE), F and P-values for linear regressions of fish growth (k_G) showing fish weight (g ww) over time (d) and ANOVAs to determine if growth was significantly greater than 0 within each dietary treatment group (control, reference, D4 and D5).**

Treatment	R^2	Mean (kg ww.d^{-1})	SE	F	P-Value	Significant (Y/N)
Control	$5.1x\ 10^{-6}$	6.5×10^{-8}	4.8×10^{-6}	0.0002	0.99	N
Reference	1.9×10^{-1}	1.5×10^{-5}	5.4×10^{-6}	7.2799	0.01	Y
D4	1.2×10^{-2}	3.5×10^{-6}	5.4×10^{-6}	0.4105	0.53	N
D5	2.4×10^{-3}	8.3×10^{-7}	3.0×10^{-6}	0.0780	0.78	N

4.3. Determining Concentration of D4 and D5 in Fish

4.3.1. Octamethylcyclotetrasiloxane (D4) in Fish

The bioaccumulation model of fish fed D4 showed an increase in concentration (g.kg^{-1} ww fish) during the 10-day uptake phase where it reached a peak of 0.026 g.kg^{-1} ww fish (Figure 4.2). After day 14, the concentration appears to decrease until the final sampling point on day 38 (depuration day 28; 0.011 g.kg^{-1} ww). See Appendix G for D4 concentration data and the standard error of the bioaccumulation model.

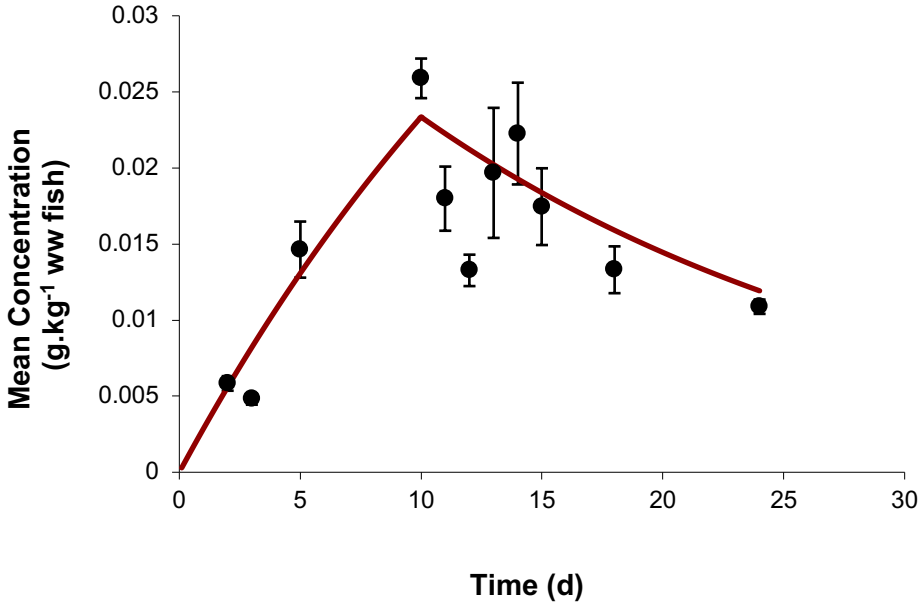


Figure 4.2. Mean concentrations (g.kg^{-1} ww fish) of octamethylcyclotetrasiloxane (D4) in *Oncorhynchus mykiss* fed diets spiked with D4 over time (d). The red line is the line of best fit and error bars represent the standard errors of the mean (n = 3).

The bioaccumulation model for reference chemicals in fish fed D4 and reference chemical-spiked diets showed an increase in concentration (g.kg^{-1} ww fish) during the 10-day uptake phase (Figure 4.3). After the onset of the depuration phase (day 10), concentrations (g.kg^{-1} ww fish) of reference chemicals showed expected decreases. Concentrations of reference chemicals in fish continued to decrease with time as expected. HCBz, PCBz, PCB-153, PCB-52 appear to reach the highest concentrations at the offset of the uptake phase (day 10) while TCBz and PCB-209 show relatively low concentrations.

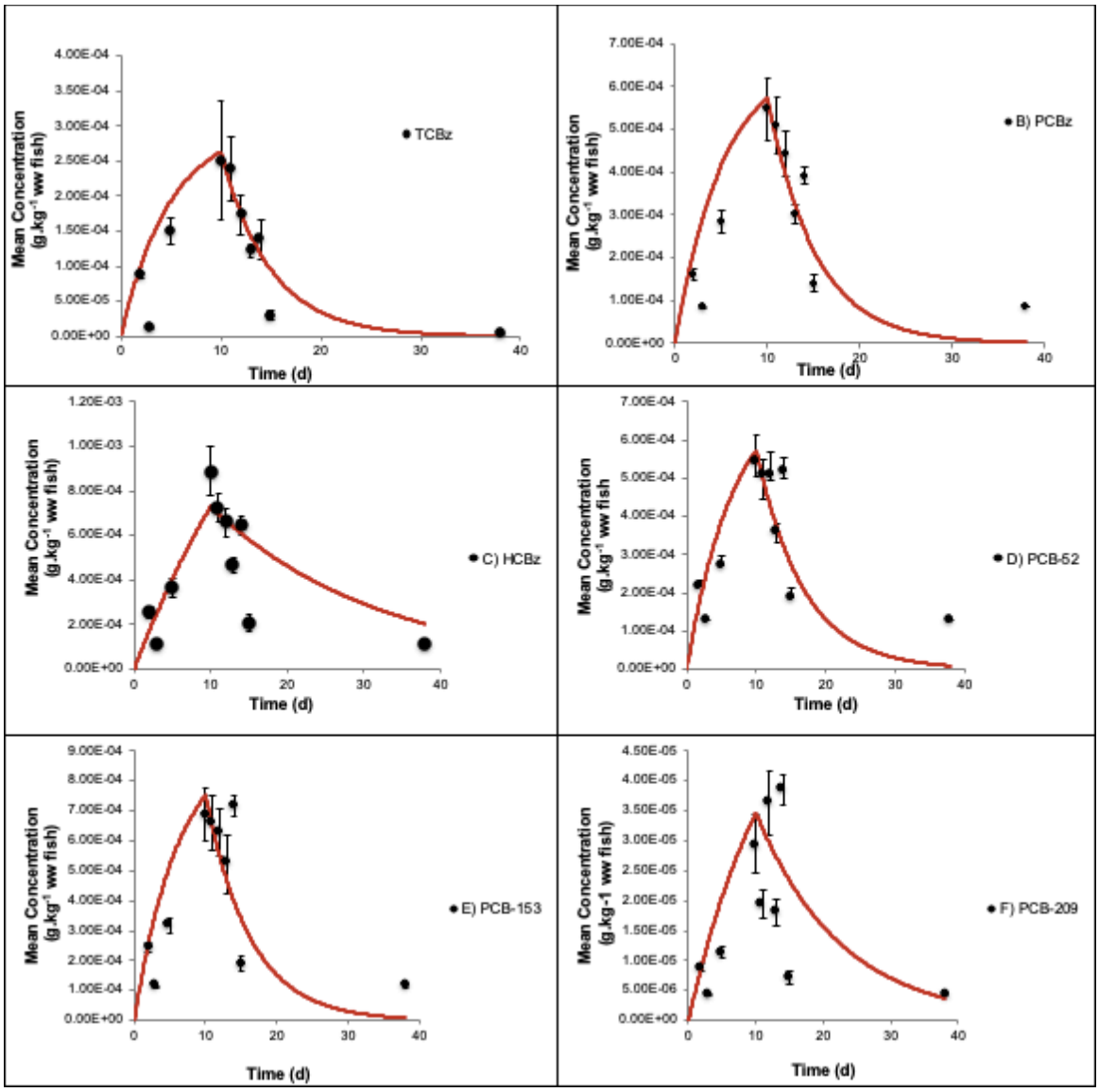


Figure 4.3. Mean concentrations (g.kg^{-1} ww fish) of octamethylcyclotetrasiloxane (D4) and the following reference chemicals in fish fed spiked diets over time (d): A) tetrachlorobenzene (TCBz), B) pentachlorobenzene (PCBz), C) hexachlorobenzene, D) 2,2',5,5'-PCB (PCB-52), E) 2,2',4,4',5,5'-PC, (PCB-153), and F) 2,2',3,3',4,4',5,5'-PCB (PCB-209). The red line represents the linear regression of the means and error bars represent the standard errors of the mean (n = 3).

4.3.2. Dietary Absorption of Octamethylcyclotetrasiloxane (D4) in Fish

Analyzing the uptake curve of the bioaccumulation model of fish fed D4-spiked diets produced a dietary absorption efficiency (E_D) of 0.322 ± 0.034 (SE) when the slope of the line of C_F versus time (i.e. 0.0027 g.kg^{-1} ww fish) was divided by C_D (i.e. 840 g.kg^{-1} feed) and multiplied by a feeding rate of 0.01 kg feed.kg fish^{-1}.d^{-1}. The uptake rate

constant (k_D) was determined to be 0.0032 ± 0.0006 (SE) d^{-1}. See Appendix G for D4 concentration data and the standard error of the dietary absorption efficiency model.

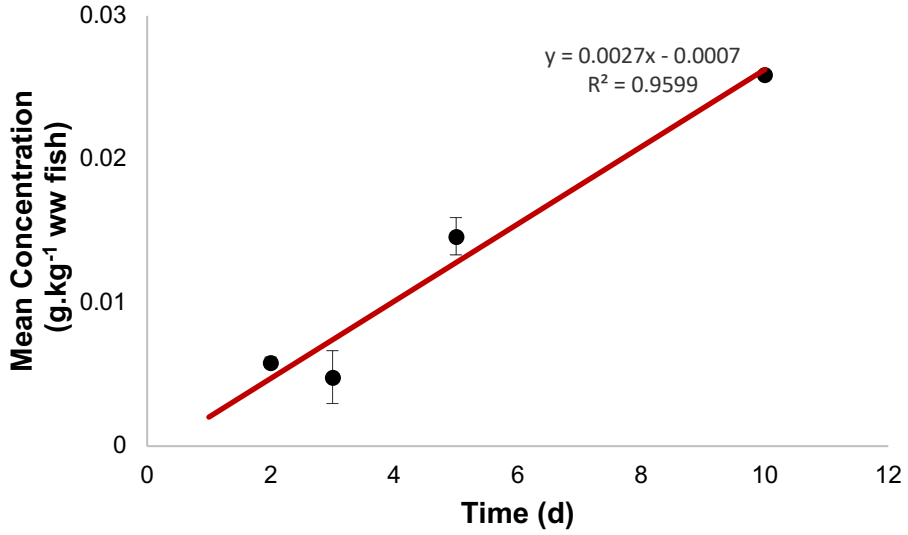


Figure 4.4. Linear regression showing the dietary absorption efficiency (E_D) of the mean concentrations (kg feed.kg ww $fish^{-1}.d^{-1}$) of octamethylcyclotetrasiloxane (D4) in *Oncorhynchus mykiss* fed spiked diets over time (d). The red line indicates a linear regression and the error bars represent the standard errors of the mean (n = 3).

4.3.3. Depuration of Octamethylcyclotetrasiloxane (D4) in Fish

The concentration ($g.kg^{-1}$ ww fish) of D4 in fish declined over time (d) during the depuration phase (Figure 4.5). The mean k_{BT} for D4 showed a significant, though slow decrease in concentration over time 0.045 ± 0.018 (SE) d^{-1} ($p = 0.034$; $R^2 = 0.993$). See Table 4.2 for k_{BT} (d^{-1}) and SE for test and reference chemicals. See Appendix G for D4 concentration data and the standard error of the depuration model. See Appendix H for a complete output of the linear regression and ANOVA.

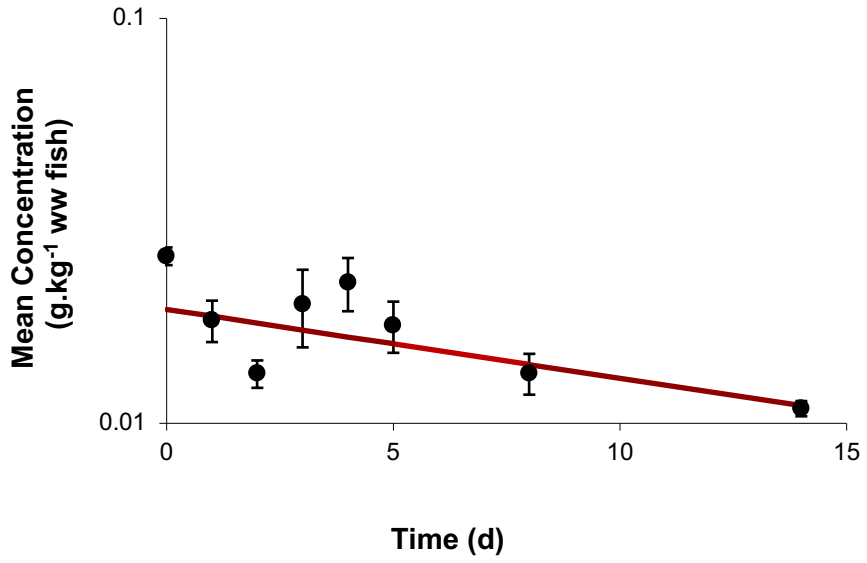


Figure 4.5. **Linear regression showing the somatic depuration rate constant (k_{BT}, d^{-1}) of the mean concentration ($g.kg^{-1}$ ww fish) of octamethylcyclotetrasiloxane (D4) in *Oncorhynchus mykiss* over time (d). The red line indicates a linear regression and error bars represent the standard errors of the mean (n = 3)**

Table 4.2. **The mean k_{BT} (d^{-1}), standard error of the mean and p-values for *Oncorhynchus mykiss* fed diets spiked with octamethylcyclotetrasiloxane (D4) and reference chemical spiked over time (d).**

Chemical	k_{BT} (d^{-1})	SE	P-value
D4	0.0446	0.01830	0.034
TCBz	0.0780	0.03000	0.020
PCBz	0.0150	0.00500	0.008
HCBz	0.0110	0.00570	0.067
PCB-52	0.0019	0.00400	0.648
PCB-153	0.0020	0.00002	0.280
PCB-209	0.0159	0.01400	0.270

The mean somatic depuration rate constants (k_{BT}, d^{-1}) for D4 and reference chemicals in fish fed spiked diets showed decreases in concentration ($g.kg^{-1}$ ww fish) with time (d) as expected (Figure 4.6). See Table 4.2 for the mean k_{BT} (d^{-1}), standard error of the mean, and p-values for test and reference chemicals.

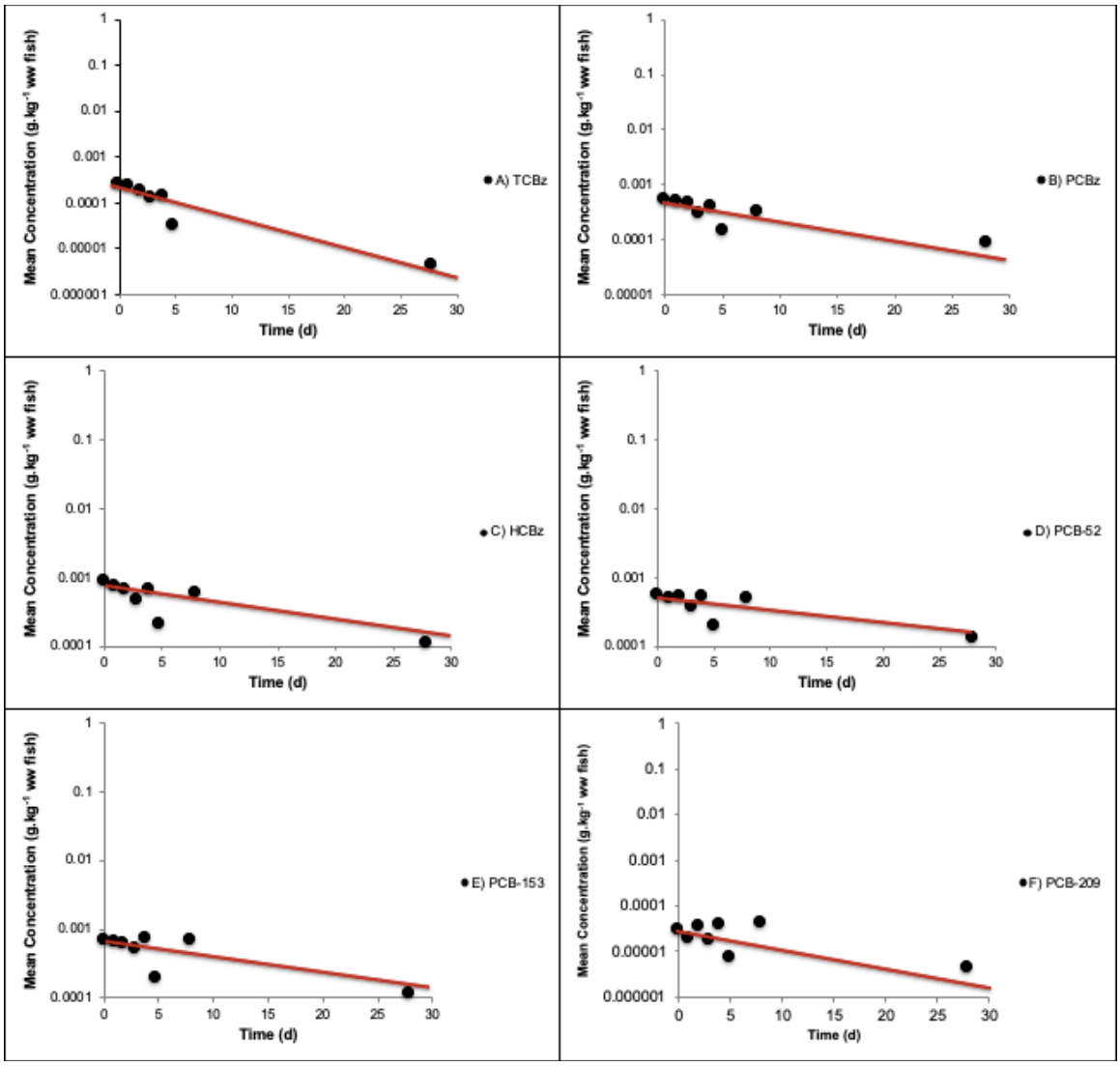


Figure 4.6. Linear regression showing the mean depuration concentrations (g.kg^{-1} ww fish) of reference chemicals in *Oncorhynchus mykiss* fed diets spiked with octamethylcyclotetrasiloxane (D4) and reference chemicals over time (d).

The relationship between the somatic depuration rate constant (k_{BT}, d^{-1}) and the K_{OW}^{-1} for the reference chemicals is depicted in Figure 4.7. Figure 4.7 indicates a significant linear relationship between the k_{BT} (d^{-1}) for all reference chemicals as K_{OW}^{-1} increases ($k_{BT,R}$, p = 0.008556, R^2 = 0.927) following the equation:

$$k_{BT,R} = 3072.079\ (\pm\ 497.6076)\ x\ (K_{ow}^{-1}) + 0.004484 \qquad (10)$$

The $k_{BT,R}$ for reference chemicals in fish fed D4 and reference chemical-spiked feed was determined to be 0.0056 $\pm$ 0.005 (SE)d^{-1} is .The Results are consistent with previous research that shows a linear relationship between reference chemical depuration rate

constants as their respective K_{OW}^{-1} increase (Lo et al. 2015). See Appendix H for a full summary output from the linear regression and ANOVA.

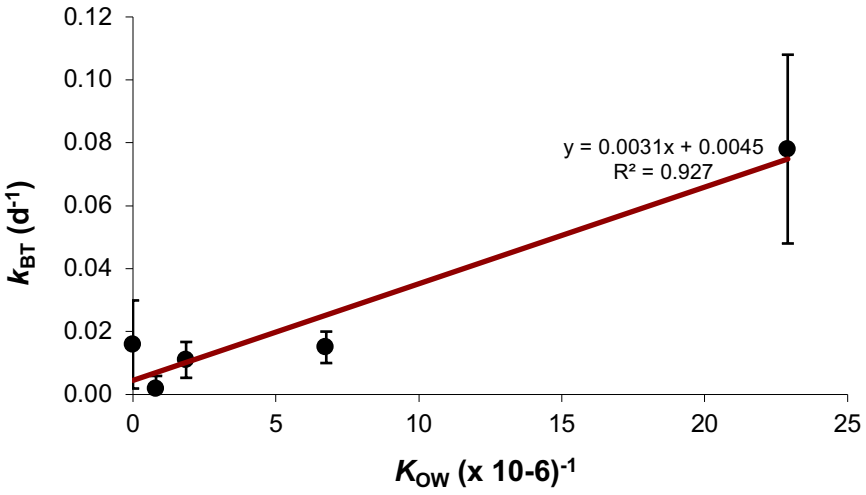


Figure 4.7. Mean somatic depuration rate constants (k_{BT}, d^{-1}) for reference chemicals in *Oncorhynchus mykiss* fed feed spiked with octamethylcyclotetrasiloxane (D4) versus the reciprocal of the octanol-water partition coefficients (K_{OW}^{-1}). Markers represent the mean, error bars represent the standard errors of the mean (n = 3) and the red line represents the linear relationship of k_{BT} and K_{OW}^{-1}. Reference chemicals appear from left to right as follows: 2,2',3,3',4,4',5',5',6,6'-PCB (PCB-209), 2,2',5,5'-PCB (PCB-52), hexachlorobenzene (HCBz), 2,2',5,5'-PCB (PCB-52), pentachlorobenzene (PCBz), and 1,2,4,5-tetrachlorobenzene (TCBz).

Developing the relationship between the log K_{OW} and k_{BT} (d^{-1}) for D4 and reference chemicals is used to determine the biotransformation rate constant (k_{BM}) of D4 (Figure 4.8) as:

$$0.0446\ (\pm\ 0.0183\ SE) \times (1/10^{xx}) - 0.005574\ (\pm\ 0.005338\ SE) \quad (12)$$

The current study determined that D4 has a k_{BM} of 0.039 ± 0.019 (SE) d^{-1}. Domoradzki et al. (2017) conducted a radio-labeled analysis of D4 metabolism from a single-dosed orally gavaged rainbow trout with a 96-hour depuration period that produced a whole body (including digesta) k_M of 0.10 d^{-1}. The differences between the current values and those from Domoradzki et al. (2017) are likely associated with the body tissue and liver analysis (current study) compared to a whole body including digesta analysis (Domoradzki et al. 2017).

Furthermore, models that assess biomagnification in aquatic food webs indicate that substances with $k_M \geq 0.05$ d^{-1} are unlikely to biomagnify (Arnot and Gobas 2003). The current study indicates that the k_{BM} does not eliminate D4 fast enough from tissue to prevent bioaccumulation. However, the cumulative k_M (including the digesta) could produce k_M's that are fast enough to prevent bioaccumulation. Future studies should research whole fish k_M to determine overall biotransformation rates in fish.

Results indicate that the k_{BT} of D4 is biotransformed from the somatic tissues of the fish at a relatively slow rate compared to other persistent chemicals including PCB-209, but slightly faster than chemicals with higher biotransformation rates including PCB-52, PCBz, and HCBz.

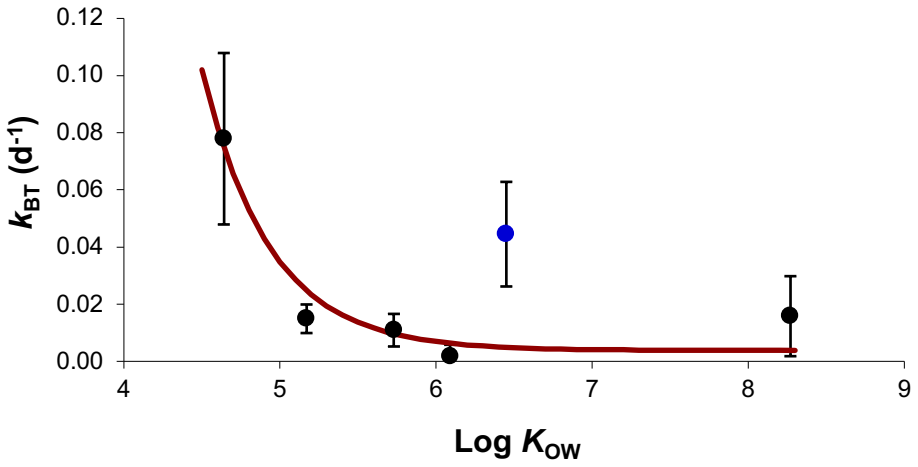


Figure 4.8. Somatic depuration rate constants (k_{BT}) for *Oncorhynchus mykiss* fed octamethylcyclotetrasiloxane (D4, blue marker) and reference chemicals (black markers as a function of the logarithm of the octanol-water partition coefficient (log K_{OW})). Markers represent the mean, error bars represent the standard errors of the mean, and the red line indicates the non-linear relationship between k_{BT} and log K_{OW}. Reference chemicals from left to right: 1,2,4,5-tetrachlorobenzene (TCBz), pentachlorobenzene (PCBz), hexachlorobenzene (HCBz), 2,2',5,5'-PCB (PCB-52), and 2,2',3,3',4,4',5',5',6,6'-PCB (PCB-209).

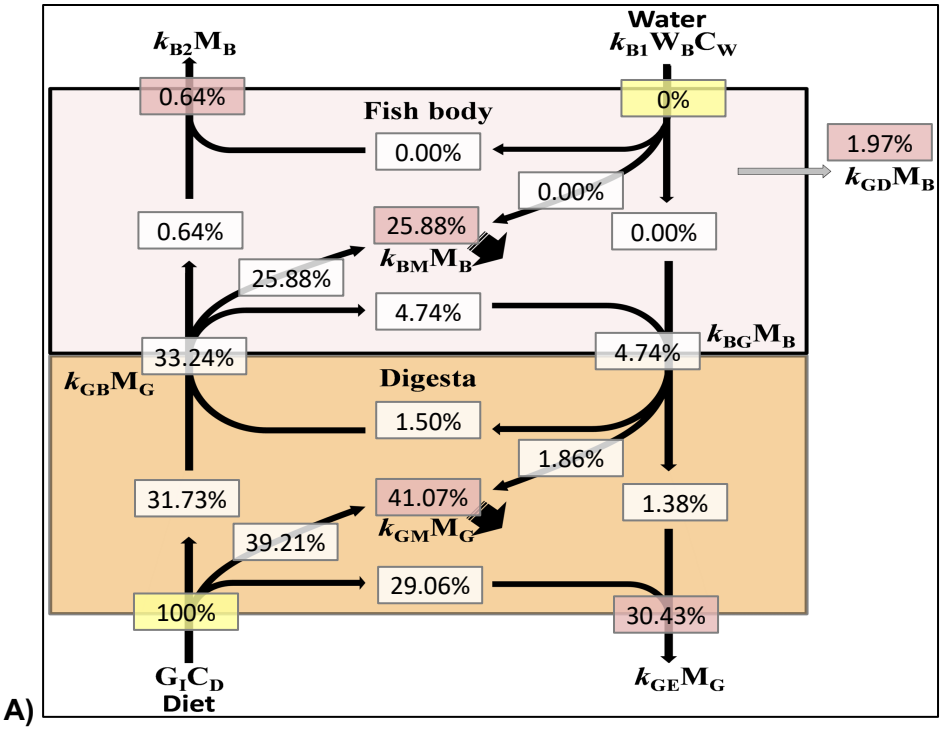
3.3.4. Bioaccumulation of Octamethylcyclotetrasiloxane (D4) in Fish

Modelling the depuration data using the ADME-B calculator produced a significant depuration rate constant (k_{BT}, d^{-1}) of 0.048 ± 0.0175 (SE) d^{-1} ($p = 0.01$). Results indicate that D4 depurates relatively slowly from the fish body tissue.

The results from the ADME-B model produced a $BCF_{ww,t}$ of 2864.6 ± 1112.1 (SE) L water.kg $fish^{-1}$ ww and a BMF_L 0.35 ± 0.08 (SE) kg lipid.kg $lipid^{-1}$, which are below CEPA 1999 B criteria (< 5000). The calculated BMF_L and BCF values are significantly less than their respective bioaccumulation values at a confidence interval of 95% (p = 0.8483 and 0.9999, respectively). BMF_L values < 1 and BCF values < 5000 indicate that D4 is unlikely to bioaccumulate in fish that are exposed via respiration or the diet according to s. 64 CEPA 1999 B criteria. See Supplementary Material 2A for a complete list of ADME-B inputs and output for D4.

Previous research on the bioaccumulation behavior of D4 places bioaccumulation metrics (BCF and BAF) just below 5000, while measured values (i.e., TMF, etc.) show biodilution (Powell et al. 2017). The measured biodilution in the environment provides evidence that biotransformation is occurring. In the current study, measuring the decrease in concentration within the body over time combined with modelling the internal distribution of D4 provides evidence that metabolism is primarily occurring in the intestinal tract.

The ADME-B model also predicts that D4's distribution and the site of biotransformation within a fish is dependent on the uptake route (i.e., via gill respiration or the diet). When exposure occurs from the diet (uptake via gill respiration = 0) the ADME-B model predicts that the majority of biotransformation (41.07%) occurs in the intestines versus the body (25.88%) (Figure 4.9A). When exposure occurs via gill respiration from water (dietary uptake = 0) the ADME-B model predicts that the majority of D4 is biotransformed in the body (81.57%) versus the intestines (5.86%) (Figure 4.9B). Thus, the ADME-B calculator predicts at least two different k_M rate constants where biotransformation in body and intestines is dependent on the exposure method (i.e., via the diet or the water).



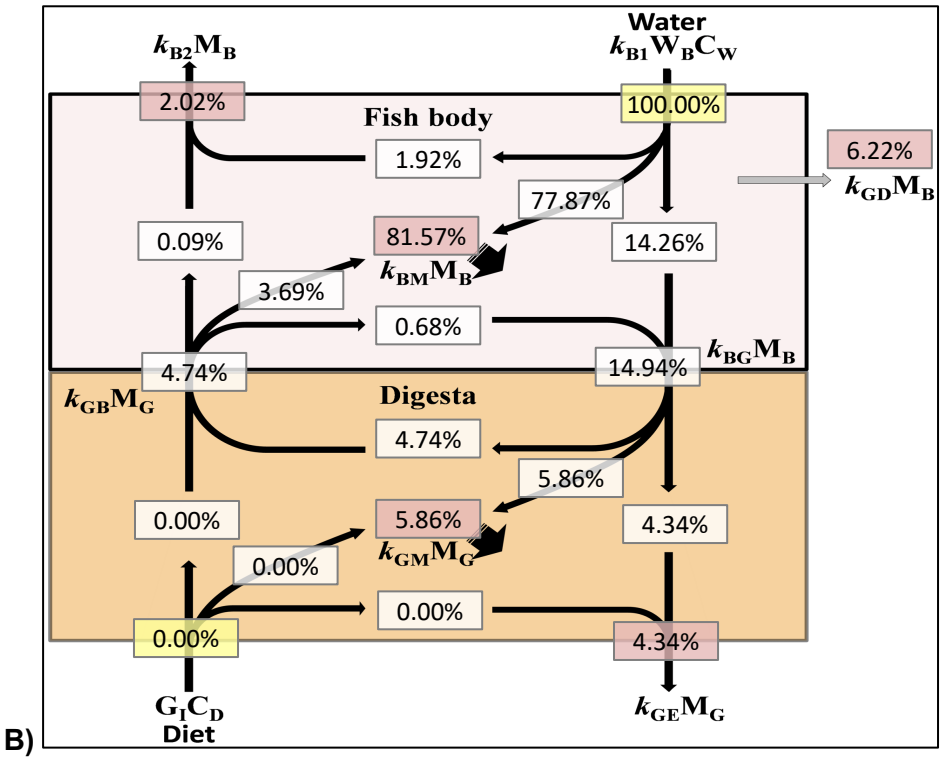


Figure 4.9. **Result output profile of a two-compartment ADME-B model showing the distribution of octamethylcyclotetrasiloxane (D4) from *Oncorhynchus mykiss* fed spiked diets under conditions where exposure is via gill respiration resulting in the bioconcentration factor (BCF) or B) the diet resulting in the bioaccumulation factor (BAF).**

4.3.5. Decamethylcyclopentasiloxane (D5) in Fish

Mean concentrations (g.kg^{-1} ww fish) during the 10-day uptake phase increased over time (d) (Figure 4.10). After the onset of the depuration phase (depuration day 0, day 10), mean concentrations (g.kg^{-1} ww fish) of D5 in the body of the fish decreased with time (d). See Appendix G for D4 concentration data and the standard error of the bioaccumulation model.

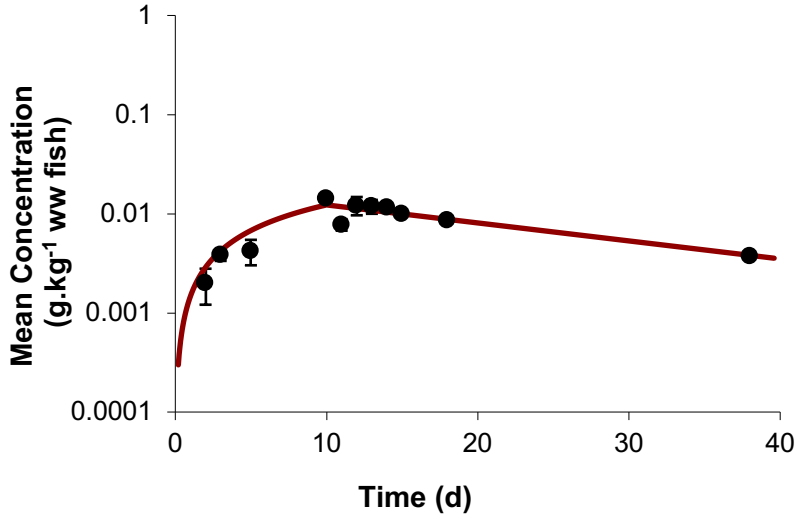


Figure 4.10. Mean concentration (g.kg^{-1} ww fish) of decamethylcyclopentasiloxane (D5) in *Oncorhynchus mykiss* over time (d). The red line represents the line of best fit and error bars represent the standard errors of the mean (n = 3).

The reference chemicals in fish fed D5 and reference chemical-spiked diets showed an increase in the concentration (g.kg^{-1} ww fish) during the 10-day uptake phase. After the onset of the depuration phase (depuration day 0, day 10 overall), concentrations of reference chemicals in fish decreased. Concentrations continued to decrease with time until the final sampling point on day 28 of the depuration phase (day 38 overall) as expected (Figure 4.11). HCBz, PCBz, PCB-153, PCB-52 appear to reach the highest concentrations at the end of the uptake phase (day 10) while TCBz and PCB-209 have relatively lower uptake concentrations compared to other reference chemicals.

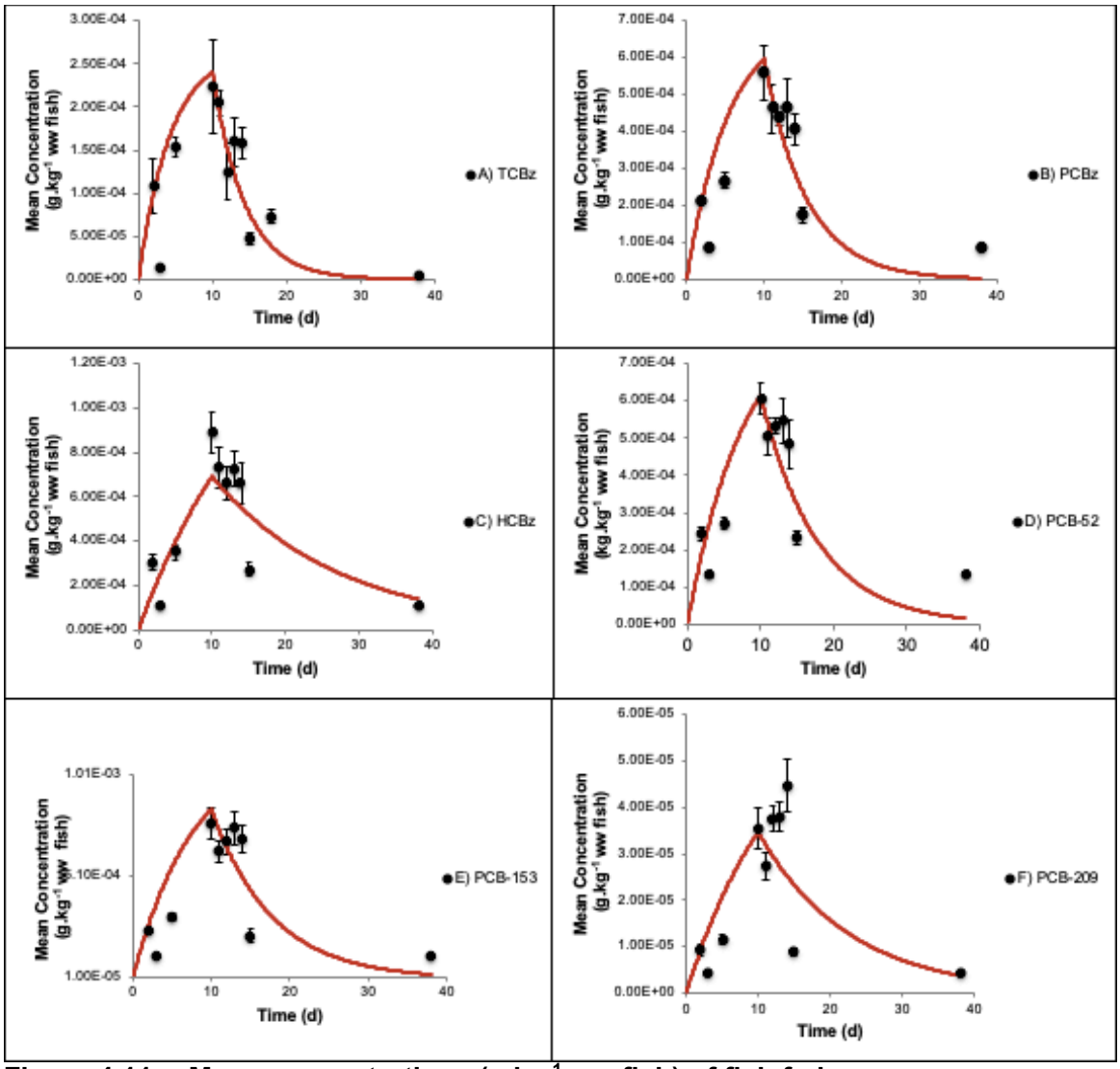


Figure 4.11. Mean concentrations (g.kg^{-1} ww fish) of fish fed decamethylcyclopentasiloxane (D5) and reference chemical spiked diets over time (d). Reference chemicals include: A) tetrachlorobenzene (TCBz), B) pentachlorobenzene (PCBz), C) hexachlorobenzene, D) 2,2',5,5'-PCB (PCB-52), E) 2,2',4,4',5,5'-PCB (PCB-153), and F) 2,2',3,3',4,4',5,5'-PCB (PCB-209). The red line represents the line of best fit and the error bars represent standard errors of the mean (n = 3)

4.3.6. Uptake of Decamethylcyclopentasiloxane (D5) in Fish

The concentration of D5 increased with time (Figure 3.13). The linear regression of the mean concentration (g.kg^{-1} ww fish) of D5 in fish fed D5-spiked diets during the 10 day uptake period explained 95% of the variance showing that increased amount of time spent exposed (d) to the spiked-diet increased the mean concentration (g.kg^{-1} ww fish) (Figure 4.12). The mean uptake absorbance efficiency (E_D) was determined to be 0.079

± 0.008 (SE) d^{-1} at the feeding rate (f) of 0.01 (kg feed.kg $fish^{-1}.d^{-1}$). The uptake rate constant (k_D) for D5 was determined to be 0.00079 ± 0.00008 (SE) d^{-1}. See Appendix G for D5 concentration data and the standard error of the uptake absorbance efficiency model.

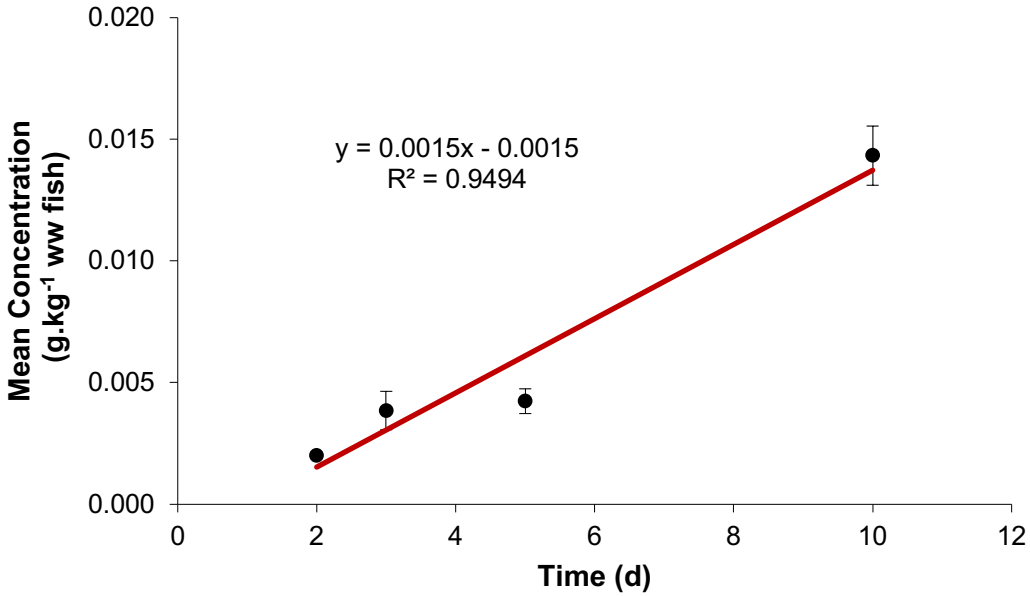


Figure 4.12. Linear regression of the dietary absorption efficiency (E_D) of the mean concentration (g.kg^{-1} ww fish) of decamethylcyclopentasiloxane (D5) in spiked feed fed to *Oncorhynchus mykiss* over time (d). The red line is the best fit linear regression and error bars represents the standard errors of the mean (n = 3).

4.3.7. Depuration of Decamethylcyclopentasiloxane (D5) in Fish

The somatic depuration rate constant (k_{BT}, d^{-1}) for fish fed D5-spiked diets showed decreases in the mean concentration with time (d) as expected (Figure 4.13). The k_{BT} for D5 was determined to be significant, but slow (k_{BT} = 0.042 ± 0.008 (SE) d^{-1}; p = 0.002). See Table 4.3 for k_{BT}, and standard error for test and reference chemicals. See Appendix G for D5 concentration data and the standard error of the depuration model. See Appendix I for a summary of the linear regression and ANOVA outputs.

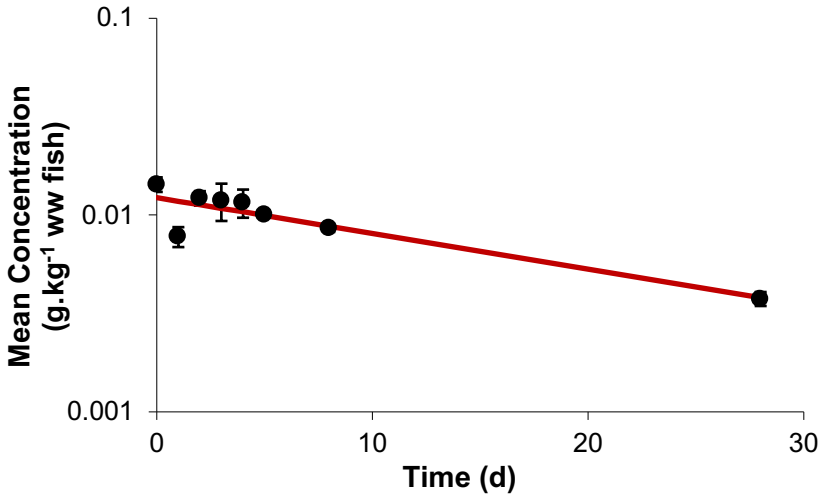


Figure 4.13. Linear regression showing the somatic depuration rate constant (k_{BT}, d^{-1}) of the mean concentration ($g.kg^{-1}$ ww fish) of decamethylcyclopentasiloxane (D5) in *Oncorhynchus mykiss* over time (d). The red line indicates a linear regression and error bars represent the standard errors of the mean (n = 3).

Table 4.3. The mean somatic depuration rate constant (k_{BT}, d^{-1}), standard error of the mean and p-values for *Oncorhynchus mykiss* fed diets spiked with decamethylcyclopentasiloxane (D5) and reference chemical spiked over time (d).

Chemical	k_{BT} (d^{-1})	SE	P-value
D5	0.0418	0.0080	0.0020
TCBz	0.0460	0.0120	0.0013
PCBz	0.0230	0.0046	0.0001
HCB	0.0230	0.0049	0.0003
PCB52	0.0130	0.0030	0.0006
PCB209	0.0230	0.0075	0.0008

The k_{BT} (d^{-1}) of reference chemicals in fish fed D5 and reference chemical-spiked diets showed decreases in mean concentration ($g.kg^{-1}$ ww fish) with time (d) (Figure 4.14). See Table 4.3 for the k_{BT}s (d^{-1}), standard error and p-values for reference chemicals.

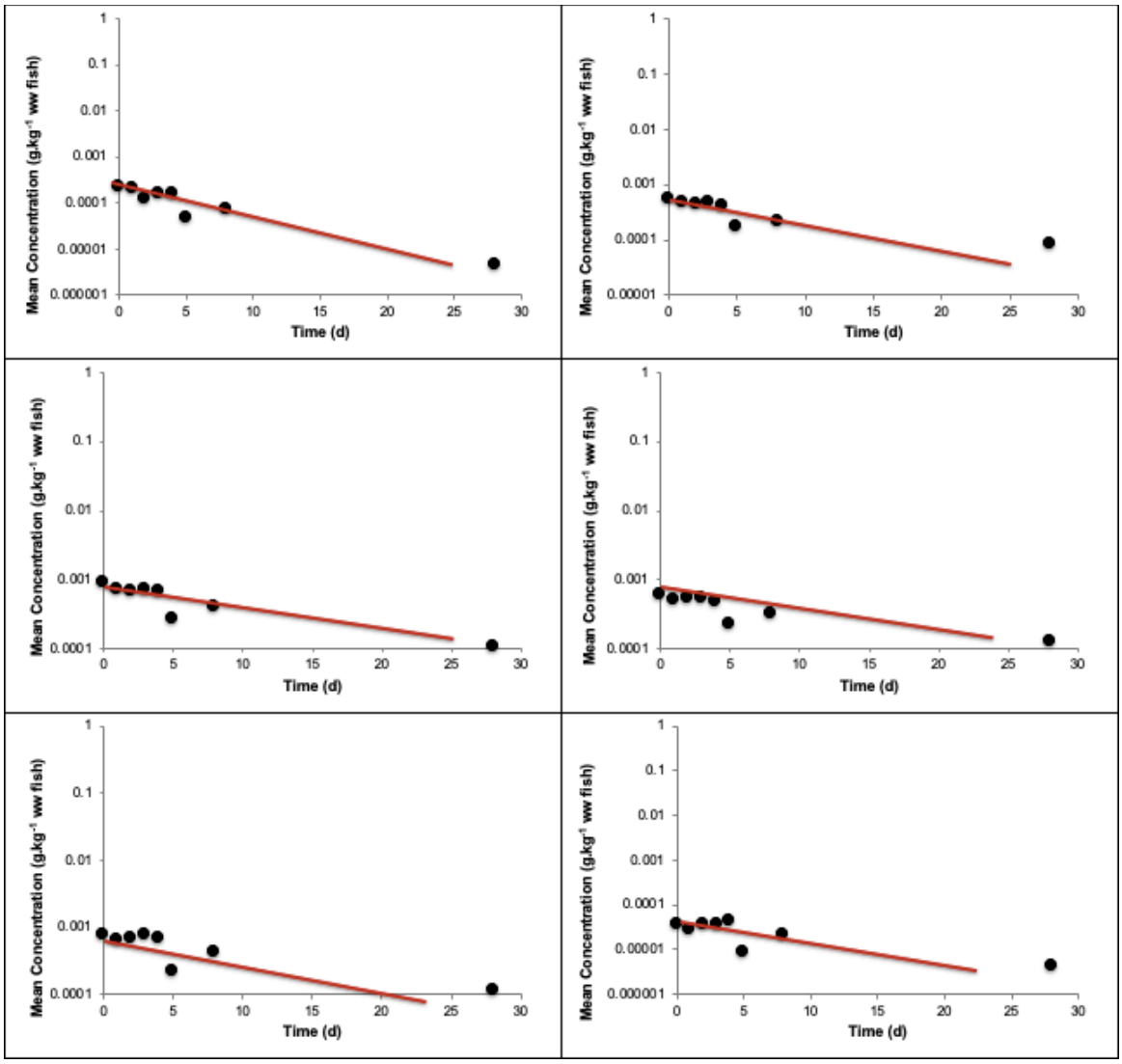


Figure 4.14. Linear regression showing the depuration of mean concentrations (g.kg^{-1} ww fish) of reference chemicals in *Oncorhynchus mykiss* fed diets spiked with decamethylcyclopentasiloxane (D5) and reference chemicals over time (d).

Comparing the log K_{OW} versus the somatic depuration rate constant (k_{BT}, d^{-1}) for D5 and reference chemicals helps to determine if D5 is being removed from a fish's body at comparable rates to reference chemicals with similar log K_{OW} values. The k_{BT} of D5 (0.0418 ± 0.008 (SE) d^{-1}) is similar to TCBz and PCB-209, suggesting that D5 is removed from fish tissue at similar rates, though the mean appears to follow faster rates associated with TCBz compared to PCB-209 (Figure 4.15; Table 4.3). The k_{BT} of D5 is similar to PCBz and HCBz. The k_{BT} of D5 is faster than PCB-52, indicating that D5 is removed from fish tissue at faster rates.

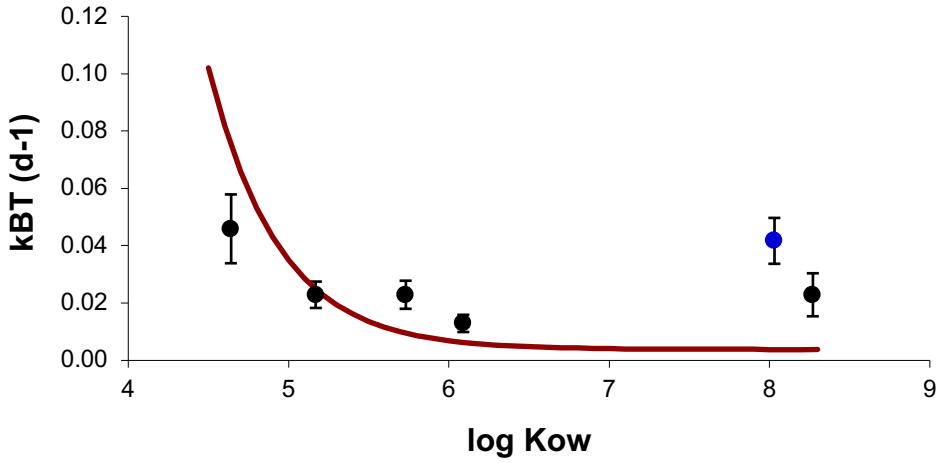


Figure 4.15. Somatic depuration rate constants (k_{BT}) for *Oncorhynchus mykiss* fed decamethylcyclopentasiloxane (D5, blue marker) and reference chemicals (black markers as a function of the logarithm of the octanol-water partition coefficient (log K_{OW})). Markers represent the mean, error bars represent the standard errors of the mean, and the red line indicates the non-linear relationship between k_{BT} and log K_{OW}. Reference chemicals from left to right: 1,2,4,5 tetrachlorobenzene (TCBz), pentachlorobenzene (PCBz), hexachlorobenzene (HCBz), 2,2',5,5'-PCB (PCB-52), and 2,2',3,3',4,4',5',5',6,6'-PCB (PCB-209).

Plotting the relationship between the somatic depuration rate constant (k_{BT}, d^{-1}) and the K_{OW}^{-1} for reference chemicals (k_{BT}, d^{-1}) helps to determine the linear relationship between the reference chemicals and thus their relative depuration constants ($k_{BT,R}$, d^{-1})), which can be used to determine their relative somatic biotransformation rates (k_{BM}, d^{-1}) (Figure 4.16). Results indicate a significant linear relationship ($p = 0.0086$, $R = 0.9270$) between the depuration constants (d^{-1}) of all reference chemicals as K_{OW}^{-1} increases following the equation:

$$y = 3072.079 \pm 497.6076\ (SE)x + 0.004484 \qquad (13)$$

The $k_{BT,R}$ for fish fed D5 and reference chemical-spiked feed was determined to be 0.0045 ± 0.0053 (SE) d^{-1}. Linear increases are as follows: PCB- 52, HCBz, PCBz, PCB-209 and TCBz. Results are consistent with previous research that shows a linear relationship between reference chemical depuration rate constants as their respective K_{OW}^{-1} increase (Lo et al. 2015). See Appendix I for a full summary output from the linear regression and ANOVA. Furthermore, solving for k_{BM} of D5 results in a 0.037 ± 0.0092 (SE) d^{-1}. It appears that both the k_{BT} and k_{BM} indicate that D5 is eliminated from tissues

at rates that are comparable to TCBz, yet faster than PCBz, HCBz, PCB-52 and PCB-209.

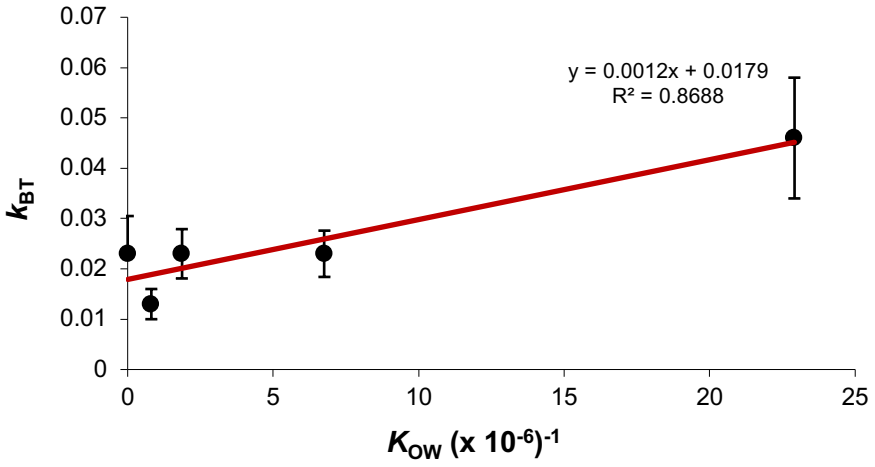


Figure 4.16. Relationship between k_{BT} (d^{-1}) measured in fish tissue sampled from fish fed diets spiked with decamethylcyclopentasiloxane (D5) and reference chemicals (black markers) versus the reciprocal of the octanol-water partition coefficient (K_{OW}^{-1}). Error bars represent standard errors of the mean and the red line represents the linear regression of k_{BT} and K_{OW}^{-1}. Reference chemicals from left to right: 2,2',4,4',5,5'-PCB (PCB-153), D5, 2,2',3,3',4,4',5',5',6,6'-PCB (PCB-209), 2,2',5,5'-PCB (PCB-52), hexachlorobenzene (HCBz), pentachlorobenzene (PCBz), and 1,2,4,5-tetrachlorobenzene (TCBz).

4.3.8. Bioaccumulation of Decamethylcyclopentasiloxane (D5) in Fish

Plotting the combined uptake and depuration concentrations and fitting it into the dietary bioaccumulation model resulted in a slow E_D of 8.4%. Inputting concentration data in the ADME-B calculator resulted in a slow dietary uptake efficiency of 8.5% (Figure 4.17). Results between the experimental results and the model results show strong model accuracy. See Supplementary Material 2B for a complete list of inputs and outputs of the ADME-B model for D5.

The results from the ADME-B calculator indicate that D5 has a $BCF_{ww,t}$ of 2289.6 ± 532.5 (SE) L water.kg^{-1} fish ww and a BMF_L 0.11 ± 0.01 (SE) kg lipid.kg^{-1} lipid ww, which is below B substance criteria outlined in s. 64 of CEPA 1999. The ADME-B BMF_L and BCF values are significantly less than 1 or 5000, respectively (p = 1.000 and 0.9822, respectively) at a confidence interval of 95%. According to s. 64 CEPA 1999, values below 1 (BMF_L) and 5000 (BCF) indicate that D5 is unlikely to bioaccumulate in aquatic environments if exposure occurs via gill respiration or from the diet.

Results from the ADME-B model indicate that the primary site of biotransformation is dependent on the route of exposure. When exposure is from the diet (i.e., gill respiration from water = 0), 81.91% of biotransformation occurs in the digesta with only 6.14% occurring in the fish body. When exposure is via gill respiration from water (i.e., dietary exposure = 0), the majority of biotransformation occurs in the fish body (70.37%) compared to the digesta (16.30%). Results are consistent with the intestines being the primary site of biotransformation in fish if the exposure route occurs via the diet. The current results are consistent with the biodilution of D5 taking place in measured aquatic environments (Powell et al. 2017).

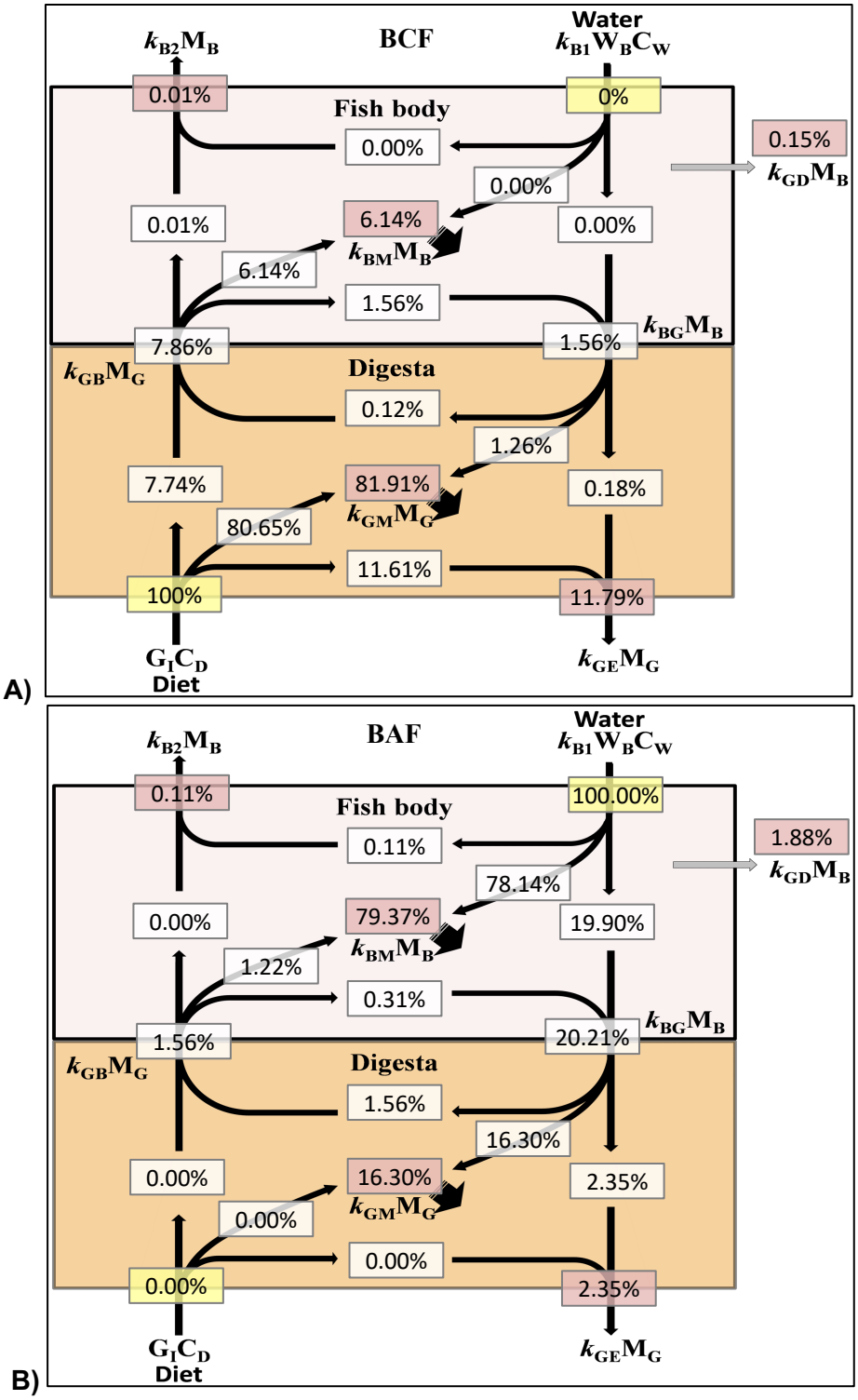


Figure 4.17. Result output profile of a two-compartment ADME-B model showing the distribution of decamethylcyclopentasiloxane (D5) from *Oncorhynchus mykiss* fed spiked diets under conditions where exposure is via gill respiration resulting in the bioconcentration factory (BCF) or B) the diet resulting in the bioaccumulation factor (BAF).

4.3.9. Comparing $E_{D,R}$ to E_D for D4 and D5

The dietary absorption efficiencies (E_D) for D4 and D5 were plotted against the relationship of the E_Ds for the reference chemicals compared to their respective log K_{OW}s ($E_{D,R}$) (Figure 4.18). The $E_{D,R}$ was sourced from Lo et al.'s (2015) bioaccumulation study and follows the equation:

$$E_{D,R}^{-1} = 5.6 \times 10^{-9} \text{ (SE } 1.8 \times 10^{-9}) * K_{OW} + 1.9 \text{ (SE } 0.1) \quad (14)$$

Comparing the E_Ds for D4 and D5 to the $E_{D,R}$ indicates that D4 and D5 are not absorbed as efficiently as the non-metabolized reference chemicals because all $E_{D,R}$s are relatively higher than the E_Ds for D4 and D5 with no overlapping error. Results imply that the reference chemicals are absorbed into the fish body at higher percentages than D4 and D5 at their respective log K_{OW}s. Given that absorption uptake follows a known relationship with their respective log K_{OW}s, the difference between the absorption of non-metabolized reference chemicals and D4 and D5 is provides evidence that D4 and D5 undergo metabolism, thus reducing the percent absorbed by the body (Lo et al. 2015).

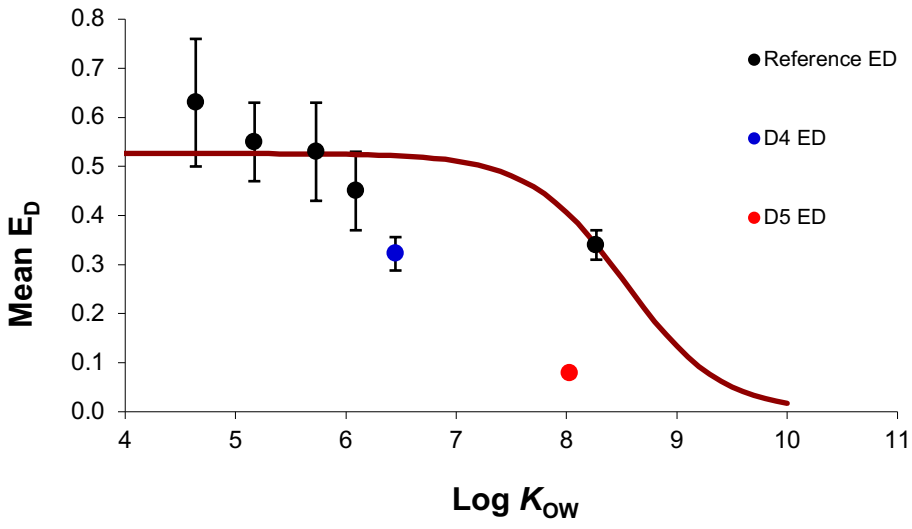


Figure 4.18. The relationship (red line) between the meane absorbance efficiencies (E_D, black markers) of the reference chemicals ($E_{D,R}$)(Lo et al. 2015) and their respective log K_{OW}S and the E_D values for octamethyltetracyclosiloxane (D4, blue marker) and decamethylcyclopentasiloxane (D5, red marker). The error bars represent the standard errors of the means.

4.3.10. Determining the Biotransformation Rate Constant of the Gut for D4 and D5 (k_{GM}, d^{-1})

Given that the current study removed and discarded the intestines to determine the somatic biotransformation and depuration rate constants, the ADME-B calculator was used to model the biotransformation rate constants in the gut (k_{GM}, d^{-1}) for D4 and D5. Results indicated relatively fast k_{GM}s of 1.26 ± 0.61 (SE) and 6.46 ± 1.12 (SE) d^{-1} for D4 and D5, respectively. Results are consisted with the ADME-B predictions that dietary exposure results in the majority of biotransformation occurring in the gut.

4.3.11. Impacts of the k_{BM} and k_{GM} on the Somatic Biotransformation Rate of D4 and D5

Given that the exposure route matters to the primary location of biotransformation (i.e., in the gut vs. the body), understanding the proportional contribution of the body and gut biotransformation rate constants on the overall somatic depuration of test chemicals from fish helps to outline risk from different exposure routes (i.e., diet vs. gill respiration). The ADME-B calculator estimated the percent of proportional contribution for the biotransformation rate of the gut k_{GM} (φ_{kGM}) and the somatic tissue k_{BM} (φ_{kBM}) to the somatic depuration of D4 and D5. When exposure is via the diet the φ_{kGM} for D4 and D5 is 61.34 and 93.0 % compared to φ_{kBM}s of 38.66 and 6.98 %, respectively. Thus, when exposure is via the diet, the majority of biotransformation of D4 and D5 occurs in the gut.

Chapter 5.

Conclusions

The first study objective was to determine the biotransformation rate constants (k_M) of D4 and D5 in rainbow trout. The current study assessed and determined that the somatic biotransformation rate constant (k_{BM}) of D4 and D5 are 0.039 ± 0.019 (SE) and 0.037 ± 0.009 (SE) d^{-1}, respectively. The biotransformation rate constants (k_{GM}) were estimated to be relatively higher than the k_{BM}s of D4 and D5 at values of 1.26 ± 0.61 (SE) and 6.46 ± 1.12 (SE) d^{-1}, respectively. This study provides support that biotransformation is a main route used to remove D4 and D5 from fish tissues.

The second study objective was to determine the contribution of biotransformation on the depuration rate and BCF of D4 and D5 in fish. The somatic depuration rate constants (k_{BT}, d^{-1}) were significant but slow for D4 and D5 (0.045 ± 0.018 (SE) and 0.042 ± 0.008 (SE), respectively. This study provides support that the exposure route affects the proportional contribution of the k_{BM} and k_{GM}. If exposure occurs via the diet and not from gill respiration, the majority of biotransformation occurs in the gut. If exposure occurs via gill respiration and not through the diet, the primary site of biotransformation occurs in the body. Therefore, the mode of exposure affects the location of biotransformation, the depuration rate, and the resulting BCF assessment.

Overall, this study provides support that biotransformation aids in increasing the depuration rate for dietary exposures of D4 and D5 in comparison to non-volatile, non-metabolized reference chemicals. Increasing the depuration rates reduces the bioaccumulation of D4 and D5 in fish. For instance, this study determined that D4 and D5 does not meet B substance criteria for BCF, BMF_L and the BAF under s. 64 of CEPA 1999 and are unlikely to bioaccumulate according to the law as values were below B criteria. The current findings are consistent with previous research measuring environmental concentrations that predict biodilution.

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Appendix A.

Experimental Set Up.

Figure A1. **Shows the tank flow-through set up containing 252 juvenile rainbow trout (*Oncorhynchus mykiss*). Tank 1 was the control (n = 36) that did not receive test or reference chemicals; tank 2 was the reference (n = 36) that only received reference chemicals; tanks 3-8 (n = 36 tank^{-1}) contained test fish ranging in three concentrations of octamethylcyclotetrasiloxane (D4) and three concentrations of decamethylcyclopentasiloxane (D5). It should be noted that the fish from tanks in low and medium concentrations of D4 and D5-spiked feed were extracted but are not discussed in the current study because concentrations were below the limit of detection.**

Appendix B.

Estimated Test and Reference Chemical Concentrations in Feed

Table B1. Estimated concentration ($g.kg^{-1}$) of reference chemicals in 'reference chemical stock fish feed' after accounting for estimated total transfer (%) from a hexane solution.

Reference Chemicals	Amount (g)	Feed (kg)	Est. Transfer (%)	Concentration ($g.kg^{-1}$ feed)
TCBz	0.0149	6.0	80	0.0248
PCBz	0.0154	6.0	80	0.0257
HCBz	0.0152	6.0	80	0.0253
PCB-52	0.0156	6.0	80	0.0260
PCB-153	0.0150	6.0	80	0.0250
PCB-209	0.0155	6.0	80	0.0258

Table B2. Predicted concentrations ($g.kg^{-1}$) of octamethylcyclotetrasiloxane (D4) added to spiked reference chemical stock to create a concentrated stock (C_{STOCK}) and a diluted stock ($C_{DILUTED}$), which were further diluted to create high concentration feed (C_{HIGH}) and corrected for a 20% chemical loss during transfer.

RBF Label	Total Test Chemical (g)	Test Feed (kg)	Reference Feed (kg)	Total Feed (g)	Predicted C ($g.kg^{-1}$)
C_{STOCK}	5.19	NA	0.0201	0.0205	258.85
$C_{DILUTED}$	1.20	0.006	0.0541	0.0602	19.93
C_{HIGH}	0.12	0.006	0.0540	0.0601	2.00

Table B3. Predicted concentrations ($g.kg^{-1}$) of decamethylcyclopentasiloxane (D5) added to spiked reference chemical stock to create a concentrated stock (C_{STOCK}) and a diluted stock ($C_{DILUTED}$), which were further diluted to create high concentration feed (C_{HIGH}) and corrected for a 20% chemical loss during transfer.

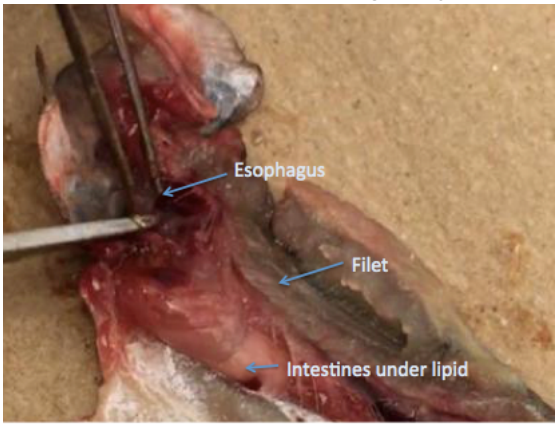
RBF Label	Total Test Chemical (g)	Test Feed (kg)	Reference Feed (kg)	Total Feed (kg)	Predicted C ($g.kg^{-1}$)
C_{STOCK}	5.190	NA	0.0201	0.0201	259.721
$C_{DILUTED}$	1.200	0.006	0.0540	0.0600	20.000
C_{HIGH}	0.120	0.006	0.0540	0.0600	2.000

Appendix C.

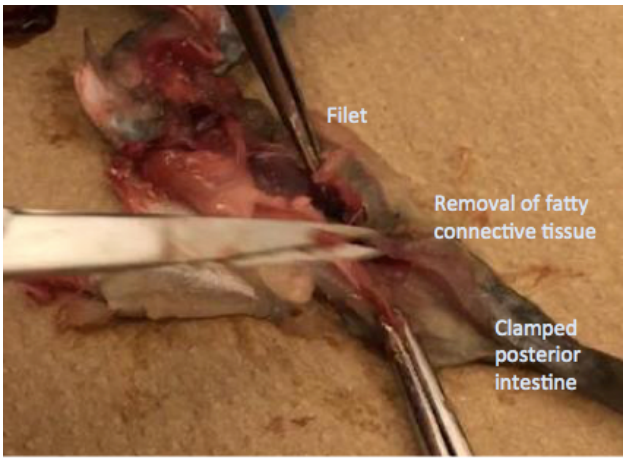
Fish Dissection Protocol

1. Weigh (kg) the fish on a clean piece of aluminum foil
2. Record the length of the fish (cm) from the rostrum to the peduncle and keeping the ruler placed along the lateral line

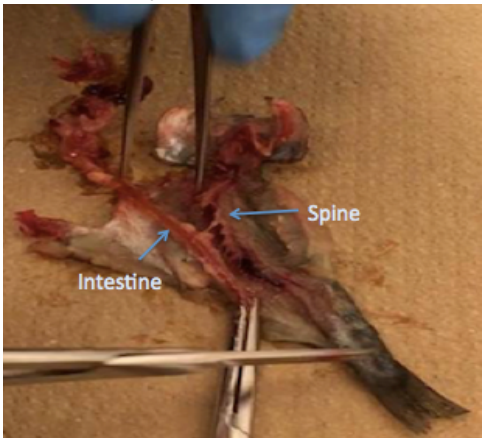
3. Place fish on large Kim wipe
4. Sever the spinal cord at the dorsal-posterior region behind the head to ensure the fish has died prior to dissection
5. Make an incision with scissors at along the ventral plane the jaw
6. Continue the incision around the anterior end of the gills, while moving towards the dorsal plane to extend the incision above the eye
7. Extend the incision from the jawline towards the posterior end, using the lateral lines as a guide along the filet
8. Be careful to cut out and away from the fish to ensure the intestinal tract is not ruptured
9. Pull back the filet to expose the organs
10. The ventral incision along the jaw will allow you to see the start of the esophagus



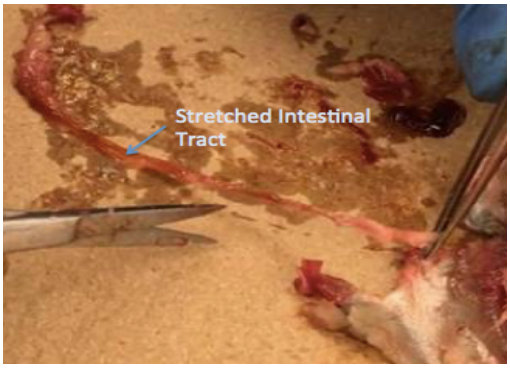
11. Use the hinged forceps to clamp the posterior end of the intestinal tract near the anus



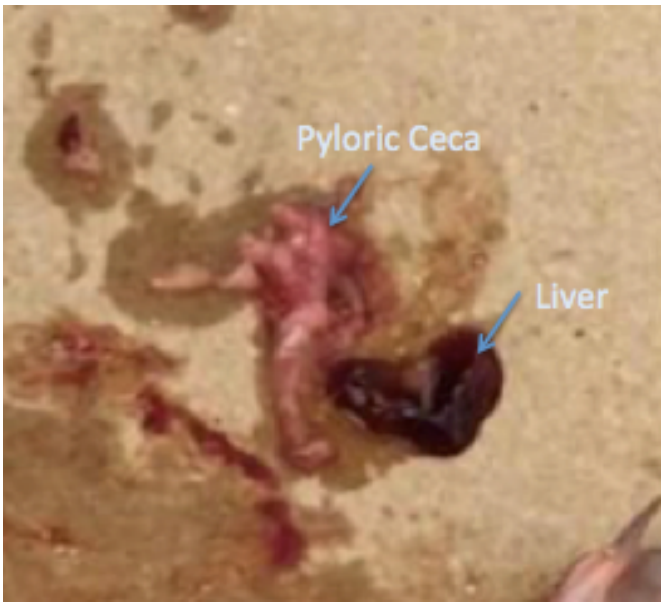
12. Remove the liver and place it in a Mason jar (label with treatment and date)
13. Slowly start cutting lipid and tissue along the spine to free the intestinal tract from the body



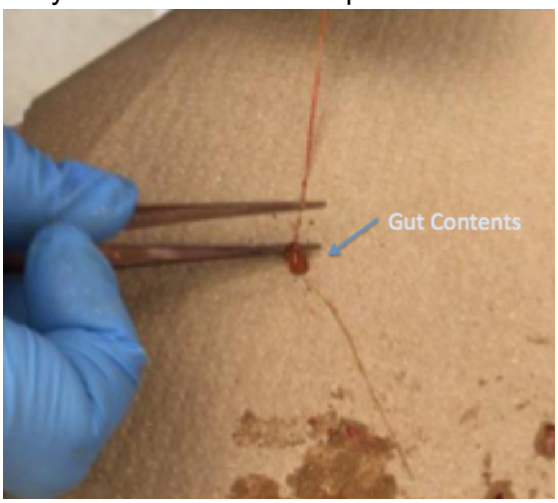
14. The intestinal tract will be somewhat rolled up with strands of fat. Cut these strands to allow the intestinal tract to lay in a straight line and extend away from the body



15. Cut the posterior end of the intestinal tract on the anterior end of the hinged forceps
16. Continue to cut strands of fat to fully remove the intestine from the body
17. Remove as much of the lipid (lighter pieces) from the intestines with forceps – it will be easy to remove and peel off
18. Remove and discard the stomach and pyloric ceca to prevent contents from contaminating the body tissue samples



19. Evacuate the gut contents into a 2 mL amber vial by holding the anterior end of the lower intestine and gently squeezing curved or straight forceps together as you move towards the posterior end of the lower intestine



20. Cap, Teflon seal and store in fridge for Dow (Label: Treatment/date)
21. Discard intestines to prevent contamination of fish tissue
22. Gather the remaining fish body and combine it in the mason jar with the liver
23. Blend (1 min) until thoroughly homogenized

Appendix D.

Rationale for Sorbent Choice

Adding C-18 and PSA was in accordance with cyclic siloxane extractions performed on seaweed (Researchgate 2017) and follows traditional QuEChERS methods (Anastassiades et al. 2003). C-18 and PSA are used in combination in order to target different coextracted materials. For instance, PSA interacts with chemicals via hydrogen bonding to remove fatty acids, organic acids, sugars and pigments from the pentane layer. C-18 has been used to target planar, non-polar matrix components that bind to non-polar test chemicals, such as lipids (Geogakopoulos et al. 2011).

$MgSO_4$ was chosen to dehydrate the sample and further promote recovery by influencing the polarity of the solvent and removes coextractants. Dehydration is necessary because it has been demonstrated that peak signals for D4 detected by GC/MS can be amplified by water. It has been hypothesized that water cleaves the Si-O bonds found in polydimethylsiloxane-based stationary columns to create D4 within the GC/MS (Varaprath et al. 2000). Signal amplification was supported during analytical tests that compared concentrations in a tetrahydrofuran solvent to concentrations from extracted feed following methods from Varaprath et al. (2000) (Figure D1). Our Preliminary results agreed with Varaprath et al. (2000) that $MgSO_4$ prevents signal amplification for cyclic siloxanes by a magnitude of almost 4 when corrected for volume (Figure D1). $MgSO_4$ may also aid in precipitating polar coextractants and reduces the polarity of ACN assist in extracting nonpolar test chemicals (Anastassiades et al. 2003).

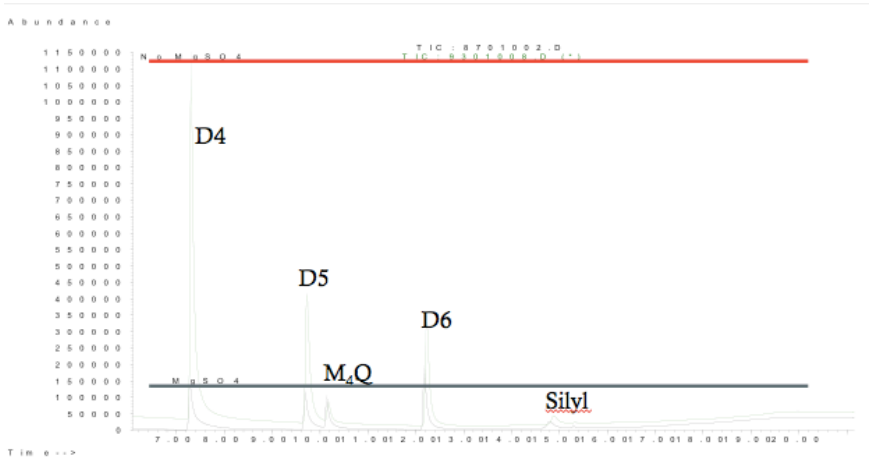


Figure D1. Chromatogram showing absolute peak height from test chemicals octamethylcyclotetrasiloxane (D4), decamethylcyclopentasiloxane (D5) and the internal standards (tetrakis(trimethlylsilyl)siloxane (SIL) tetrakis(trimethylsiloxy)silane (M4Q) extracted from feed without $MgSO_4$ (red line) and with the addition of $MgSO_4$ (grey line) during the extraction process following methods from Varaprath et al. (2000). This chromatogram does not represent adjusted solvent volume between extractions with $MgSO_4$ and without $MgSO_4$. When corrected for solvent volume, the 10x over amplification of extraction without $MgSO_4$ is reduced to ~ 4x higher than extraction with $MgSO_4$.

Appendix E.

Lipid Analysis

E1. Lipid Analysis Protocol

Lipid analysis followed modifications to Bligh and Dyer's (1959) lipid extraction method. Fish tissues were thawed and approximately 4 g of fish tissue from each sampling date was transferred to 50 mL falcon centrifuge tubes and ran in triplicate. Average weights were calculated for tissues from each sample date. The thawed samples were homogenized for 60 s. 4 mL of chloroform was added to each centrifuge tube followed by 8 mL of methanol. A 4 mL solution (0.05 g chloroform.L^{-1} butylated hydroxyl toluene) was a lipophilic organic compound that was added to reduce lipid oxidation. 4 mL of dH_2O was then added and the mixture was homogenized (60 s). The mixture was allowed to settle for 10 min and then centrifuged (1500 rpm, 20 min). The upper alcohol layer was aspirated and discarded. A Pasteur pipette was used to penetrate the protein layer in order to access and remove the upper chloroform layer containing the lipid content. The chloroform layer was then deposited into a graduated cylinder where the volume was recorded. A 1 mL aliquot of the lipid layer was transferred into a disposable glass test tube in triplicate. Each of the triplicate samples were weighed twice, averaged, and then placed under a nitrogen stream to dry. Each tube was then re-weighed, and averages were recorded. Total lipid (kg) calculations are as follows: $L_{TOTAL} = W_L * V_L / V_{aliquot}$. Where L_{TOTAL} refers to the total lipid (kg), $W_{Laliquot}$ refers to the weight (kg) of the lipid in an aliquot sample, V_L refers to the average volume (mL) of total lipid layer taken from triplicate samples and the $V_{aliquot}$ refers to the volume (mL) of the aliquot sample. The percent lipid ($kg.kg^{-1}$ tissue) was calculated as follows: % lipid content = $(L_{TOTAL} / W_{TOTAL}) * 100\%$, where W_{TOTAL} refers to the average of the triplicate tissues sampled.

E2. Lipid Analysis Results

Table E1. Total average % lipid sampled from fish at three different sampling points (uptake day 2, depuration day 14 and depuration day 28) from each of the given tanks.

Tank	Uptake (Day 2)	Depuration (Day 14)	Depuration (Day 28)	Mean	SD	CV	Significance (Y/N)
Control	3.82	3.28	3.58	3.56	0.27	0.08	N
Reference	2.25	3.31	3.58	3.05	0.70	0.23	N
D4high	2.43	4.28	4.37	3.69	1.10	0.30	N
D5high	3.22	1.14*	3.49	3.36	0.19	0.06	N

*Note: Lipid analysis for $D5_{HIGH}$ on Depuration Day 14 was omitted because the available tissue was primarily composed of scale and tail, thus likely underestimating the amount of lipid. Thus, the average total for Depuration Day 14 was based on Control, Reference, $D4_{HIGH}$ and $D5_{HIGH}$ only.

Appendix F.

Method Validation and Rationale for Sorbent Choice

F1.1. Method Development: Feed

Standard curve were created to determine the relationship between the signal strength and known concentrations of test and reference chemical in fish feed. Concentrations of test and reference chemicals were created by extracting clean feed and then spiking the extracted pentane (i.e., the matrix-matched pentane) after the extraction took place (POST XT) (Figure F1). Linear regressions produced high coefficients of determination for both D4 and D5 (R^2 = 0.995 and 0.997).

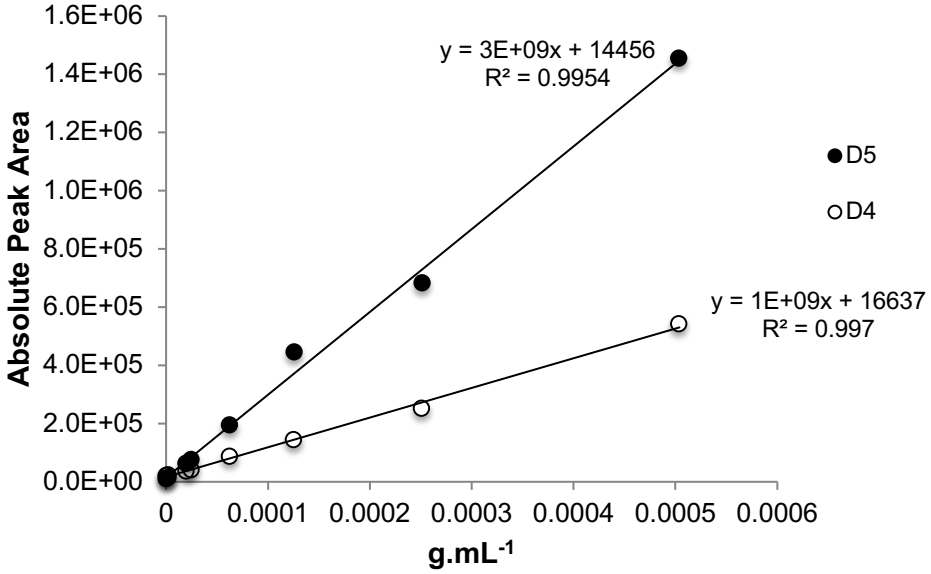


Figure F1. Standard curves showing the relationship between absolute peak area and the concentration (g.mL^{-1}) of test chemical in the fish feed extract standard curve that was created by spiking extracted pentane with reference chemicals and octamethylcyclotetrasiloxane (D4) or decamethylcyclopentasiloxane (D5) after the feed had undergone the extraction process (POST XT).

F1.2. Method Development: Fish Tissue

Chemical recoveries from fish tissue were determined by comparing standard curves made from tissue that underwent the extraction process to produce matrix-matched pentane, which was then spiked with known concentrations of test, reference and IS (POST XT) (Figure F2) to standard curves created by spiking fish tissues with known concentrations before the extraction took place (PRE XT) (Figure F3).

POST XT standard curves for D4 and D5 showed high coefficients of determination (R^2 = 0.988; 0.956). Results indicate strong linear relationships between the concentration of D4 and D5 in fish tissue (g.kg^{-1} ww fish) and signal strength expressed by the peak area ratio (PAR). PAR was determined by comparing the peak areas of D4 and D5 with the peak areas of M4Q or SIL, respectively. The high coefficient of determination also accounts for a majority of observed variance (Figure F2).

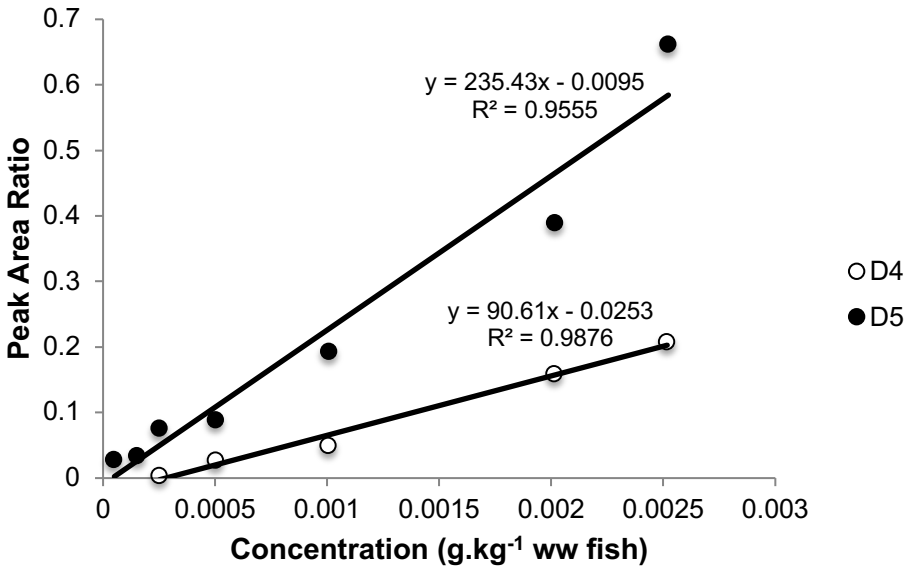


Figure F2. Standard curves showing the peak area ratio (PAR) and the concentration (g.kg^{-1} ww fish) of test chemicals in fish tissues from matrix-matched pentane that was spiked after the extraction occurred (POST XT). PAR was calculated by dividing the peak area of octamethylcyclotetrasiloxane (D4) by the peak area of tetrakis(trimethylsiloxy)silane (M4Q) and the peak area of decamethylcyclopentasiloxane (D5) by the peak area of tetrakis(trimethylsilyl)siloxane (SIL).

Linear regressions were run on standard curves that were created by spiking fish tissue with known concentrations of test and reference chemicals and then extracting the chemicals from the tissue (PRE XT). Coefficients of determinations for D4 and D5 followed a linear increase in PAR signal strength with increasing concentration (g.kg^{-1} ww fish) (R^2 = 0.9775; 0.9968) (Figure F3). Results indicate a linear relationship that explains the majority of variation in signal strength based on concentration.

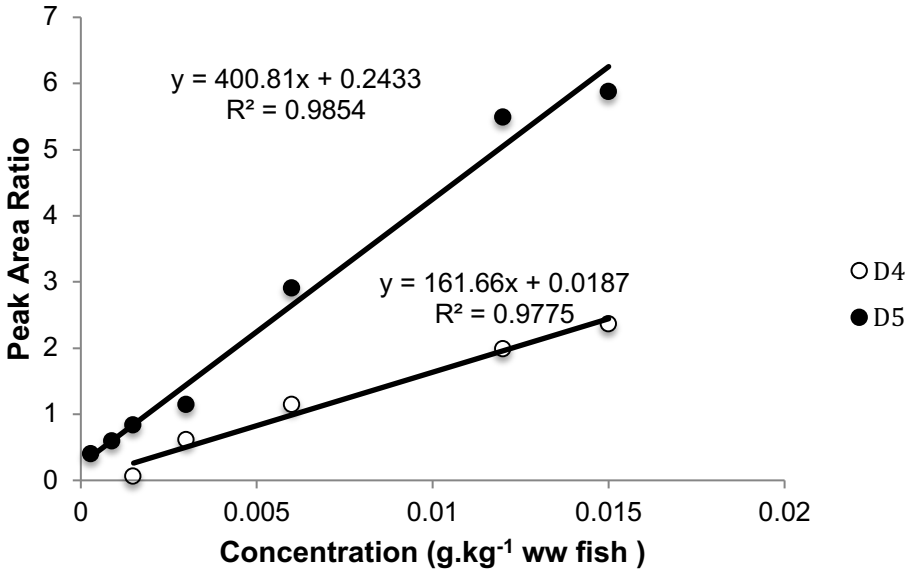


Figure F3. Standard curves showing the peak area ratio (PAR) and concentration (g.kg^{-1} ww fish) of test chemicals in fish tissues that were spiked before the extraction occurred (PRE XT). PAR was calculated by dividing the peak area of octamethylcyclotetrasiloxane (D4) with the peak area of tetrakis(trimethylsiloxy)silane (M4Q) and the peak area of decamethylcyclopentasiloxane (D5) with the peak area of tetrakis(trimethylsilyl)siloxane (SIL).

The average percent recovery for D4 and D5 from fish tissue was determined by comparing the PAR associated with each concentration in the PRE XT standard curve to the POST XT standard curve. Results indicate that D4 and D5 are recovered at 2346.4 ± 758.0 and 1797.6 ± 386.8% respectively. All reference chemicals were recovered at ≥ 43%. See Table G4 for average percent recovery and associated standard deviations.

Results indicate that the PRE XT curve overestimated D4 and D5 recoveries compared to POST XT curves. Overestimates may indicate that the test chemicals undergo matrix effects when water, heat, and equipment-based polydimethylsiloxanes are combined during the extraction process. Overestimating D4 and D5 recovery is consistent with findings from Varaprath et al. (2000) where cyclic volatile methyl siloxanes (cVMS) are overestimated during the ion elution process in the GC. Varaprath et al. (2000) postulates that interactions between the polydimethylsiloxane components of the GC, high temperatures, and water can generate cVMS within the GC. This rationale may help explain overestimates in the PRE XT standard curves in the current experiment. For instance, the POST XT was created using matrix-matched pentane (i.e., dehydrated pentane that had undergone the extraction process and was then spiked) and is predicted to create fewer cVMS within a sample. In contrast, the PRE XT was created using the tissues that were spiked and then extracted using salts to dehydrate the samples. However, the final concentrations in the PRE XT were diluted using non-dehydrated pentane to reduce concentrations to those compatible with the GC/MS. Future research on cVMS (i.e., D4 and D5) should use dehydrated solvents when performing all dilutions to prevent overestimations if spontaneous cVMS creation is occurring within the GC.

Furthermore, the discrepancies between the recoveries calculated by comparing the POST XT and PRE XT standard curves of test chemicals led the current study to use the PRE XT to determine unknown concentrations. Though the PRE XT overestimates the concentration of D4 and D5, the design of the PRE XT curve should encompass variations in matrix effects that occur during the extraction process.

Results also indicate that the reference chemicals did not experience overestimates given the relatively normal (0-100% range), yet low, recoveries reported ("LC-MS Method Validation", n.d.). Low recoveries support that reference chemicals did not experience overestimates. Relatively high test-chemical (> 1000%) recoveries indicate that test chemicals were overestimated. Both test and reference chemicals underwent the same extraction methods using the same tissue samples. Discrepancies between recoveries of test and reference chemicals from tissue indicate that the current method may be more effective at extracting (though overestimating) test chemicals compared to reference chemicals.

Appendix G.

Concentration Data from Fish Tissue

Table G1. The mean concentrations ($g.kg^{-1}$ ww fish) and standard errors of the mean (SE) of octamethylcyclotetrasiloxane (D4) and decamethylcyclopentasiloxane (D5) in fish fed spiked diets over time (d).

Uptake Day	Depuration Day	D4 ($g.kg^{-1}$ ww fish)	D4 - SE ($g.kg^{-1}$ ww fish)	D5 ($g.kg^{-1}$ ww fish)	D5 - SE ($g.kg^{-1}$ ww fish)
2		6.10×10^{-4}	4.77×10^{-5}	2.00×10^{-3}	2.80×10^{-5}
3		3.27×10^{-4}	3.76×10^{-5}	3.85×10^{-3}	7.90×10^{-4}
5		9.65×10^{-4}	4.00×10^{-5}	4.24×10^{-3}	5.08×10^{-4}
10	0	3.12×10^{-3}	5.46×10^{-4}	1.43×10^{-2}	1.22×10^{-3}
	1	1.37×10^{-3}	2.07×10^{-4}	7.78×10^{-3}	9.04×10^{-4}
	2	1.21×10^{-3}	1.01×10^{-4}	1.22×10^{-2}	1.03×10^{-3}
	3	1.91×10^{-3}	4.22×10^{-4}	1.19×10^{-2}	2.55×10^{-3}
	4	2.19×10^{-3}	3.28×10^{-4}	1.16×10^{-2}	1.89×10^{-3}
	5	1.78×10^{-3}	7.40×10^{-4}	1.01×10^{-2}	2.03×10^{-4}
	8	1.29×10^{-3}	1.53×10^{-4}	8.58×10^{-3}	$3. \times 10^{-4}$
	14	1.07×10^{-3}	4.61×10^{-5}	3.91×10^{-4}	4.54×10^{-5}
	28	1.18×10^{-3}	8.51×10^{-5}	3.76×10^{-3}	3.07×10^{-4}

Table G2. Standard error of the models of the concentrations ($g.kg^{-1}$ ww fish) of octamethylcyclotetrasiloxane (D4) and decamethylcyclopentasiloxane (D5) in fish fed spiked diets over time (d).

SEM ($g.kg^{-1}$ ww fish)	D4	D5
Bioaccumulation Model	5.80×10^{-6}	9.15×10^{-5}
Dietary Absorption Model	1.81×10^{-4}	3.36×10^{-4}
Depuration Model	2.35×10^{-5}	1.27×10^{-4}

Appendix H

Summary Output of linear regressions and ANOVAs conducted on D4 in fish tissues from fish fed spiked diets.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.962826
R Square	0.927033
Adjusted R	0.902711
Standard E	0.009511
Observatio	5

ANOVA

	df	*SS*	*MS*	*F*	*ignificance F*
Regressior	1	0.003448	0.003448	38.11454	0.008556
Residual	3	0.000271	9.05E-05		
Total	4	0.003719			

	Coefficients	*andard Err*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*ower 95.0%*	*Ipper 95.0%*
Intercept	0.004484	0.005335	0.840509	0.462331	-0.01249	0.021461	-0.01249	0.021461
X Variable	3072.079	497.6076	6.173697	0.008556	1488.469	4655.688	1488.469	4655.688

Figure H1. Summary output of linear regression and ANOVA for the depuration rate constants (k_{BT}, d^{-1}) of reference chemicals compared to their respective K_{OW}^{-1} (x 10^{-6}).

Appendix I

Summary Output of linear regressions and ANOVAs conducted on D5 in fish tissues from fish fed spiked diets.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.905098
R Square	0.819202
Adjusted R	0.789069
Standard E	0.192807
Observatic	8

ANOVA

	df	*SS*	*MS*	*F*	*ignificance F*
Regressior	1	1.010633	1.010633	27.18616	0.001988
Residual	6	0.223047	0.037175		
Total	7	1.233681			

	Coefficients	*standard Erı*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*ower 95.0%*	*Ipper 95.0%*	*Ipper 95.0%*
Intercept	2.507016	0.085213	29.42065	1.02E-07	2.298508	2.715524	2.298508	2.715524	2.715524
X Variable	-0.04182	0.008021	-5.21403	0.001988	-0.06145	-0.02219	-0.06145	-0.02219	-0.02219

Figure I1. Summary output of linear regression and ANOVA for the depuration rate constants (k_{BT}, d^{-1}) of reference chemicals compared to their respective K_{OW}^{-1} (x 10^{-6}) for fish fed D5.

Supplementary Material

Supplementary Material 1.

Determination of Recovery Efficiency Using a Corn Oil Carrier
Report ver. 1.3 - Experimental extraction of octamethylcyclotetrasiloxane (D4) from fish feed using a modified QuEChERS method and absolute peak height standard controls.

By: Karen L. Compton
Date: April 29th, 2017

METHOD.

Fish feed (Alcan EWOS Pacific for salmonids in a 1.5 mm pellet diameter containing 50% protein, 18% lipid, 2% fiber, 2.1% calcium, 1.1% potassium and 0.5% sodium) was spiked with the highly volatile cyclic siloxane octamethylcyclotetrasiloxane (D4) [CAS no. 556-67-2; Dow Corning, Inc.] and then extracted in order to estimate the dose that could be fed to rainbow trout (*Oncorhynchus mykiss*). Analytical methods were developed to improve extraction recovery from feed by mitigating volatilization and contamination.

Preliminary investigations not reported here dictated that traditional methods that use solvents to spike feed and columns to clean extracted samples (Lo et al. 2015) may not be effective for highly volatile chemicals such as D4. Furthermore, sample extraction following methods that reduced evaporation and solvent use were either time consuming (Varaprath et al. 2000) or resulted in low process efficiencies at our facility (Varaprath et al. 2000; the Norwegian Institute for Air Research as described in McGoldrick et al. 2010). Low recoveries were likely a combination of spiking feed with solvents that required evaporation, and thus loss of highly volatile siloxanes, and the method's inability to fully remove D4 from high fat coextractants associated with feed.

As a result, this protocol prevents evaporation and reduces headspace when storing feed and/or test chemical to prevent volatilization. Furthermore, additional steps were taken to reduce contamination. These included banning the use of siloxane-containing personal care products 12-hrs before lab personal worked on the study and the addition of dehydrating components to extracted samples. Method details are recorded below.

Spiking Solution

Spiking solution was prepared following methods from the OECD test no. 305 for highly volatile chemicals. D4 (0.0267 g) and the reference chemical pentachlorobenzene (PCBz) [CAS no. 608-93-5] (0.0256 g) were added to corn oil (CO) (0.2582 g, ~ 345 µl) in a 2 mL amber vial, vortexed for (20 s), placed on a shaker at high (30 min) and then mixed overnight on the rotor rack at 60 rpm (Stuart – SRT9D). The contents were stored in the fridge if use was not immediate. A control solution was created by following the abovementioned methods and used a similar mass for the reference chemical and vehicle (PCBz = 0.0252 g; CO = 0.2681 g).

Spiking Feed

Spiking fish feed followed methods from the OECD test no. 305 (2012). Fish feed (0.0757 kg) was portioned into a weigh boat. Approximately half of the feed was added to a 125 mL round bottom flask (rbf). The prepared CO-spiking solution was vortexed (15 s) and added to the rbf containing feed. The amber vial was washed with CO and the contents were added to the rbf (w_{total} = 0.00053 kg). The vial was then capped and hand

shaken (30 s) to ensure equal mixing of CO throughout the feed. The remaining half of the feed was added to the rbf, capped with a glass stopper, and sealed using Teflon tape. The rbf was shaken by hand (30 s) to further homogenize the CO throughout the feed then placed on the rotor rack at 10 rpm and allowed to mix overnight. Calculated concentrations for test, reference and vehicle in feed are as follows: D4 = 0.353 $g.kg^{-1}$; PCBz = 0.338 $g.kg^{-1}$; CO = 7.00 $g.kg^{-1}$). A control was prepared using comparable amounts of un-spiked CO (w = 0.55 g) in feed (w = 0.0756 kg) and with the above-mentioned procedure. CO concentration did not exceed 5% in accordance in with OECD test no. 305 (2012) in test or control feed. See Supplementary Appendix SA for concentration and mass values.

QuEChERS Extraction

A Quick Easy Cheap Effective Rugged Safe (QuEChERS) method was modified from Wang et al.'s (2017) cyclic siloxane extraction from plasma. Feed (0.001 kg) was weighed into 15 mL conical centrifuge tubes (Falcon - 352096). The tube intended for the pre-extraction spiked feed received feed spiked with D4 and PCBz in a CO vehicle and were ran in triplicate (REP1, REP2 and REP3). Control (CTRL) and post extraction spiked feed standard (POST XT) tube received control feed spiked with PCBz in CO vehicle. Centrifuge tubes labeled pre extraction spiked solvent (PREXT) and no-extraction spiked solvent (noXT) did not receive feed. Mildly polar acetonitrile (ACN) [CAS no. 75-05-8; Fisher Chemical] (1 mL) was added to all centrifuge tubes except for noXT and then shaken by hand (1 min) to aid in a single – phase extraction (Anastassiades et al. 2003). See Appendix SB for detailed assembly of sample tubes.

Aliquots of the internal standards (IS) tetrakis(trimethylsilyl)siloxane (SIL) [CAS no. 4098-98-0; Gelest, Inc.] and tetrakis(trimethylsiloxy)silane (M4Q) [CAS no. 3555-47-3; Gelest, Inc.] from a 10 mL stock in toluene [CAS no. 108-88-3; Fisher Chemical] solution were added to all tubes. SIL was added at appropriate volumes to achieve equal mass of D4 as expected in 0.001 kg of spiked feed, while the mass of M4Q was reduced by a factor of 4. M4Q was chosen in accordance with previous research (Varaprath et al. 2000; Woodburn et al. 2013). Aliquots of D4 and reference chemicals were then added to match concentrations to those expected in 0.001 kg of spiked feed (Table S1). REP1-3, CTRL, POS TXT and PRE XT were placed on a shaker at high (30 min).

Table S1. Concentration of chemicals in toluene stock solution compared to expected mass of D4 in 0.001 kg of spiked fish feed as used to spike POSTXT and PREXT standard controls (test and reference) and all centrifuge tubes with IS.

Use	Chemical	C_{stock} ($g.mL^{1-}$)	V (ul)*	M (g)
Reference	PCBz	0.00197	181	0.00036
Test	D4	0.00167	305	0.00036
Internal Standard	SIL	0.00147	251	0.00036
Internal Standard	M4Q	0.00126	71	8.9×10^{-5}

*V (mL) was calculated to predict the amount required to match the mass of reference or internal standards in standard controls to the expected mass (g) of D4 in 0.001 kg of feed.

Salts, sodium chloride (NaCl) [CAS no. 7647-14-5; Fisher Chemical] (0.2 g) and anhydrous magnesium sulfate ($MgSO_4$) [CAS no. 7487-89-9; Fisher Chemical] (0.25 g) were added to all centrifuge tubes to aid in liquid-liquid partitioning and forces ACN into

organic content as the mixture is shaken by hand (1 min) (Anastassiades et al. 2003). Pentane [CAS no. 109-66-0; Caledon Laboratories, Ltd.] (4 mL) was added to all centrifuge tubes, placed on a shaker (30 min) to further separate the liquid-liquid layer, and then centrifuged (5000 rpm, 10 min) (Wang et al. 2017). Pentane is a nonpolar solvent that is immiscible with ACN and allows the extraction of nonpolar test chemicals in combination with varying amounts of NaCl (Anastassiades et al. 2003). See Appendix SB for detailed sample preparation per sample tube.

Pentane (3 mL) per sample was pipetted into a new corresponding centrifuge tube. All new tubes contained the sorbents octadecysilane (C-18 = 0.18 g; Agilent Technologies Part No. 5982-5752), silica-bonded primary secondary amine (PSA = 0.18 g; Agilent Technologies Part No. 5982-8382), and $MgSO_4$ (0.25 g) to aid in lipid-chemical separation. It is important to note that noXT was not pipetted into a new tube and was only exposed to $MgSO_4$ in the original tube for dehydration purposes. All samples were centrifuged (10 min, 5000 rpm) to separate liquids from sorbents and completed the first extraction in a series of 3. See Appendix F for rationale for the use of PSA, C-18 and $MgSO_4$ as sorbents. Fresh pentane (3 mL) was added to the original working tube-containing feed, shook by hand (5 min) and centrifuged (10 min, 5000 rpm). The top 3 mL of pentane was transferred into a new 12 mL centrifuge tube containing sorbents and centrifuged (10 min, 5000 rpm) to complete the second extraction in the series. This process was repeated two more times for a total of 3 extractions that were not pooled in order to determine the extraction recovery of each successive extraction.

REP1-3 and CTRL centrifuge received 486 µL of toluene to compensate for matrix-induced changes in GC/MS retention time between samples that received test and reference chemicals from a CO vehicle (i.e., REP1-3) versus standard controls that were spiked with test and reference chemicals from toluene stock solutions (i.e, POST XT, PRE XT and noXT). See Appendix F for chromatogram of matrix-induced changes in retention time and additional rationale for adding toluene.

The POST tXT centrifuge tube received test and reference chemicals from toluene stocks in amounts equal to those expected in REP1-3 (D4 = 305 µL; PCBz = 181 µL). Finally, an aliquot of 10 µL.tube^{-1} was pipetted into a 2 mL amber vial containing 990 µL of pentane. See Appendix SB for detailed description of individual sample preparation, concentrations and masses.

Analysis

GC/MS

Extracts were blank corrected using pentane to account for laboratory contamination and analyzed to determine the absolute peak height in samples compared to standard controls using an Agilent 6890 gas chromatograph (GC) connected to an Agilent 5973N mass spectrometer (MS). The MS contains a cool-on column auto-injector port, a 5% phenyl methyl siloxane-coated column (30 m x 250 µm x 0.25 µm HP – 5MS; Agilent) and deactivated guard column comprised of fused silica (5 m x 530 µm x 0.25 µm; Agilent). The oven was programmed at an initial temperature of 30°C for 2 min, increased by 10 °C min^{-1} to 160°C for 15 min and then finally increased by 30°C min^{-1} to 300°C. The GC/MS uses helium as a carrier gas under a flow rate of 1mL min^{-1} with an initial injection of 1.0 µL from a 10-µL gas-tight glass syringe (Agilent). MS data was collected by selecting test, reference and IS ions as follows: D4 = 281, PCBz = 250, M4Q = 281, SIL = 232). D4, M4Q and PCBz ion selection was in accordance with previous research that had high signal recovery with reduced interference (Varaprath et al. 2000; Woodburn et al. 2013; Lo et al. 2015). Ion selection for SIL was determined by evaluating a diluted amount of SIL in solvent under scan mode within a retention time

(RT) of 10 min to determine which ions have high intensity and few interference peaks. Absolute peak heights were integrated and quantitated via Chemstation software in order to compare between standards of known concentration and unknown concentrations extracted from spiked feed.

Matrix Matched Analysis

Mathematical relationships have been established between each sample preparation described above and were used to measure process efficiency (PE), matrix effect (ME), extraction recovery (ER) and extraction yield (EY). The process efficiency is the truest term for how a GC/MS process is functioning and encompasses the product of the percent recovery and the matrix effect (Leito [date unknown]).

$$PE = (\%\ Recovery \times \%\ ME) / 100 \quad (S1)$$

or in this case:

$$PE = c / a = Avg.\ (REP1 – 3) / noXT * 100\% \quad (S2)$$

The ME measures the influence that a given matrix (i.e., the feed and solvents) has on the signal strength and elution in the GC/MS. Among other factors, high lipid content matrices, analyte stability during extraction and analysis, analyte hydrophobicity, and concentration could affect the signal for peak area or height (Patel 2011). In turn, the matrix effect is a measure of the signal strength where values equal to 100% imply that the ME is virtually non-existent. Values > 100 % imply signal over amplification and < 100% suggest signal suppression (Leito [date unknown]; Patel 2011). There is currently no guideline for what an acceptable value for the ME is, but some researchers have set ± 15% as a reasonable limit (Petal, 2011). The ME is measured by comparing the signal strength of the absolute peak height for the following prepared samples:

$$ME = b / a = (POST\ XT / noXT) * 100\% \quad (S3)$$

The RE measures the amount of chemical recovered from the extraction process when chemical reactions do not take place. The RE compares the pre extraction spiked matrix (i.e., feed in REP1-3) in comparison to the same concentration expected in an equivalent amount of a matrix (i.e., feed in POST XT) that is spiked after the extraction process has taken place (Leito [date unknown]; Patel 2011).

$$Extraction\ Recovery\ (RE) = c / b = (Avg.\ REP1\text{-}3) / POST\ XT \quad (S4)$$

The YE measures the amount of chemical recovered from the extraction process when chemical reactions take place by comparing equal concentrations in solvent that is spiked before the extraction process takes place (PRE XT) and when the extraction does not take place (noXT) (Leito [date unknown]; Patel 2011).

$$YE = d / a = PRE\ XT / noXT \quad (S5)$$

RESULTS/DISCUSSION.

Feed Extractions

The absolute extraction recovery from fish feed was assessed via three sequential extractions that were analyzed individually via GC/MS. Individual analysis determined the proportionate recovery per extraction to determine if multiple extractions were necessary. Figure S1 depicts that approximately 75.18% of D4 is recovered during the first extraction when comparing blank-corrected REP1-3 to blank-corrected postXT (absolute peak height: REP 1 = 68037; REP2 = 64946; REP3 = 34165; postXT = 74106). Extraction recoveries were reproducible between REP1 and REP2, where REP3 resulted in a lower recovery. As such, average absolute peak height was reduced to 55716 ± 18727 (SD). The internal standard (M4Q) shown in Figure S1 displayed somewhat consistent absolute peak heights between all samples (Avg. = 58405 ± 4332.67 SD).

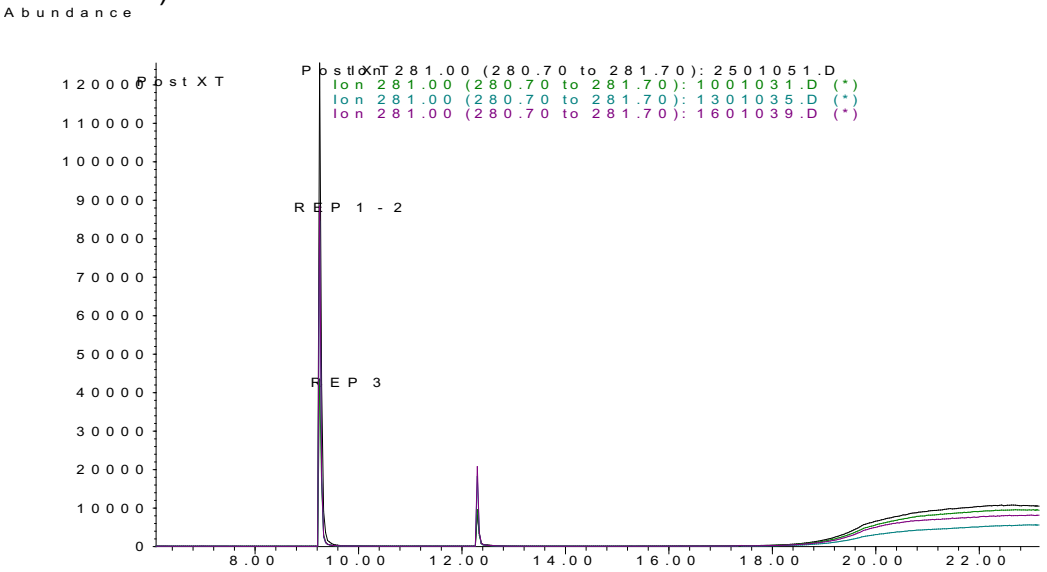


Figure S1. Chromatogram showing absolute peak height extraction recovery of the test chemical octamethylcyclotetrasiloxane (D4, RT = 9.248) and the internal standard, tetrakis(trimethylsiloxy)silane (M4Q, RT = 12.35) from the first extraction in a three part extraction series.

The second serial extraction recovered 11.03% of D4 when averaged blank-corrected REP1-3 (Avg. absolute peak height = 16900 ± 2521.25 (SD)) was compared to blank-corrected POST XT (Figure S2). Figure S3 shows that 2.65% of D4 was extracted when comparing average absolute peak height of REP1-3 (Avg. = 4604.50 ± 811.05 SD) to blank-corrected POST XT. If the three extractions are considered together, the total RE All three figures provide support that multiple extractions are necessary to recover adequate amounts of D4 from feed in order to determine dosing concentration.

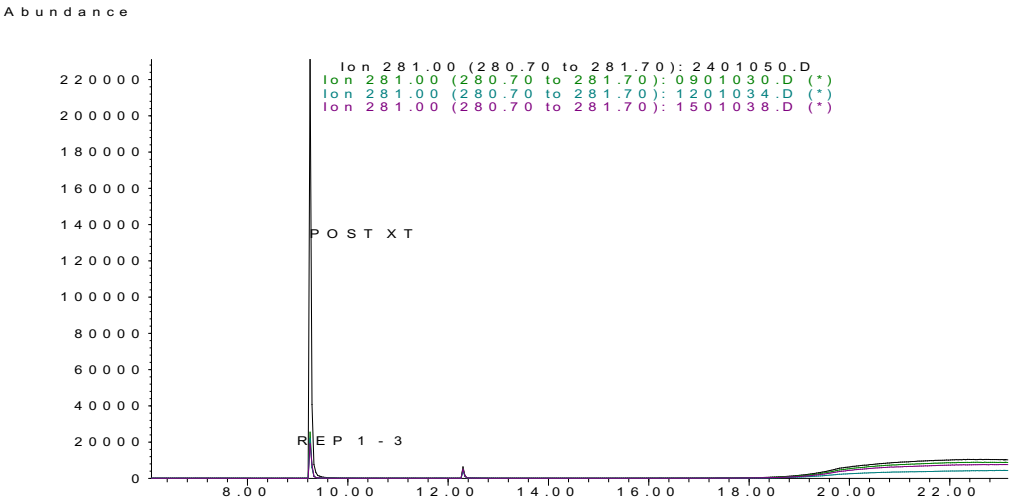


Figure S2. Chromatogram showing absolute peak height extraction recovery of the test chemical octamethylcyclotetrasiloxane (D4, RT = 9.248) and the internal standard, tetrakis(trimethylsiloxy)silane (RT = 12.335) from the second extraction in a three part extraction series.

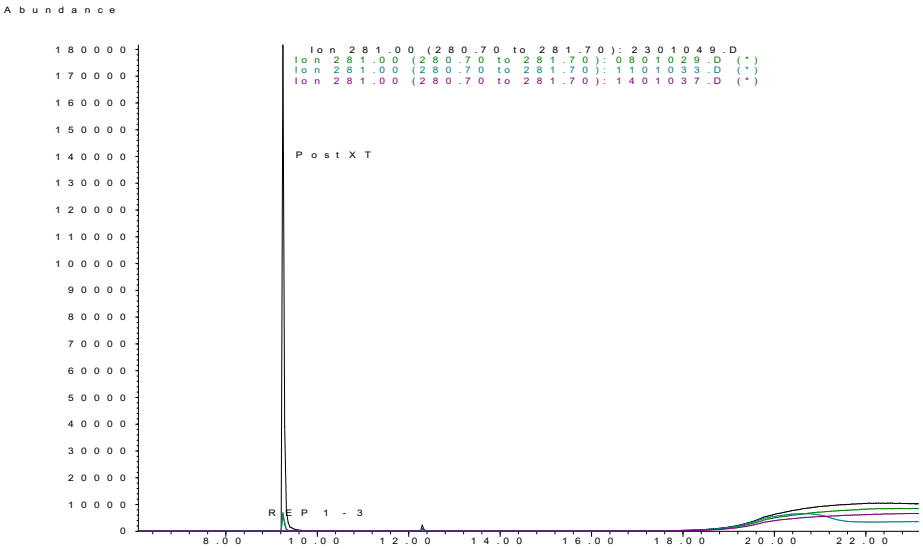


Figure S3. Chromatogram showing absolute peak height extraction recovery of the test chemical octamethylcyclotetrasiloxane (D4, RT = 9.248) and the internal standard, tetrakis(trimethylsiloxy)silane (RT = 12.335) from the third extraction in a three part extraction series.

Matrix Effects

Variations of the above-mentioned method were ran several times with modifications to the protocol, including the amount and addition of salts, sorbents and corrective toluene addition to reduce the matrix effect. In turn, shaking and centrifugation increased as the method became more complex in order to successfully extract chemicals and ensure separation between liquid and solid layer additions.

The addition of $MgSO_4$ and PSA brought the PE for absolute peak height up from 44.18% to 89.15%. However, actual RE remained uncertain at 249% with a 35.75% ME

and a EY of 53.99%. Results suggest that the process was working, but the impact of the matrix largely affected the signal over amplification for recovery. Controlling the volume of pentane removed from the original working tube-containing feed, rather than removing as much pentane as possible, removed inconsistencies between replicates. In turn, controlling the volume of pentane that reached the sorbent tube increased the process efficiency to 109.08%. However, strong matrix effects were still present (178.35%) with relatively poor recoveries (61.16%). These results prompted the investigation of the analysis of sequential extractions and their associated ER.

Furthermore, results for the EY (53.99%) of the $MgSO_4$ and PSA experiment also indicated that inconsistencies between the solvent-based POST XT and noXT might be causing additional reactions to take place and skewed results (Petal 2011). In turn, $MgSO_4$ was added to the noXT sample tube and compared to a noXT without $MgSO_4$ during the sequential extraction analysis. Results from this study showed that the addition of $MgSO_4$ increased PE in the first extraction from 86.86% to 139.14% and ME improved from 71.53% to 114.59%. RE, which is not a function of the noXT, was still unlikely to be a result of signal amplification at a value of 142.32%. These results coincide with previous research that shows $MgSO_4$ has an impact on the ability to successfully recover test chemicals from extracts while combating the matrix effects (Anastassiades et al. 2003) and addresses specific complications associated with D4 over amplification when water interacts with internal components of the GC/MS to produce D4 (Varaprath et al. 2000).

Finally, the addition of toluene to REP1-3 helped addressed ME in the sequential extractions and resulted in the aforementioned highest extraction recoveries (75.18% from the first extraction in a series of three and an estimated additive RE 90.19% from all three extractions). Toluene reduced the ME to within reasonable values of ± 15% with a value of 114.97% for the first extraction. ME increased with each sequential extraction, which may suggest that lower concentrations of test chemicals are subject to high ME (second extraction = 237.79%; third extraction = 179.51%). This is likely evident of our experimental design where the ratio of pentane increases as the test chemical decreases via extraction and future research should take to take this in consideration when analyzing pooled samples.

The addition of toluene also positively affected the PE that decreased with each sequential extraction. The first extraction resulted in an average PE of 86.44% ± 20.39 (SD), with the second and third extractions showing averages of 26.22% ± 2.48 (SD) and 7.14% ± 0.46 (SD). Variation could be associated with the relatively low absolute peak height of REP3 as shown in Figure S1.

Additional Considerations

The analytical results indicate that D4 can be added and removed from contents with high amounts of lipid. This process requires the addition of salts and sorbents in order to accurately determine the amount of extracted D4. These values and protocol alterations will be accounted for when determining the concentrations for the *in vivo* experiment. Furthermore, a limit of detection (LOD) of ~ $2e^{-9}$ $g.mL^{-1}$ has been derived from a standard curve in pentane. This amount equates to $5.95613e^{-10}$ $mol.kg^{-1}$ ww fish. These measures will be repeated using fish feed and fish tissue to determine if the LOD is altered by a given matrix and applied to determine the extraction recovery on a matrix-basis if significant differences between the slopes and LOD are found.

Supplementary Material 2.

Digital ADME-B models.

S2A.

Digital Document 1: Please see 'ADME Fish Bioaccumulation Calculator Beta Version 1.14 (clean) D4 Karen Kompton-3 means-b.xlsx' for ADME-B model inputs and outputs for test and field conditions for fish fed octamethylcyclotetrasiloxane (D4) over time.

S2B.

Digital Document 2: Please see 'KCedits_ADME Fish Bioaccumulation Calculator Beta Ver 1.14 (clean) D5-3 Means.xlsx' for ADME-B model inputs and outputs for test and field conditions for fish fed decamethylcyclopentasiloxane (D5) over time.

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Supplementary Material - APPENDICES.

Appendix SA.

Predicted and actual mass of test, reference and vehicle substances in corn oil spiking solutions.

Table SA1. Predicted and actual mass (g) of test, reference and vehicle substances used in spiking solution and associated concentrations in test feed.

Chemical	Chemical (g)	Feed (kg)	C ($g.kg^{-1}$ feed)	Total CO (g)	C_{CO} ($g.kg^{-1}$ feed)	% CO	≤ 5%
PCBz	0.0256	0.0757	0.338	NA	NA	NA	NA
D4	0.0267	0.0757	0.353	NA	NA	NA	NA
Corn Oil	0.2582	0.0757	3.412	0.53*	7.00	0.7	Y
Total	0.3105						

*Total mass (g) of CO added to feed after CO wash.

Table SA2. Predicted and actual mass (g) of test, reference and vehicle substances used in spiking solution and associated concentrations in control feed ($g.kg^{-1}$ feed).

Chemical	Chemical (g)	Feed (kg)	C ($g.kg^{-1}$ feed)	Total CO (g)	C_{CO} ($g.kg^{-1}$ feed)	% CO
PCBz	0.0252	0.0756	0.333	NA	NA	NA
D4	NA	NA	NA	NA	NA	NA
Corn Oil	0.2681	0.0756	3.547	0.55*	7.00	0.728
Total	0.2933					

*Total mass (g) of CO added to feed after CO wash.

Appendix SB.

Detailed Sample Preparation

Instructions for the preparation of samples used to determine process efficiency, matrix effect, extraction recovery and yield. Refer to Table SB1 for a list of test, reference and internal standard concentrations and masses and Table SB2 for the mass of feed, solvents, salts and sorbents added in each sample preparation.

- **No extraction solvent spiked standard (noXT - a)**: prepared by spiking 4 mL of pentane with test, reference, and IS that were previously diluted in individual 10 mL toluene stock solutions (i.e., 10 mL toluene / chemical). The volumes of test, reference, and IS that were added to noXT were intended to yield equal masses that matched those expected in 0.001 g of spiked feed. Matching mass allows a comparison between concentrations in the noXT (a) and REP1-3 (c) in order to determine the process efficiency between unknown concentrations in feed that undergoes the extraction process (i.e., REP1-3) and equal concentrations in a solvent that does not experience extraction or matrix effects (i.e., noXT). The noXT did not receive ACN, salts or sorbets (except for $MgSO_4$), or undergo extraction via the shaker. The noXT was centrifuged twice (5000 rpm, 10 min) with the remaining samples. A 10 µL mL aliquot of the pentane extracts were pipetted into a 2 mL amber vial containing 990 µL of pentane for analysis.

- **Post-extraction spiked matrix (POST XT)**: the post-extraction spiked matrix was prepared by adding 1 g of control fish feed that was not spiked with D4 but received the reference chemical (PCBz). The fish feed underwent the normal extraction protocol including shaking and centrifugation (2 x) in 4 mL pentane. ACN, salts and sorbents were used in the extraction process. After the extraction was completed, the pentane extraction from un-spiked feed was spiked with the same concentrations as noXT (A). A 10 µL mL aliquot of the pentane extracts were pipetted into a 2 mL amber vial containing 990 µL of pentane for analysis.

- **Pre-extraction spiked matrix (REP1-3)**: was prepared by adding 0.001 g of fish feed that was previously spiked with D4 and reference chemical (PCBz). The fish feed followed the normal extraction protocol including shaking and centrifugation (2 x) in 4 mL pentane. ACN, salts and sorbents were used in the extraction process. REP1-3 also received an addition of toluene to match with postXT, preXT and noXT standards. A 10 µL mL aliquot of the pentane extracts were pipetted into a 2 mL amber vial containing 990 µL of pentane for analysis.

- **Pre-extraction solvent spiked standard (PRE XT):** was prepared without using any feed. It was comprised of 4 mL of pentane extract that was spiked with equal concentrations of IS, reference, and test chemicals as expected in REP1-3 before it was subjected to the shaker and centrifugation (2 x) extraction process. ACN, salts and sorbents were used in the extraction process. A 10 µL mL aliquot of the pentane extracts were pipetted into a 2 mL amber vial containing 990 µL of pentane for analysis.

Table SB1. Amount of mass (g) and concentration in feed ($g.kg^{-1}$) in each sample tube for the test chemical, octamethylcyclotetrasiloxane (D4), reference chemical, pentachlorobenzene (PCBz), and internal standards (tetrakis(trimethylsilyl)siloxane (SIL) and tetrakis(trimethylsiloxy)silane (M4Q). Letters (a) – (d) relate the specific sample to equations to solve for process efficiency, matrix effect, extraction recovery and yield.

Sample	D4 M (g)	D4 C ($g.kg^{-1}$)	PCBz M (g)	PCBz C ($g.kg^{-1}$)	SIL M (g)	SIL C ($g.kg^{-1}$)	M4Q M (g)	M4Q C ($g.kg^{-1}$)
CTRL	NA	NA	2.52×10^{-2}	3.33×10^{-1}	3.56×10^{-4}	3.56×10^{-1}	8.90×10^{-5}	8.90×10^{-2}
POST XT (b)	3.56×10^{-4}	3.56×10^{-1}	3.56×10^{-4}	3.56×10^{-1}	3.56×10^{-4}	3.56×10^{-1}	8.90×10^{-5}	8.90×10^{-2}
REP 1 (c)	2.67×10^{-2}	3.53×10^{-1}	2.56×10^{-2}	3.38×10^{-1}	3.56×10^{-4}	3.56×10^{-1}	8.90×10^{-5}	8.90×10^{-2}
REP 2 (c)	2.67×10^{-2}	3.53×10^{-1}	2.56×10^{-2}	3.38×10^{-1}	3.56×10^{-4}	3.56×10^{-1}	8.90×10^{-5}	8.90×10^{-2}
REP 3 (c)	2.67×10^{-2}	3.53×10^{-1}	2.56×10^{-2}	3.38×10^{-1}	3.56×10^{-4}	3.56×10^{-1}	8.90×10^{-5}	8.90×10^{-2}
PRE XT (d)	3.56×10^{-4}	3.56×10^{-1}	3.56×10^{-4}	3.56×10^{-1}	3.56×10^{-4}	3.56×10^{-1}	8.90×10^{-5}	8.90×10^{-2}
noXT (a)	3.56×10^{-4}	3.56×10^{-1}	3.56×10^{-4}	3.56×10^{-1}	3.56×10^{-4}	3.56×10^{-1}	8.90×10^{-5}	8.90×10^{-2}

Table SB2. Amount of feed (kg), solvents (mL), salts (g), and sorbents (g) added to each sample. Letters (a) – (d) relate the specific sample to equations to solve for process efficiency, matrix effect, extraction recovery and yield.

Sample Tube	Feed (kg)	ACN (mL)	Pentane (mL)	MgSO4 (g)	NaCl (g)	C-18 (g)	PSA (g)	MgSO4 (g)
CTRL	0.001	1	4	0.25	0.2	0.18	0.18	0.25
POST XT (b)	0.001	1	4	0.25	0.2	0.18	0.18	0.25
REP1 (c)	0.001	1	4	0.25	0.2	0.18	0.18	0.25
REP2 (c)	0.001	1	4	0.25	0.2	0.18	0.18	0.25
REP3 (c)	0.001	1	4	0.25	0.2	0.18	0.18	0.25
PRE XT(d)	0.001	1	4	0.25	0.2	0.18	0.18	0.25
noXT (a)	NA	NA	4	0.25	NA	NA	NA	NA