BIOACCUMULATION, TISSUE DISTRIBUTION AND MATERNAL TRANSFER OF IONOGENIC ORGANIC CONTAMINANTS IN ZEBRAFISH (*DANIO RERIO*)

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DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

Chen Fangfang

21 Aug 2014

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TABLE OF CONTENTS

SUMMARY

The occurrence of pharmaceuticals and personal care products (PPCPs) and perfluorinated compounds (PFCs) in the environment has raised public health concerns in recent years. The bioaccumulation behavior of these ionogenic organic contaminants (IOCs) in aquatic organisms is not well understood. This thesis involves a series of laboratory investigations to assess the bioaccumulation behavior of several PPCPs and PFCs in zebrafish (*Danio rerio*), a common aquatic vertebrate model used for aquatic and human toxicology studies. PPCPs investigated included carbamazepine, diclofenac, ibuprofen, naproxen, triclosan, diphenhydramine, gemfibrozil, sertraline, risperidone and simvastatin, fluoxetine and bisphenol A (BPA). PFCs included perfluoroalkyl carboxylic acids (PFCAs), perfluoroalkyl sulfonic acids and mono- and di-perfluoroalkyl phosphonic acids (PFPAs). Continuous flow-through exposure experiments were designed and implemented to assess uptake and elimination kinetics, half-lives, tissue distribution and maternal transfer of these compounds in adult female zebrafish. The main objectives were to (i) evaluate the toxicokinetics and bioconcentration factors (BCFs) of PPCPs and PFCs in zebrafish and (ii) assess the role of key biological constituents (proteins, phospholipids and neutral lipids) and influence of octanol-water, membrane-water and protein-water distribution coefficients (D_{ow}, D_{mw} and D_{pw}) on the bioaccumulation behavior of these IOCs in zebrafish. The results showed rapid uptake and depuration of PPCPs and relatively slow uptake and depuration for PFCs. For PPCPs, half-lives ($t_{1/2}$) ranged between 0.2 to 3.8 days. The whole body steady state bioconcentration factors (BCF_{ss}) of PPCPs ranged between 0.2 and 465. The highest tissue-specific BCF_{ss} was observed for triclosan in liver (6,464 ± 1,565). The BCF_{ss} of

triclosan was tissue dependent, with BCF values in liver (6,464 ± 1,565) exceeding those in muscle (136 ± 37) by approximately 50 times, which highlights the importance of conducting tissue-specific analysis in order to avoid under or over prediction of bioaccumulation potential and exposure. For PFCs, the whole body BCF_{ss} after 24 days of exposure ranged between 9.6 and 1.7×10^9. The data indicate that di-PFPAs are highly bioaccumulative, exhibiting BCFs higher than PFOS and long-chain (C_9 - C_{14}) PFCAs. Positive linear relationships were observed between log BCF_{ss} and carbon chain length in all tissues. For the same perfluoroalkyl carbon chain length, the bioaccumulation potential was ordered PFSAs > PFCAs > mono-PFPAs, indicating functional groups contribute to bioaccumulation to some degree. The observed potein-, phospholipid- and neutral lipid-distribution coefficient (D_{pw} / D_{lipw} / D_{lw}) values varied in different tissues for both PPCPs and PFCs indicating these constituents in different tissues are not equal with respect to sorptive capacity. Application of a mechanistic bioaccumulation model for IOCs demonstrates that proteins, phospholipids and neutral lipids all likely contribute to the sorptive capacity and tissue distribution of the studied IOCs in zebrafish. The study provides novel information regarding the mechanisms governing bioaccumulation and tissue distribution of ionogenic organic chemicals (IOCs) in aquatic organisms.

LIST OF ABBREVIATIONS

BAF	Bioaccumulation Factor
BCF	Bioconcentration Factor
BFRs	Brominated Flame Retardants
BMF	Biomagnification Factor
BPA	Bisphenol A
BSAF	Biota-Sediment Accumulation Factor
CUPs	Current-Use Pesticides
EDCs	Endocrine Disrupting Chemicals
ESI	Electrospray Ionization
FW	Fish Weight
FWMF	Food Web Magnification Factor
HPLC	High-Performance Liquid Chromatography
HPV	High Production Volume
IOCs	Ionogenic Organic Chemicals / Compounds
ISTD	Injection Internal Standards
LC/MS-MS	Liquid Chromatography Tandem Mass Spectrometry
LFABP	Liver Fatty Acid Binding Protein
LSI	Liver Somatic Index
MDL	Method Detection Limit
OAT	Organic Anion Transporter
OCPs	Organochlorine Pesticides

PBT	Persistent, Bioaccumulative and Toxic
PCA	Principal Component Analysis
PCBs	Polychlorinated Biphenyls
PFCAs	Perfluorocarboxylic Acids
PFCs	Perfluorinated Compounds
PFOA	Perfluorooctanoic Acid
PFOS	Perfluorooctane Sulfonate
PFPAs	Perfluoroalkyl Phosphonic Acids
PFSAs	Perfluoalkyl Sulfonic Acid
ppb	Parts Per Billion
PPCPs	Pharmaceuticals and Personal Care Products
ppt	Parts Per Trillion
QSAR	Quantitative Structure–Activity Relationship
RRFs	Relative Response Factors
SPE	Solid Phase Extraction
TCPAA	2,4,5- Trichlorophenoxyacetic Acid
TMF	Trophic Magnification Factor
US EPA	United States Environmental Protection Agency
WWTP	Wastewater Treatment Plant

LIST OF SYMBOLS

C_B	Chmical Concentration in Biota
C_d	Chemical Concentration in the Diet
χ_I	Fraction of Chemical in Charged Form at pH 7
χ_N	Fraction of Chemical in Neutral Form
C_W	Chemical Concentration in Water
D_{BW}	Sorptive Capacity of the Biota to Water Ratio
D_{lipw}	Liposome-Water Partitioning Coefficient
D_{MW}	Memberane-Water Distribution Coefficient
Δ_{MW}	Scaling Factors relating the Membrane-Water Partition Coefficients
D_{OW}	Octanol-Water Distribution Coefficient
Δ_{OW}	Scaling Factors relating the Octanol-Water Partition Coefficients
D_{pw}	Protein-Water Distribution Coefficient
f_{NLOM}	Volume Fraction of Nonlipid Organic Matter
f_{PL}	Volume Fraction of Phospholipids
f_{SL}	Volume Fraction of Neutral (Storage) Lipids
f_W	Volume Fraction of Phospholipids
g	Fish Growth Rate
K_1	Uptake Rate Constant
K_2	Elimination Rate Constant
K_d	Elimination Rate Constant

K_e	Fecal Egestion Rate Constant
K_{oa}	Octanol-Air Partitioning Coefficient
K_{OW}	Octanol-Water Partitioning Coefficient
K_{PW}	Protein-Water Partitioning Coefficient
K_u	Uptake Rate Constant
ρ_{NLOM}	Protein Contribution Factor to Sorptive Capacity

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1 INTRODUCTION

1.1 Background

There is increasing public concern regarding environmental and human health effects associated with commercial chemicals in the environment. It is imperative that chemical contamination and public health risks stemming from hazardous materials released into the environment are well characterized and understood. The presence of potentially hazardous contaminants in aquatic environments, even at trace levels, is often highly undesired due to potential ecological and human health impacts. Proper environmental assessment and management includes understanding of contaminant sources, transport pathways, environmental behavior and exposure risks.

Well-studied “legacy” contaminants such as dioxins, Polychlorinated Biphenyls (PCBs) and DDT have been highly regulated since the 1970’s. However many of these chemicals continue to present threats to ecosystem and human health. In recent years, several contaminants of concern have likewise emerged as potentially hazardous environmental contaminants. “Emerging contaminants” can be broadly defined as any synthetic or naturally occurring chemical or any microorganism that is not commonly monitored in the environment but has the potential to enter the environment and cause known or suspected adverse ecological and (or) human health effects [1]. These emerging contaminants are commonly from municipal, agricultural, and industrial wastewater sources and pathways [1]. Key chemical contaminants of emerging concern include perfluorinated chemicals (PFCs), current-use pesticides (CUPs), brominated flame retardants (BFRs), commonly used pharmaceuticals and personal care products (PPCPs)

such as carbamazepine, ibuprofen, diclofenac, dipenhydramine and fluoxetine.

Many of these substances are high production volume (HPV) chemicals and thus may attain toxicologically significant concentrations in the environment [2-5]. It has been demonstrated that many of these new emerging chemicals behave endocrine-disrupting properties and potentially interfere with the sexual development and reproductive function [6-10]. Also, certain PFCs (e.g., perfluorooctanoic acid (PFOA), perfluorooctanesulfonates (PFOS)) are carcinogens [11-15]. Thus, the available data suggest that many of these chemicals of emerging concern can be persistent in the environment and cause toxic effects in organisms.

Among these emerging contaminants of concern, many are ionogenic organic compounds (IOCs), such as organic acids and organic bases. Currently most studies of organic contaminants of emerging concern in aquatic systems have focused on neutral organic compounds, such as PCBs, DDT etc. However, the IOCs behavior in the environment has not been well documented. Present study intended to investigate the bioaccumulation behavior and the tissue distribution mechanisms of the IOCs in fish, including PFCs and PPCPs.

PFCs are organofluorine compounds with all hydrogens replaced by fluorine on a carbon chain - but the molecule also contains at least one different atom or functional group [16]. PFCs are highly persistent in the environment. Several PFCs are present in the blood of virtually everyone in the general population [17,18]. PPCPs as defined by United States Environmental Protection Agency (US EPA) are referring to any products that are employed by individuals and agribusinesses for purposes such as cosmetic reasons, to

enhance personal health or physical conditions of livestock [19]. PPCPs are composed of a diverse collection of thousands of chemical substances, including prescription and over-the-counter therapeutic drugs, veterinary drugs, fragrances, cosmetics, insect repellent and detergents for personal care products [19].

Due to their global occurrence in different environment, investigating bioaccumulation potential and distributions among tissues is important for many of these emerging contaminants to understand their pharmacokinetic and toxic effects on aquatic organisms which has not been well demonstrated [20,21].

Although some PFCs, such as PFOA and PFOS have been banned in USA and Europe, they still widely exist in the environment, due to their large volume productions, persistence in the environment, and emissions from old products and precursors. However, the data regarding bioaccumulation behavior of PFCs is still lacking.

1.2 Levels of PPCPs and PFCs in the aquatic environment and biota

1.2.1 PPCPs in the aquatic environment and biota

PPCPs enter into the environment through manufacturing, agribusiness, veterinary use, , hospital and community use or individual human activity [22]. PPCPs often end up in soil and water bodies because of their good water solubility [22].

1.2.1.1 PPCPs in the aquatic environment

In general, PPCPs in WWTP effluents and surface waters are present at 0.001- 1 μg/L [23]. Due to the presence at such trace levels, PPCPs are commonly referred to as micro-

pollutants. Recent years, PPCPs were detected regularly in the aquatic environment. A review [24] listed the compounds with highest detection frequency in recent EDC / PPCP survey of U.S. streams in which, coprostanol, N-N-diethyltoluamide, caffeine and triclosan et al. had the most frequency of detection. Another review [25] summarized a list of the PPCPs together with their structures and some representative environmental occurrence and effects data. A WHO report [26] regarding to PPCPs in drinking water summarized several classes of pharmaceuticals present in wastewater influent in the United Kingdom. The values reported in the WHO report in UK were within the same range as those reported in Europe and the USA. Data in the literature also suggest that annual usage of PPCPs are positively associated with concentrations of PPCPs measured in water bodies [26]. A monitoring programme [27] in the United Kingdom reported occurrence of 12 PPCPs or their metabolites in surface waters. The results showed that different classes of pharmaceuticals were present in both WWTP effluents and receiving waters in England.

1.2.1.2 PPCPs in the aquatic biota

PPCPs occurrence is being increasingly reported in a variety of aquatic organisms in recent years [28], including marine mussels [29] and fish tissues [30,31]. For example, the Gizzard Shad tissues in Hamilton Harbour were determined with Paroxetine 0.58 ng/g, fluoxetine 1.02 ng/g and norfluoxetine 1.08 ng/g [31]. 0.3 ng/mL of carbamazepin was detected in fish blood plasma in Stockholm and 102 ng/mL of ibuprofen in Gothenburg exposed to treated sewage effluents [32]. A national pilot study was conducted to assess the bioaccumulation of PPCPs in fish sampled from five effluent-dominated rivers in the United States which receive direct discharge from WWTP in Chicago, Illinois; Dallas,

Texas; Orlando, Florida; Phoenix, Arizona; and West Chester, Pennsylvania, USA [30]. In this pilot study, the concentrations of sertraline were as high as 19 and 545 ng/g in fillet and liver tissue, respectively. It has been found that most of PPCPs distributed more in liver than in fish fillet in this study. The authors claimed that the levels of PPCPs investigated were dependent on the degree of wastewater treatment employed [30]. Schultz et al. [33] have determined the antidepressant pharmaceuticals in two U.S. effluent-impacted streams. The antidepressants, venlafaxine, bupropion, and citalopram were the highest concentration in both streams, with concentrations of at least one order of magnitude greater than fluoxetine and sertraline which are the more commonly investigated antidepressants. In this study, the antidepressants, fluoxetine and sertraline, along with their metabolites, were observed in fish brain tissue, typically at ng/g levels [33].

1.2.2 PFCs in the aquatic environment and biota

A number of PFCs have been detected in aquatic and terrestrial environments, as well as fish, wildlife and humans, worldwide [13,17,18,21,34,35]. Exposure to some PFCs has been confirmed adverse effects on animals and human, e.g. carcinogenic, liver toxicantic, and immune system toxicantic effects [36]. Herein, we only overview the occurrence of PFCs in the aquatic environment and biota.

1.2.2.1 PFCs in the aquatic environment

Due to PFCs are very stable and persistent, they eventually turn up in the environment, especially in water. Currently they can be found in the deep sea and even in the Arctic because of wide spread along with water currents [37]. Table 1.1 summarizes several

regular PFCs of their occurrence in natural waters.

Table 1.1 Examples of concentrations (ng/L) of perfluorinated compounds measured in the aquatic environment.

Type of water	Country (site)	PFOS	PFOA	PFHpA	PFNA	PFDA	Ref.
Wastewater							
Effluent	USA (New York)	3-68	58-1050	-	0-376	0-47	[38]
Effluent	USA (Kentucky)	8-993	8.3-334	-	0-15.7	0-201	[39]
Effluent	USA (Georgia)	0-70	7-227	-	0-54	0-86	[39]
Effluent	Japan (Tsurumi)	78.7-639.9	17.8-24.9	5.5-7.2	27.5-41.8	3-4.5	[40]
Effluent	China (Shanghai)	73.7-746	44.4-429	2.1-8.7	0-7.67	1.87-5.9	[41]
River							
Tokyo Bay	Japan	5.8	6.7	-	20.1	-	[42]
Tsurumi	Japan	179.6-179.9	13.4-15.9	3.1-4.4	15.4-38	2.1-3.9	[40]
Pearl	China	0.9-99	0.85-13	<0.13-5.6	<0.13-3.1	<0.13-0.67	[43]
Yantze	China	<0.01-14	2-260	0.074-9.2	<0.005-10	<0.005-3.8	[43]
Geylang	Singapore	1.3-12.5	6-16.3	0.7-1.9	1.5-4.9	-	[44]
kallang	Singapore	1.3-6.4	5.4-7.1	0.7-1.5	1.3-3.7	-	[44]
Ganges	India	<0.04-1.81	<0.04-0.201	-	<0.04-0.176	<0.04-<0.1	[45]
Oder	Poland	-	3.8	0.73	0.73	-	[46]
Po	Italy	-	200	6.6	1.46	-	[46]
Danuve	Romania/Ukraine	-	16.4	0.95	0.27	-	[46]
Seine	France	-	8.9	3.7	1.26	-	[46]
Thames	UK	-	23	4.1	0.79	-	[46]
Rhine	Germany	-	12.3	3.3	1.5	-	[46]
Guadalquivir	Spain	-	4.6	1.58	1.02	-	[46]
Moehne	Germany	193	3640	148	-	-	[47]
Lake							
Taihu	China	3.6-394	10.6-36.7	0-18.4	-	-	[48]
Shihwa	Korea	89.11	19.22	2.5	3.26	1.98	[49]
Maggiore	Italy	7.8	2.4	2.4	0.6	3.7	[50]
Ontario	Canada	3.9	2.6	-	3.1	-	[51]
Michigan	Canada	3.8	3.4	-	-	-	[51]

The reports of perfluorinated phosphonic acids (PFPAs) in the environment are rare. To our knowledge, D'eon et al. have been the first to report the presence of PFPAs in surface water and wastewater treatment plant (WWTP) effluent in Canada [52]. Zhushi has

determined the concentration of perfluorohexane phosphate (PFHxPA) in the rivers of Tokyo Bay, Janpan [42]. Table 1.2 summarizes the occurrence of PFPAs in natural waters.

Table 1.2 Concentrations (ng/L) of perfluorinated phosphonic acids measured in the aquatic environment.

Type of water	Country (site)	PFHxPA	PFOPA	PFDPA	Ref.
Wastewater					
Effluent 1	Canada	2.1	2.5	0.46	[52]
Effluent 2	Canada	0.47	0.77	0.42	[52]
Effluent 3	Canada	0.33	1	0.38	[52]
Surface water					
Tokyo Bay	Japan	< 0.15	-	-	[42]
Ijmuiden	Netherland	-	1	-	[53]
Dolson Creek	Canada	0.058	0.19	0.075	[52]
Flat Creek	Canada	-	0.088	0.14	[52]
Grand River	Canada	0.27	0.26	0.05	[52]
Eagle Creek	Canada	-	0.99	0.73	[52]
Wascana River	Canada	-	1.1	0.87	[52]
Pembina River	Canada	-	1.6	0.3	[52]
India Creek	Canada	1.2	3.4	0.66	[52]
Souris River	Canada	-	0.66	0.3	[52]
Rouge River	Canada	0.095	1.3	0.26	[52]

1.2.2.2 PFCs in aquatic biota

PFCs have been reported in the organisms at all levels of aquatic benthic food webs including algae, zebrafish mussel, round goby, as well as smallmouth bass [54]. PFOS was the most frequently detected compound in these food webs [54]. In a Canadian Arctic marine food web, concentrations of PFOS, perfluorooctansulfoamide (PFOSA), and C_7-C_{14} perfluorocarboxylic acids (PFCAs) observed ranged between 0.1 and 40 ng /g wet weight in biota. PFOS and PFCA concentrations were observed higher in liver and

blood which are protein-rich compartments in beluga whales indicating the potentially proteinophillic properties of PFCs [55]. In the marine mammalian food web, observed concentrations of PFOSA, PFOS and C_8-C_{14} PFCAs, increased significantly with trophic level indicating biomagnification [55]. In a food web of Lake Ontario, PFOS was the most widely detected compound in all samples in the study. The concentrations of C_8-C_{15} PFCAs ranged between <0.5 and 90 ng/g biota [56]. Concentrations of PFCs in fish blood and liver were reported in a study which investigated fish from different coastal regions of Japan. PFOS concentrations in blood ranged between 1 and 834 ng/mL and in liver from 3 to 7900 ng/g wet weight. Perfluorobutane sulphonate (PFBS) was not observed in all fish samples [57]. PFPAs were detected in lake trout from Lake Ontario, Lake Erie, and Lake Huron with concentration ranging from 0 to 0.032 ng/g wet weight [58].

1.3 Bioaccumulation in aquatic organisms

1.3.1 Bioconcentration

Bioconcentration is the process of accumulation of water-borne chemicals by fish and other aquatic animals through non-dietary routes [59]. The potential of bioconcentration for a chemical is often expressed by a bioconcentration factor (BCF) which is measured under controlled laboratory conditions. BCF is the proportionality constant of the chemical concentration in an aquatic organism at steady-state equilibrium (C_B) to the chemical concentration in ambient water (C_w) according to equation 1 [60],

$$BCF_{steady\ state} = C_B / C_w \qquad (1)$$

BCF can also be calculated from a one-compartment model, where k_1 is the

uptake rate constant and k_2 is the elimination rate constant [59],

$$BCF_{kinetic} = k_1 / k_2 \quad (2)$$

1.3.2 Bioaccumulation

Bioaccumulation is a process that leads to higher concentration of a chemical in organisms than in the surrounding environmental media. It is measured under field conditions and hence all possible sources and routes of exposures (respiratory, dermal, and dietary) are considered [61]. Several indices are generally used to express the extent to which a chemical can bioaccumulate in an organism, including bioconcentration factor (BCF), bioaccumulation factor (BAF, the ratio of chemical concentration in an organism to that in water), biota-sediment accumulation factor, BSAF (the ratio of concentration of a chemical in an organism to that in sediment), biomagnifications factor (BMF) and the trophic or food web magnification factor (TMF) [61,62].

1.3.3 Biomagnification

US EPA defines biomagnification as the "result of the process of bioaccumulation and biotransfer by which tissue concentrations of chemicals in organisms at one trophic level exceed tissue concentrations in organisms at the next lower trophic level in a food chain." It is typically measured under field conditions and is expressed by biomagnification factor (BMF) [63]. BMF is a proportional ratio between the concentration of a chemical in an organism (C_B) to that in its diet (C_d). The other less commonly used indices, food web magnification factor (FWMF) and trophic magnification factor (TMF), are used to examine a chemical's potential to biomagnify in a food web [63].

1.3.4 Bioaccumulation regulations

There are millions of existing chemicals and around 2000 new chemicals are added each year [63,64]. Among these, the compounds with persistent (P), bioaccumulative (B) and toxic (T) characteristics are of major concern. In order to identify or restrict the use of these PBT substances, regulatory efforts such as implementation of Registration, Authorization and Evaluation of Chemicals (REACH) are conducted [65]. Similar assessments for PBT characteristics of approximately 23,000 chemicals are being added in the Domestic Substances List (DSL) in Canada [66]. 610 new potential PBT chemicals were identified by Howard and Muir [67].

BAF / BCF assessments are increasingly employed in regulatory criteria [68]. The bioaccumulation potential serves as a valuable tool to predict possible adverse effects of PBT chemicals on the organisms. Therefore, evaluation of bioaccumulation potential of a chemical is significantly important.

Specific criteria to identify bioaccumulative substances are employed in the bioaccumulation regulations in Canada, the United States, and the European Union (listed in Table 1.3) [61]. It can be seen from Table 1.3, all regulations are on the basis of BCF, BAF or K_{ow}. The use of data from field studies or controlled laboratory studies is not stated in the criteria. In addition, the criteria apply to aquatic organisms (e.g., fish) or nonaquatic organisms are not considered in these regulations [61].

Table 1.3 An overview of regulatory bioaccumulation assessment endpoints and criteria (adapted from Gobas et al. [61,63])

Regulatory agency	Category	Bioaccumulation endpoint	Criteria	Program
Environment Canada	-	K_{ow}	≥100000	CEPA 1999 [a]
Environment Canada	-	BCF	≥5000	CEPA 1999
Environment Canada	-	BAF	≥5000	CEPA 1999
European Union	bioaccumulative	BCF	≥2000	REACH [b]
European Union	very bioaccumulative	BCF	≥5000	REACH
US EPA	bioaccumulative	BCF	1000-5000	TSCA, TRI [c]
US EPA	very bioaccumulative	BCF	≥5000	TSCA, TRI
United Nations Environment Program	-	K_{ow}	≥100000	Stockholm Convention [d]
United Nations Environment Program	-	BCF	≥5000	Stockholm Convention

[a] CEPA= Canadian Environmental Protection Act, 1999 (Government of Canada 1999, 2000).
[b] Registration, Evaluation, and Authorisation of Chemicals (REACH) Annex XII (European Commission 2001).
[c] Currently being used by the US Environmental Protection Agency in its Toxic Substances Control Act (TSCA) and Toxic Release Inventory (TRI) programs (US EPA 1976).
[d] Stockholm Convention on Persistent Organic Pollutants (UNEP 2001).

Due to the limited information of experimental BCFs of chemicals, the application of the regulations for screening the bioaccumulative compounds has shown difficulties. For example, Arnot and Gobas reported that empirical BCFs and BAFs were available for only 4% and 0.2% of the chemicals, respectively [63]. Thus, K_{ow} is used more for screening the bioaccumulative compounds. However, K_{ow}, sometimes, is not a reliable discriptor to identify the bioaccumulative compounds. According to above regulation, the chemicals with log K_{ow} greater than 5 will be considered bioaccumulative, however there is evidence that many substances with log K_{ow} greater than 5 have no or less bioaccumulative potential than expected because of rapid biotransformation [65]. Therefore, biotransformation is an important factor that should be considered in chemical evaluation schemes. In addition, it is reported that some substances with lower log K_{ow}

and high log K_{oa} (octanol-air partitioning coefficient) may bioaccumulate in air-breathing organisms [69,70].

Due to bioaccumulation models consider both biological and environmental factors, they are better tools for identifying potential PBT substances than chemical properties only, such as K_{ow} and K_{oa} [61].

1.3.5 Bioaccumulation of PPCPs

The data of the bioaccumulation potential of PPCPs in biota are not substantial [71]. A previous study has reported that PPCPs in fish plasma can reach significantly higher concentrations than in ambient water [72]. An Algal bioaccumulation study of triclocarban, triclosan, and methyl-triclosan in a North Texas WWTP showed the BAFs were approximately 10^3 level [73]. Zhang et al. [74] conducted a bioconcentration study of PPCPs in rainbow trout using a novel space-resolved solid-phase microextraction. In fish muscle, the BCFs for 8 days were 0.49 ± 0.06 for atrazine, 0.40 ± 0.21 for gemfibrozil, 0.52 ± 0.11 for carbamazepine, 1.50 ± 0.25 for ibuprofen, and 58.98 ± 16.81 for fluoxetine, respectively. In adipose tissue, the BCFs exposed for 8 days were 4.94 ± 0.45 for atrazine, 20.75 ± 3.74 for gemfibrozil, 4.16 ± 0.87 for carbamazepine, 23.69 ± 2.23 for ibuprofen, and 143.36 ± 21.50 for fluoxetine. Zhou et al. [75] reported the BAF for fluoxetine in fish muscle following 7 and 14 d exposure were 62 and 84, respectively. BCFs have also been found to have a positive correlation with log K_{ow} [75]. A study of antibiotics in Lake Baiyangdian in north China showed that enrofloxacin bioaccumulated in shrimp and river snail with mean BAFs of 16600 and 6140 L/kg, respectively; and roxithromycin accumulated in crucian carp with a mean BAF value of 7410 L/kg. In

addition, enrofloxacin was potentially bioaccumulated in common carp with a mean BAF value of 4490 L/kg and sulfamethazine was potentially bioaccumulated in topmouth gudgeon with a mean BAF value of 3870 L/kg [76]. It has been reported that BAFs of antibiotics in mussel are in accordance with log K_{ow}. In a study, the BCFs of 30 pharmaceuticals and anthropogenic waste indicators (AWIs) were detected in earthworm and soil with BAF ranged from 0.05 (galaxolide) to 27 (triclosan) [77,78].

1.3.6 Bioaccumulation of PFCs

Unlike many bioaccumulative environmental pollutants (e.g. POPs, PCBs), PFCs do not accumulate in lipids of the organisms. Instead, these chemicals accumulate more in the blood, liver and gallbladder compared to lipid [79-81]. A controlled laboratoary bioconcentration study of rainbow trout exposed to a variety of PFCs, showed that PFC concentrations in fish were obseved to be greatest in the blood followed by the kidney then the liver, gallbladder, adipose tissue and finally muscle tissue. BCFs of PFCAs and PFSAs in the blood and liver in the rainbow trout ranged from 4.0 to 23 000 and BCFs generally increased with increasing of carbon chain length [79]. In another study on fish, the BCF for PFOS in the common shiner was reported to range between 6 300 and 125 000 [82]. In a field study on fish from different coastal regions of Japan, BCFs for PFOS in livers vary from 274 to 41 600 [57]. An experimental study on carp found that the BCFs of PFOA and PFOS which have the same carbon chain length, differed by more than two orders of magnitude (BCF of PFOA $\leq$ 5.1 to 9.4; BCF of PFOS = 720 to 1300). In this study, The obseved BCFs of perfluorododecanoic acid were from 10,000 to 16,000 and BCFs of perfluorotetradecanoic acid were from 16,000 to 17,000 [83].

Another study was conducted to examine dietary accumulation of PFCs in juvenile rainbow trout for 34 days [84]. The results showed that BMFs of PFCs ranged from 0.038 to 1.0 in fish, which suggested that PFCs will not biomagnify in this fish [84]. It is consistent with another study that PFCAs do not biomagnify in the Arctic piscivorous food web [55]. However, in another bottlenose dolphin marine food web, BMFs varied from < 1 to 156 at Sarasota Bay and < 1 to 30 at Charleston indicating biomagnification in this marine food web. But the authors claimed that using PFC concentrations in the plasma and liver as surrogate to whole body burden overestimates the BMFs [85]. It has been observed that PFOS biomagnified in the Arctic marine food web when liver concentrations of PFOS were used for seabirds and marine mammals. However, the authors claimed that transformation of precursors to PFOS may contribute to overestimation of biomagnification values. Whereas, the BMF values of precursors of PFOS, PFOSA and N-EtPFOSA, were often greater than 1 indicating biomagnification potential for these compounds [86]. An experimental study on captive mink liver reported that BMFs ranged from 11 to 23 [87].

1.4 Bioaccumulation Models

The potential for bioconcentration of a compound in the controlled condition is commonly assessed using the OECD Technical Guidance 305 test [88]. This test requires a large number of fish and long exposure duration. Conducting this test on all potential bioaccumulative chemicals is not practicable. Hence it is necessary to employ in silico models to predict BCF.

1.4.1 Bioaccumulation Models for Neutral Compounds

Lots of studies have shown a relationship between BCF and K_{ow} [59]. Thus, most of BCF models are based on hydrophobicity (K_{ow}) of the compounds. The hydrophobicity model considers bioconcentration as the partitioning of a chemical between the ambient water and the lipid portion of an aquatic organism [59]. This one compartment model assumes uptake kinetics is limited only by diffusion and metabolism of conpounds is negligible. Additionally, the BCF value is independent of exposure concentration [59]. The hydrophobicity model can be expressed as a regression equation [89],

$$\log BCF = a \log K_{ow} + b \qquad (3)$$

where a and b are empirical constants calculated from the regression analysis of BCF-K_{ow} data sets. Equation (3) was first proposed to predict the BCF of nonionic hydrophobic organic chemicals by Neely et al. [89]. Models based on the hydrophobicity as the only factor may be applicable to compounds which are non-ionic, low molecular weight compound and have little or no degradation [65]. Thus, they underestimate the potential for metabolic elimination.

Later, BCF models have been improved by including several correction or description factors using quantitative structural activity relationship (QSAR) approaches [90]. For example, BCFBAF, formerly called BCFWIN, developed by the US EPA and Syracuse Research Corporation (SRC), which is part of the Estimation Programs Interface (EPI) Suite, a publicly available program integrated into the PBT profiler. Two different methods are used for BCFBAF estimation for fish. The first is based on BCF-log K_{ow} regression plus any correction factors. The other is the Arnot-Gobas model, which calculates BCF values from mechanistic first principles [91]. The BCFBAF program is very extensive with around

700 chemicals including some ionic compounds [92]. However, for ionic compounds, BCFs is often being overestimated.

Gobas-Arnot model is dependent on differences in absorption, distribution, metabolism and elimination (ADME) processes (see Figure 1.1). Specifically, chemical BCFs can be calculated as equation (4) [93].

$$BCF = (k_1 + k_D) / (k_2 + k_G + k_M + k_E) \quad (4)$$

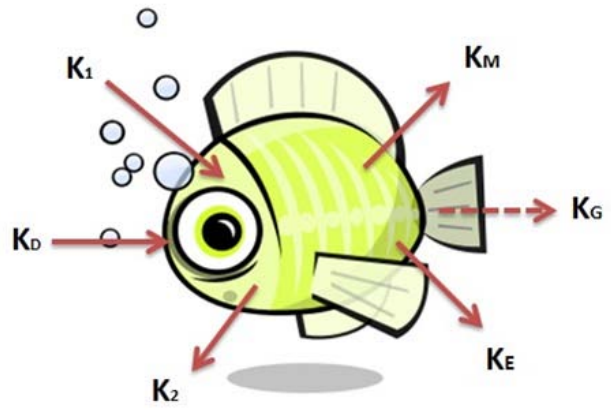


Figure 1.1 A conceptual diagram representing the major route of chemicals uptake and elimination in aquatic organisms (adapted from Arnot et al. [93]).

Where,

k_D = dietary uptake rate constant (d^{-1})

k_1 = gill uptake rate constant (d^{-1})

k_2 = gill elimination rate constant (d^{-1})

k_M = metabolic transformation rate constant (d^{-1})

k_E = fecal egestion rate constant (d^{-1})

k_G = growth dilution rate constant (d^{-1})

Gobas-Arnot model estimates the bioaccumulation potential of organic chemicals with a log K_{ow} between 4.0 and 12.2. One of the limitations of the model is that it is not

appropriate for predicting BCF potential of IOCs and surface-active chemicals. The other is that this model only applies to bioaccumulation in aquatic food webs [93]. There are empirical data showing that although some chemicals which do not biomagnify in aquatic food webs have biomagnification potential in terrestrial food webs. Hence the octanol-air partition coefficient (K_{oa}) should be considered in QSARs for predicting the bioaccumulation potential of organic chemicals in terrestrial food webs [94,95].

1.4.2 Bioaccumulation Models for IOCs

New emerging environmental contaminants, such as ionizable PPCPs and PFCs, cannot be assumed to behave the same as the neutral, lipophilic contaminants. The ionization of these ionizable compounds should be considered for bioaccumulation estimation [96]. It is, therefore, necessary to consider the pH dependent distribution coefficient, log D_{ow} (log K_{ow} at pH 7) for bioconcentration assessments of them. Moreover, it is proposed that phospholipids-water portioning coefficient (K_{lipw}) in the tissues and protein-water partitioning coefficient (K_{pw}) should be included in the BCF model [97,98]. In addition, chemical speciation, electrostatic interactions with other charged species, adsorption to non-biotic and biotic surfaces, and transport across biological membranes which are some of the crucial fate processes involved in bioaccumulation and distribution of ionizable compounds [99], are also proposed to be included in the BCF model.

Fu et al. [100] have compared performance of 3 models for estimating the BCFs of organic acids and bases. The first is the method recommended by European Union in the Technical Guidance Document (TGD) for chemical risk assessment [101] for IOCs. It employs corrected log K_{ow} with the fraction of neutral molecules or log D (sum of log

K_{ow} of neutral and ionic molecule) as predictors. The other two models are those of Meylan et al. [92] for IOCs, and the dynamic cell model based on the Fick–Nernst–Planck equation [100]. Fu et al. have found the TGD method gave good estimates when log *D* is used as the predictor. The model of Meylan et al. has performed less satisfactorily, whereas the results obtained by the cell model have been obseved to perform well for bases but not for acids [100]. The authors also have proposed an original regression model for estimating the BCFs of IOCs using log K_{ow} and pK_a (acid dissociation constant at logarithmic scale) as predictors [100].

Dimitrov et al. [99] have developed the BCF base-line model which includes up to 705 chemicals in the model for predicting BCFs. Water solubility of chemicals is considered in this base-line model. This model set up the maximum BCF potential as a base-line based on log K_{ow}, and mitigating factors that can reduce bioconcentration potential, such as molecular size and flexibility, ionization, biotransformation, etc., are used as reducing factors of the base-line [96,99]. In a further analysis, pK_a, log *P* and log *D* have been used for evaluating the impact of ionization on bioaccumulation. The authors found that the pK_a was not sufficiently adequate to be included in the model, while the log *P* and log *D* performed well [99].

Trapp et al. [102] developed a model based on activity to describe the fate in environment and partitioning of IOCs in biota. The model includes dissociation, electrical interactions in solids, liquid water, in air, and salinity in seawater to describe the environmental fate of IOCs. However the authors claimed that the empirical data applied in the model were rare leading to uncertainties of application of the model [102]. This activity-based approach can be also used to predict the accumulation in biota. The apparent bulk activity

capacity of biota in this model is composed of the capacity of water and lipids content [102].

Erickson et al. [103,104] established a mechanistic model for the uptake and elimination of IOCs at fish gill surfaces through water and blood exchange. Three processes are included in this model. First, the pH change at gill surfaces affects the relative amounts of neutral and ionized molecules. Second, ionized molecules help maintain high diffusion gradients of neutral molecules across epithelial cell membranes. Third, limited permeability of ionized molecules can have appreciable effects on chemical flux [103]. Relationship of water pH, alkalinity, and chemical properties with chemical uptake and elimination are discussed in this model [104].

Liu et al. [105] proposed a novel kinetic model based on adsorption for description of the bioaccumulation of PFCs. The empirical data showed the bioaccumulation of PFCs in biota is concentration dependent. The authors suggested the accumulation mechanism for PFCs be probably protein binding.

More recently, a mechanistic mass balance BCF model for IOCs in fish was developed [98]. The model estimates the overall sorption capacity of the biota using distribution ratios, such as membrane–water distribution ratios (D_{mw}) are used to characterize sorption to phospholipids instead of considering D_{ow} only [98]. At the same time, the sorption capacity of animal protein is as well included in this model [98]. Regarding to $D_{mw,}$ Escher et al. [106] developed a phospholipid sorption model to evaluate the liposome-water partition coefficients (D_{mw} or named D_{lipw}) for organic acids and bases which are mostly IOCs. Debruyn and Gobas [107] recommended estimating the sorptive capacity of

protein as 5% of lipid to predict the concentrations of hydrophobic chemicals in biota. Hence Armitage et al. employed the Escher model for D_{mw} calculation and Debruyn and Gobas model for protein sorption capacity. As a result, Armitage model works well for organic acids and bases. The model supports the hypothesis that phospholipids contribute substantially to the sorption capacity of organism for IOCs [98].

Based on above various models, there are two prevailing hypotheses for the bioaccumulation and tissue distribution mechanisms for IOCs in recent years. One assumes that interactions with animal proteins including serum albumin or liver fatty acid binding proteins (L-FABP) etc. regulate the distribution, accumulation of IOCs [108-113]. The other assumes that partitioning to membrane phospholipids, which have a higher affinity for ionizable species than neutral storage lipids, contribute to the bioaccumulation potential of IOCs [98,106,114-116]. Hence, this thesis aims to assess the role of key biological constituents (proteins, phospholipids etc.) and influence of octanol-water, membrane-water and protein-water distribution coefficients (D_{ow}, D_{mw} and D_{pw}) on bioaccumulation potential of IOCs in fish to better understand the bioaccumulation mechanism of IOCs in aquatic organisms.

In summary, assessment of bioconcentration has been considered an important endpoint in risk assessment, thus there is a growing need to generate reliable and useful bioconcentration data or models.

1.5 Study Objectives

The specific aim of the present study is to conduct controlled exposure experiments using zebrafish (*Danio rerio*) to evaluate pharmacokinetics of several representative IOCs of

emerging concern. Knowledge generated from the study will help to improve mechanistic understanding of uptake/elimination kinetics and tissue distribution of xenobiotic chemicals in fish. This study involves laboratory investigations to assess the bioaccumulation behavior and tissue distribution of various PPCPs and PFCs chemicals in zebrafish (*Danio rerio*), a common aquatic vertebrate model used for aquatic and human toxicology studies.

Specific aims include:

1. To develop robust analytical methods to identify and quantify concentrations of a variety of PPCPs and PFCs of concern in water and zebrafish tissues, e.g. muscle, liver, ovary and blood plasma.

2. To evaluate the aqueous exposure, bioaccumulation kinetics, tissue distribution and maternal transfer of PPCPs and PFCs in the zebrafish.
3. To assess the role of key biological constituents (proteins, phospholipids and neutral lipids) and the influence of octanol-water, membrane-water and protein-water distribution coefficients (D_{ow}, D_{mw} and D_{pw}) on bioaccumulation potential of IOC in aquatic organisms.
4. To better understand the mechanisms governing the bioaccumulation behavior of ionogenic organic contaminants (IOCs) in aquatic organisms.

1.6 Significance of the Study

This research aims to provide novel methods and information regarding the toxicokinetics and bioaccumulation behavior of IOCs. The study will help development

of mechanistic models for assessing the bioaccumulation of IOCs in aquatic systems, which will assist in future risk assessments and more effective management of these chemicals.

1.7 Study Design and Approach

1.7.1 Test Chemicals

Chemicals of emerging concern investigated in this study include pharmaceuticals and personal care products (PPCPs), perfluorinated chemicals (PFCs) such as perfluorooctanoic acid (PFOA) and perfluoro-octane-sulfonate (PFOS). The physicochemical properties of various PPCPs and PFCs are shown below in Table 1.4 and Table 1.5.

Table 1.4 Physicochemical properties of selected PPCPs

Chemical	Acidic/Basic	pK_a [a]	Charged Fraction of molecules (%) (pH 7)	Log K_{ow} [a]	Log D_{ow} (pH 7) [a]	Log K_{pw} [b]	Log K_{lipw} [c]
Ibuprofen	acidic	4.4	99.7	3.56	1.09	2.72	1.5
Naproxen	acidic	4.2	99.8	3.24	0.76	2.54	1.2
Diclofenac	acidic	4	99.9	4.45	1.26	3.23	1.65
Gemfibrozil	acidic	4.7	99.5	4.77	1.93	3.41	2.26
Fluoxetine	basic	10	99.9	4.05	1.59	3	1.95
Risperidone	basic	8.24	94.5	3.59	1.04	2.74	1.46
Sertraline	basic	9.5	99.7	4.66	2.88	3.35	3.11
Diphenhydramine	basic	9	99.1	3.77	2.03	2.84	2.35
Triclosan	neutral [d]	7.8	13.7	5.35	5.31	3.74	5.26
Bisphenol A	neutral	9.5	0.3	4.25	4.25	3.11	4.14
Carbamazepine	neutral	15.4	4×10^{-7}	3.64	3.52	2.76	3.38
Simvastatin	neutral	13.5	3×10^{-5}	4.68	4.17	3.36	4.06

[a] Estimated values, obtained from SPARC.
[b] Estimated from equation 3 in Chapter 4
[c] Estimated from equation 1 and 2 in Chapter 4
[d] We assume this neutral because the major fraction is neutral.

Table 1.5 Acronym, structure and physicochemical properties of selected PFCs

Test Compound	Acronym	Structure	C#	pK_a	Log K_{ow}	Log D_{ow} (pH 7)	Log K_{pw} [d]	Log K_{lipw} [h]
Perfluoroalkyl sulfonates								
Perfluorobutane sulfonic acid	PFBS	$CF_3(CF_2)_3SO_3H$	4	-3.31 [f]	3.9 [e]	0.25 [f]	2.91	0.75
Perfluorohexane sulfonic acid	PFHxS	$CF_3(CF_2)_5SO_3H$	6	-3.32 [b]	4.34 [c]	1.7 [b]	3.16	2.05
Perfluorooctane sulfonic acid	PFOS	$CF_3(CF_2)_7SO_3H$	8	0.14[d]	4.49 [c]	3.05 [g]	3.25	3.27
Perfluorocarboxylic acids								
Perfluorobutyric acid	PFBA	$CF_3(CF_2)_2CO_2H$	4	0.37 [a]	2.14 [c]	-0.36 [a]	1.91	0.20
Perfluorohexanoic acid	PFHxA	$CF_3(CF_2)_4CO_2H$	6	0.42 [a]	3.48 [c]	1.24 [a]	2.67	1.64
Perfluorooctanoic acid	PFOA	$CF_3(CF_2)_6CO_2H$	8	0.50 [a]	4.81[c]	2.69 [a]	3.43	2.94
Perfluorononanoic acid	PFNA	$CF_3(CF_2)_7CO_2H$	9	0.52 [a]	5.48 [c]	3.42 [a]	3.81	3.60
Perfluorodecanoic acid	PFDA	$CF_3(CF_2)_8CO_2H$	10	0.52 [a]	6.15 [c]	4.15 [a]	4.20	4.26
Perfluoroundecanoic acid	PFUnDA	$CF_3(CF_2)_9CO_2H$	11	0.52 [a]	6.82 [c]	4.88 [a]	4.58	4.91
Perfluorododecanoic acid	PFDoDA	$CF_3(CF_2)_{10}CO_2H$	12	0.52 [a]	7.49 [c]	5.61 [a]	4.96	5.57
Perfluorotridecanoic acid	PFTrDA	$CF_3(CF_2)_{11}CO_2H$	13	0.52 [a]	8.16 [c]	6.34 [a]	5.34	6.23
Perfluorotetradecanoic acid	PFTeDA	$CF_3(CF_2)_{12}CO_2H$	14	0.52 [a]	8.83 [c]	7.07 [a]	5.72	6.88
Perfluoroalkyl phosphonic acids								
Perfluorohexylphosphonic acid	PFHxPA	$CF_3(CF_2)_5PO_3H_2$	6	0.74 [a]	3.48 [e]	0.96 [a]	2.67	1.38
Perfluorooctylphosphonic acid	PFOPA	$CF_3(CF_2)_7PO_3H_2$	8	0.78 [a]	4.73 [e]	2.43 [a]	3.39	2.71
Perfluorodecylphosphonic acid	PFDPA	$CF_3(CF_2)_9PO_3H_2$	10	0.78 [a]	5.98 [e]	3.89 [a]	4.10	4.02
Bis(tridecafluorohexyl)phosphinic acid	6/6 di-PFPA	$CF_3(CF_2)_5CF_3(CF_2)_5PO_2H$	12	-1.41 [b]	6.96 [c]	6.05 [b]	4.66	5.97
(Heptadecafluorooctyl)(tridecafluorohexyl)phosphinic acid	6/8 di-PFPA	$CF_3(CF_2)_5CF_3(CF_2)_7PO_2H$	14	-1.41 [b]	8.3 [c]	7.4 [b]	5.42	7.18
Bis(heptadecafluorooctyl)phosphinic acid	8/8 di-PFPA	$CF_3(CF_2)_7CF_3(CF_2)_7PO_2H$	16	-1.41 [b]	9.64 [c]	8.8 [b]	6.18	8.44
(Henicosafluorodecyl)(tridecafluorohexyl)phosphinic acid	6/10 di-PFPA	$CF_3(CF_2)_5CF_3(CF_2)_9PO_2H$	16	-1.41 [b]	9.64 [c]	8.8 [b]	6.18	8.44
(Henicosafluorodecyl)(heptadecafluorooctyl)phosphinic acid	8/10 di-PFPA	$CF_3(CF_2)_7CF_3(CF_2)_9PO_2H$	18	-1.41 [b]	10.98 [c]	10.2 [b]	6.95	9.70
(Pentacosafluorododecyl)(tridecafluorohexyl)phosphinic acid	6/12 di-PFPA	$CF_3(CF_2)_5CF_3(CF_2)_{11}PO_2H$	18	-1.41 [b]	10.98 [c]	10.2 [b]	6.95	9.70

C#,perfluoroalkyl chain length

[a] Estimated values, obtained from database of Scifinder.

[b] Estimated values, obtained from database of Chemicalize, http://www.chemicalize.org/
[c] Estimated values, obtained from EPI Suite V 4.1(KOWWIN V1.68)
[d] Estimated from equation 3 in Chapter 4
[e] values obtained from Wang et al. [117]
[f] Estimated values, obtained from database of Chembase, http://en.chembase.cn/molecule-8864.html
[g] Estimated values, obtained from database of Chemspider
[h] Estimated from equation 2 in Chapter 4

1.7.2 Zebrafish as a Model Organism

Common test organisms used for bioaccumulation or risk assessment include fathead minnow, guppy, common carp, mussels, rainbow trout, zebrafish and grass shrimp. Among these organisms, zebrafish (*Danio rerio*) has special characteristics as vertebrate model organism [118]. Zebrafish is a kind of tropical freshwater fish which belongs to family of minnow (Cyprinidae) of order Cypriniformes [119]. They live in the rivers of northern India, northern Pakistan, Nepal and Bhutan in Southeast Asia at the temperature range from 20^{o}C to 32^{o}C [120]. Some of characteristics have made zebrafish to be popular test organism in laboratory and environmental study area [121]. Firstly, zebrafish is very small with approximately 3-5 centimeters long for an adult fish. This tiny size can greatly reduce housing space and husbandry costs, as well as the amount of chemicals exposed and the volume of hazardous waste generated [121]. Secondly, the embryonic development of zebrafish is very rapid. Its major organs can be developed within three days and become sexually mature after four months, which enables the use in transgenerational studies and mutagenesis analyzes [121]. Thirdly, zebrafish embryo has strong ability to survive even if there are severe morphological malformations or organ dysfunction, which is important for the study of human diseases. In addition, genes of zebrafish exhibite high similarities as those of human, which is important to the study of human diseases. With these characteristics, zebrafish can serve as an important model subject in laboratory and environmental study.

1.7.3 Experimental Setup

1.7.3.1 Flow-through Exposure System

Figure 1.2 has shown the schematic flow through exposure system. Dechlorinated tap water is delivered into mixing chambers where stock solutions of the analytes or methanol (solvent control) in 60 mL polycarbonate syringes were infused at 35 μL/min using a syringe pump (New Era Pump Systems, Inc., NY, USA). Tygon lab tubing (Masterflex, Cole-Parmer, Veron Hills, IL) was used in all the exposure experiments. From mixing chambers, the targeted concentration of a chemical was delivered into 60 L of exposure tanks with peristaltic pumps (Masterflex, Cole-Parmer, Veron Hills, IL) under flow through turnover conditions of 5 volume replacements per day. The outflow of exposure tanks were delivered into an activated carbon column to remove organic chemicals and then flowed into drainage.

1.7.3.2 Bioaccumulation Experimental Setup

Details regarding the experimental setup for flow-through bioaccumulation studies, animal care and housing and chemical analysis will be described in Chapter 3.

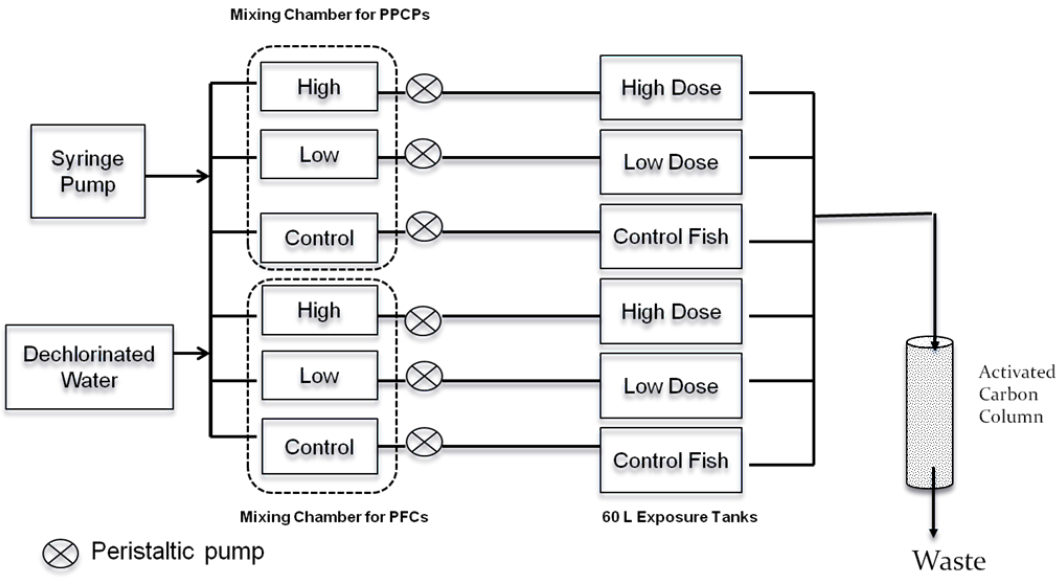


Figure 1.2 Schematic illustration of flow through exposure system.

1.8 Thesis Scope and Organization of Chapters

The general scope of this thesis involves investigation into the bioaccumulation behavior of PPCPs and PFCs in fish. This work included completion of a method paper (***Chapter 2)*** which presents a novel analytical method for rapid detection of PPCPs in fish plasma micro-aliquots. The method was evaluated by analysis of 20 µL plasma micro-aliquot samples collected from zebrafish (*Danio rerio*) from a controlled bioaccumulation study, including fish from a control (no exposure), as well as fish exposed to environmentally relevant concentrations of PPCPs. The developed method may be useful for future biomonitoring programs, especially those employing small fishes with low blood compartment volumes, as well as toxicokinetic and/or toxicodynamic studies, requiring fast analysis of PPCPs residues in fish plasma. ***Chapter 3*** investigated the uptake and elimination kinetics and tissue-specific bioconcentration of PPCPs. The study provides measures of key bioaccumulation metrics, including uptake rates (k_u), depuration rate

constants (k_d), half-life and kinetically derived and apparent steady-state bioconcentration factors (BCF_k and BCF_{ss}). To further evaluate the bioaccumulation mechanism of IOCs, influence of octanol-water, membrane-water and protein-water distribution coefficients (D_{ow}, D_{mw} and D_{pw}) on bioaccumulation potential of PPCPs in fish was investigated in ***Chapter 4***. The protein / membrane / lipid-water distribution coefficients (D_{pw} / D_{lipw} / D_{lw}) in the specific tissues of zebrafish were calculated in this study which provides important empirical data for future risk assessment or modeling prediction. The observed BCF data of weak acids and bases were applied to the Armitage BCF model for IOCs. Comparison of the observed BCF data to modeled data was discussed. The relationship between physicochemical properties, plasma and tissue protein and lipid composition and bioaccumulation behavior is explored and evaluated. ***Chapter 5*** assessed the uptake and elimination kinetics and tissue-specific bioconcentration of 3 classes of PFCs, including PFCAs and PFSAs and PFPAs. The study provides measures of key bioaccumulation metrics, including uptake rates (k_u), depuration rate constants (k_d), half-life and kinetically derived and apparent steady-state bioconcentration factors (BCF_k and BCF_{ss}). To our knowledge, it is the first report of PFPAs bioaccumulation data on fish. Furthermore, influence of octanol-water, membrane-water and protein-water distribution coefficients (D_{ow}, D_{mw} and D_{pw}) on bioaccumulation potential of PFCs in fish was investigated in ***Chapter 6***. The observed BCF data of PFCs applied to the Armitage BCF model were discussed. Comparison of the observed BCF data to modified K_{pw} model data was also investigated. ***Chapter 7*** is a summary of the findings presented in the preceding chapters.

CHAPTER 2 RAPID ANALYSIS OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS IN FISH PLASMA MICRO-ALIQUOTS USING LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMERY

2.1 Introduction

The occurrence of pharmaceuticals and personal care products (PPCPs) in the environment has received increasing attention in recent years. They usually enter into the environment through human activity and as residues from manufacturing, agriculture, veterinary use, as well as community and hospital use [122]. Numerous studies have reported PPCP residues in waste water treatment plant (WWTP) influents and effluents, as well as surface waters, with concentrations generally ranging between 0.001 and 1 μg/L [23,123-129].

PPCPs can accumulate in tissues of aquatic organisms [28,31,32,130,131] and toxicity tests indicates many of these compounds can cause chronic low-level effects [132-137]. Paroxetine and fluoxetine, commonly prescribed selective serotonin reuptake inhibitor (SSRI) antidepressants, have been detected in fish muscle tissue from Hamilton Harbour, Canada, at concentrations of 0.58 ng/g and 1.02 ng/g, respectively [31]. A recent national pilot study in the United States to assess the accumulation of PPCPs in fish demonstrated the presence of norfluoxetine, sertraline, diltiazem, diphenhydramine and carbamazepine at ng/g concentrations in fillet composites from effluent-dominated surface waters [30]. The survey showed the anticonvulsant, carbamazepine, was present at a concentration of 2.3 ng/g in fish fillets in Chicago, USA. Sertraline was detected at concentrations as high

as 19 and 545 ng/g in fish fillets and liver, respectively.

While these and other studies have focused on assessing the occurrence and levels of PPCPs in fish muscle and liver tissue, there are relatively few studies of these contaminants of emerging concern in fish plasma. Previously, carbamazepine and ibuprofen residues were detected at 0.3 pg/μL and 102 pg/μL, respectively, in plasma of fish exposed to treated sewage effluents in Sweden [32]. Tanoue et al. [131] reported concentrations of several PPCPs in plasma of cyprinoid fish from an effluent-dominated stream in Japan, with concentrations ranging between 0.03 and 110 pg/μL.

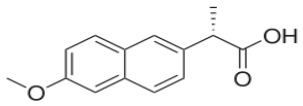
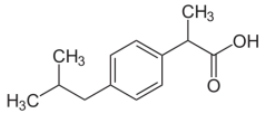
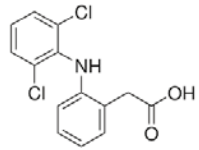
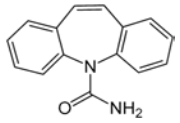
The concentration of xenobiotic compounds in blood plasma is a key parameter for comparative toxicology and pharmacology studies. Plasma concentrations can provide important information regarding recent exposure conditions [21,22]. The "Fish Plasma Model" has been proposed for prioritizing pharmaceuticals for in-depth ecological risk assessment [138,139]. The model utilizes estimated drug concentrations in fish plasma and human therapeutic plasma concentration data to assess exposure risks in fish. Implementation of this approach will require fast, reliable measurements of PPCP residue concentrations in fish plasma.

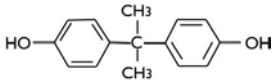
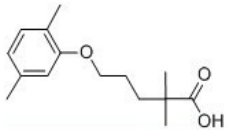
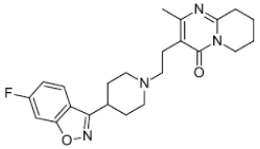
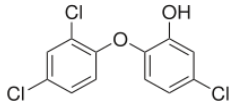
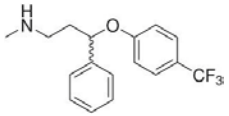
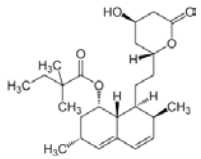
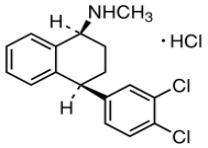
Currently, there are relatively few multi-residue analytical methods for trace quantification of PPCPs in fish plasma. Previous methods [32,131] have employed sample preparation techniques such as ultrasound assisted extraction (UAE), gel-permeation chromatography (GPC) and solid-phase extraction (SPE), prior to analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS). These methods require relatively large amounts of plasma for analysis (0.5 to 2 mL). In many cases however, only microliter volumes of

plasma can be attained from fish. This is especially true for small fish species.

The objective of the present study was to develop a multi-residue analytical method for rapid analysis of PPCPs in fish plasma micro-aliquots (20 μL). Target analytes included eleven key PPCPs that are commonly detected in environmental and biological samples, including bisphenol A (BPA), carbamazepine, diclofenac, fluoxetine, gemfibrozil, ibuprofen, naproxen, risperidone, sertraline, simvastatin and triclosan. The developed method was evaluated and further applied for the analysis these contaminants of emerging concern in plasma micro-aliquots of zebrafish (*Danio rerio*) subjected to environmentally relevant (parts per billion) levels.

Table 2.1 List of target PPCP compounds, along with the compound class, molecular structure, acid dissociation constant (pK_a), octanol-water distribution coefficient (log D_{ow}), name and spiking amount (ng) of internal surrogate compounds (IS) used for isotope dilution quantification.

Compound	**Class**	**Structure**	**pK_a**[a]	**Log D_{ow}**[a] **(pH 7)**	**Internal Surrogate Compound (IS)**	**Amount of IS spiked (ng)**
Naproxen	Non-steroidal anti-inflammatory drug (NSAID)		4.4[b]	0.76	Wafarin-d_5	99.1
Ibuprofen	NSAID		4.2[b]	1.09	Ibuprofen-d_3	233.2
Diclofenac	NSAID		4.0[b]	1.26	Ibuprofen-d_3	233.2
Carbamazepine	Anti-seizure		15.4[c]	3.52	Carbamazepine-d_{10}	25.1

Bisphenol A	Plasticizer		9.5[c]	4.25	Bisphenol A-d_6	248.7
Gemfibrozil	Fibrates		4.7[b]	1.93	Gemfibrozil-d_6	7.5
Risperidone	Atypical anti-psychotic		8.2[d]	1.04	Proponolol-d_7	249.6
Triclosan	Antibacterial and antifungal		7.8[b]	5.31	$^{13}C_{12}$-Triclosan	12.5
Fluoxetine	Anti-depressant		10.0[d]	1.59	Fluoxetine-d_5	256.8
Simvastatin	Antilipidemic		13.5[c]	4.17	$^{13}C_{12}$-Triclosan	12.5
Sertraline	SSRI Anti-depressant		9.5[d]	2.88	Fluoxetine-d_5	256.8

[a] Estimated values, obtained from SPARC
[b] Weak acid
[c] Neutral molecule
[d] Weak base

2.2 Experimental Section

2.2.1 Materials

High purity (> 97%) standards of diclofenac, naproxen, triclosan, ibuprofen, gemfibrozil, sertraline, risperidone, simvastatin were obtained from Sigma-Aldrich Co. LLC. (St Louis, USA). Bisphenol A was obtained from Merck (Hohenbrunn, Germany). Carbamazepine was purchased from TCI-EP (Tokyo, Japan). Fluoxetine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Isotopically labeled compounds, propanolol-d_7, ibuprofen-d_3, fluoxetine-d_5, carbamazepine-d_{10} and warfarin-d_5, were purchased from CDN Isotopes Inc. (Pointe-Claire, Quebec, Canada). Bisphenol A-d_6, $^{13}C_6$-2,4,5-trichlorophenoxyacetic acid ($^{13}C_6$-TCPAA) and gemfibrozil-d_6 were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, US). $^{13}C_{12}$-Triclosan was purchased from Wellington Laboratories Inc. (Ontario, Canada). HPLC grade Acetone, methanol and acetonitrile were purchased from Fisher Scientific Inc. (Loughborough, UK). All standards were prepared in methanol and stored at -20^{o}C.

2.2.2 Collection fish plasma micro-aliquots

Adult zebrafish were purchased from a local fish farm in Singapore (Mainland Tropical Fish Farm, Singapore). A flow-through experimental system was employed to expose fish to the studied PPCPs. Fish were separated into three groups, a low dose group, a high dose group and a control group. The low dose fish were exposed to a mixture of the studied PPCPs, with water concentrations ranging between 0.01 and 5 μg/L. Concentrations of individual PPCPs in water for the high dose group ranged between

0.04 and 12 μg/L. Control fish were subjected to the same conditions but without any exposure to the PPCPs. During the exposure period all fish were fed a diet consisting of artemia, twice daily. At the end of exposure, zebrafish were euthanized by submersion in ice water for 5 min. It cannot be submersed for too long otherwise blood clots will occur. The tail of the fish was cut off using sterilized spring scissors to expose caudal vein and blood was collected via pipette (Eppendorf Research, Germany). To maximize blood collection efficiency, we used 200 μL Prot/Elec tips with an elongated capillary tip of 10 μL (#223-9915, Bio-Rad, USA). Tips were fully filled with 18 mg/mL of EDTA (E5134, Sigma-Aldrich) and immersed in the same solution for at least 24 hours, completely dried at 60 °C, then cooled to room temperature. The anticoagulant coated tips were prepared within one week prior to use. It was found that approximately 10-20 μL of whole blood can be collected from one adult zebrafish. The whole blood was placed in a BD microtainer lithium heparin tube (#422-365971, BD, USA). The plasma was separated by centrifugation at 3000 g for 10 min at 4^{o}C (Eppendorf Centrifuge 5417R). Then plasma was transferred into a 2 mL centrifuge tube and stored at -80^{o}C prior to extraction.

2.2.3 Sample Preparation

For extraction of target analytes from the fish plasma micro-aliquots (20 μL), we tested and evaluated two different protocols, including LLE and SPE. To check for background contamination, a procedural blank consisting of 20 μL of Milli-Q water was processed with every batch of 10 samples.

For LLE, 20 μL of thawed plasma (pooled from five individual fish) was added to a 2 mL centrifuge tube and spiked with eight isotopically labeled internal surrogate (IS)

compounds (propanolol-d_7, ibuprofen-d_3, fluoxetine-d_5, carbamazepine-d_{10}, warfarin-d_5, bisphenol A-d_6, gemfibrozil-d_6 and $^{13}C_{12}$-triclosan). For six of the eleven target analytes, a matching mass labeled compound was available, while five of the target compounds were assigned a labeled compound exhibiting similar physicochemical properties, matrix effect and method recovery. A list of the corresponding internal surrogate compounds used to quantify the various target analytes is shown in Table 2.1. The amount of internal surrogate compounds spiked varied between 7.5 to 256.8 ng, depending on analyte sensitivity (Table 2.1). 1 mL of ice-cold acetone was added to extract target compounds and to precipitate proteins. Centrifuge tubes were sonicated for 3 min, then centrifuged at 12,000 rpm under 4^{o}C for 5 min. Extraction was repeated a second time with an additional 1 mL of acetone. The combined supernatant was evaporated under a gentle nitrogen stream to dryness. The test tube was reconstituted with 120 μL of methanol : water (1:4, v/v). Final extracts were transferred into LC vials and spiked with 5 μL of the injection internal standard, $^{13}C_6$-TCPAA (5 mg/L). The main purpose of the injection internal standard, $^{13}C_6$-TCPAA, is to determine the absolute recovery of internal surrogate compounds spiked prior to extraction [140]. All extracts were stored in darkness at 4 ^{o}C prior to LC-MS/MS analysis.

For SPE, 1.5 mL of 1% formic acid was added to 20 μL plasma micro-aliquots, then spiked with internal surrogate standards. The mixture was diluted to 5 mL with Milli-Q water before SPE. SPE was performed using Phenomenex X-33u 200 mg SPE cartridges. The conditioning of the SPE cartridges was performed with 5 mL of methanol followed by 5 mL of Milli-Q water. A vacuum manifold system was employed. The 5 mL samples were passed through the cartridges at a flow rate of 5 mL/min. The cartridges were then

washed with 5 mL of 5% methanol in water and then eluted with 3 mL of acetone, followed by 5 mL of methanol. The extracts were evaporated to dryness under a gentle nitrogen stream. The test tubes were reconstituted in 120 μL of methanol : water (1:4, v/v), transferred to LC vials and spiked with the injection internal standard (25 ng of $^{13}C_6$-TCPAA).

2.2.4 Identification and Quantification of Target Analytes using LC-ESI-MS/MS

Liquid chromatography was performed with a DIONEX Ultimate 3000 HPLC system. Compounds were separated chromatographically using a high efficiency Agilent Poroshell 120 SB-C18 column (2.1×50 mm, 2.7 μm). The high-efficiency Poroshell 120 column can provide high efficiency at lower pressures, thus avoiding the need for ultra-high performance liquid chromatography and sub 2 μm columns. The column was maintained at a temperature of 40°C for the entire run. Chromatographic separations were carried out using the following mobile phase gradient: solvent (A) 5 mM ammonium acetate in Milli-Q water, solvent (B) methanol : acetonitrile (1:1, v/v) at a flow rate of 0.3 mL/min. Solvent B was held at 20% for 0.5 min. Solvent B held at 20% for 0.5 min, increased linearly to 100% by 4 min and held till 6.5 min, then decreased to 20% by 6.7 min and held until 9 min. The sample volume injected was 10 μL.

The HPLC was coupled to a QTRAP 5500 tandem mass spectrometer (ABSCIEX) with electrospray ionization (ESI) source. The mass spectrometer was operated with a Turbo V™ source and ESI probe, capable of fast polarity switching and analysis of positive and negative ions. Source-dependent parameters for compounds analyzed in ESI positive mode were: curtain gas, 30 psi; nitrogen collision gas, high; source temperature was 550

◦C; ion spray voltage was 5500 V; ion source gases GS1 and GS2 were set at 55 and 60 psi, respectively. For compounds analyzed in negative mode, parameters were as follows: curtain gas, 15 psi; ion spray voltage was −4500 V; while all other parameters were the same as in ESI positive mode. Identification and quantification of all target analytes and internal surrogate compounds were conducted via tandem mass spectrometry. Ions were acquired in multiple reaction monitoring (MRM) mode, with a dwell time of 20 ms. Optimum ion transitions and MS operating conditions were determined by infusion with a syringe pump. A summary of the monitored ion transitions and corresponding operating parameters is shown in Table 2.2. For tandem MS, the overall number of MRM transitions monitored influences the degree of data points acquired. For the purpose of this study, only one MRM transition was monitored for labeled internal standards, as well as analytes which structurally similar isotopically labeled internal surrogates were available (e.g., ibuprofen, carbamazepine, bisphenol A, gemfibrozil, triclosan, fluoxetine). For analytes that were assigned an alternative internal surrogate compound, (e.g., diclofenac, naproxen, risperidone, sertraline and simvastatin), two MRM transitions were monitored.

Following Method 1694 by US EPA [140], we utilized an isotope dilution calibration approach. Specifically, a series of five calibration standard solutions (CS1-CS5) was prepared, with varied concentrations of the target analytes (range: 0.5-300 pg/μL) and a fixed concentration of the internal surrogate compounds. The relative response (RR) for each target analyte was computed for the five calibration solutions. Mean RR values were used for quantification only if the relative standard deviation (RSD) of the five observed RR values was below 20%, thereby indicating good linearity [140].

2.2.5 Method performance assessment

Method validation experiments involved extraction and analysis of plasma samples fortified with target analytes at the anticipated concentration level (10-100 ppb in plasma), (Table 2.1). Relative and absolute recoveries were determined and assessed according to US EPA protocols [140].

To evaluate matrix effects for the target compounds we employed the approach proposed by Matuszewski et al. [141]. Specifically, 40 μL of matrix (zebrafish plasma from control fish) was spiked with 5 μL of a target analyte mixture, then extracted and reconstituted in 80 μL of methanol. This extract was divided into two sub-samples, A and B. Sub-sample A (40 μL) was spiked with 10 μL of the target analyte mixture, along with 50 μL of Milli-Q water. Sub-sample B (40 μL) was diluted with 10 μL of methanol, along with 50 μL of Milli-Q water. A separate analytical standard (*S*) was prepared by combining 40 μL of methanol with 10 μL of the target analyte mixture and 50 μL of Mill-Q water. By comparing analyte peak areas of sub-samples and the analytical standard, a matrix effect (ME) value was calculated as:

$$ME\ (\%) = 100\ \times \frac{(A_i - B_i)}{S_i} \qquad (1)$$

where *Ai*, *Bi* and *Si* are the peak areas of the analytes (*i*) in subsample *A*, subsample *B* and the analytical standard (*S*), respectively. An ME of 100% indicates no matrix effect. Signal suppression or enhancement are indicated by ME values < 100% or > 100%, respectively [31].

2.3 Results and Discussion

2.3.1 Recovery of analytes by LLE and SPE

Both the Phenomenex X-33u SPE cartridge and LLE extraction protocols performed relatively well. Absolute recoveries of spiked native target analytes were above 55 % (Figure 2.1). The highest recoveries were observed for simvastatin, while triclosan exhibited the lowest. For example, using LLE the absolute recoveries of spiked simvastatin and triclosan were 137 ± 16 % and 55 ± 1.89 %, respectively. Using isotope dilution quantification to correct for losses, relative recoveries of nearly 100% (85-120%) were achieved for all compounds, demonstrating good accuracy when using the assigned internal surrogate compounds.

The investigated target analytes are from multiple compound classes and exhibit a variety of functional groups and range widely in physicochemical properties such as acid dissociation constant (pK_a) and octanol-water distribution coefficient (D_{ow}). It is often difficult to find a single extraction protocol that can universally work to capture multi-class PPCPs. With the exception of sertraline and fluoxetine, absolute recoveries observed with LLE approach were comparable to SPE. The LLE method provides satisfactory recoveries of multi-class PPCPs, spanning a range of pK_a between 4 and 13 and log D_{ow}'s between 0.7 and 5.3. The results indicate the relatively faster and cheaper LLE protocol is an effective strategy for extraction of these target PPCPs from fish plasma micro-aliquots.

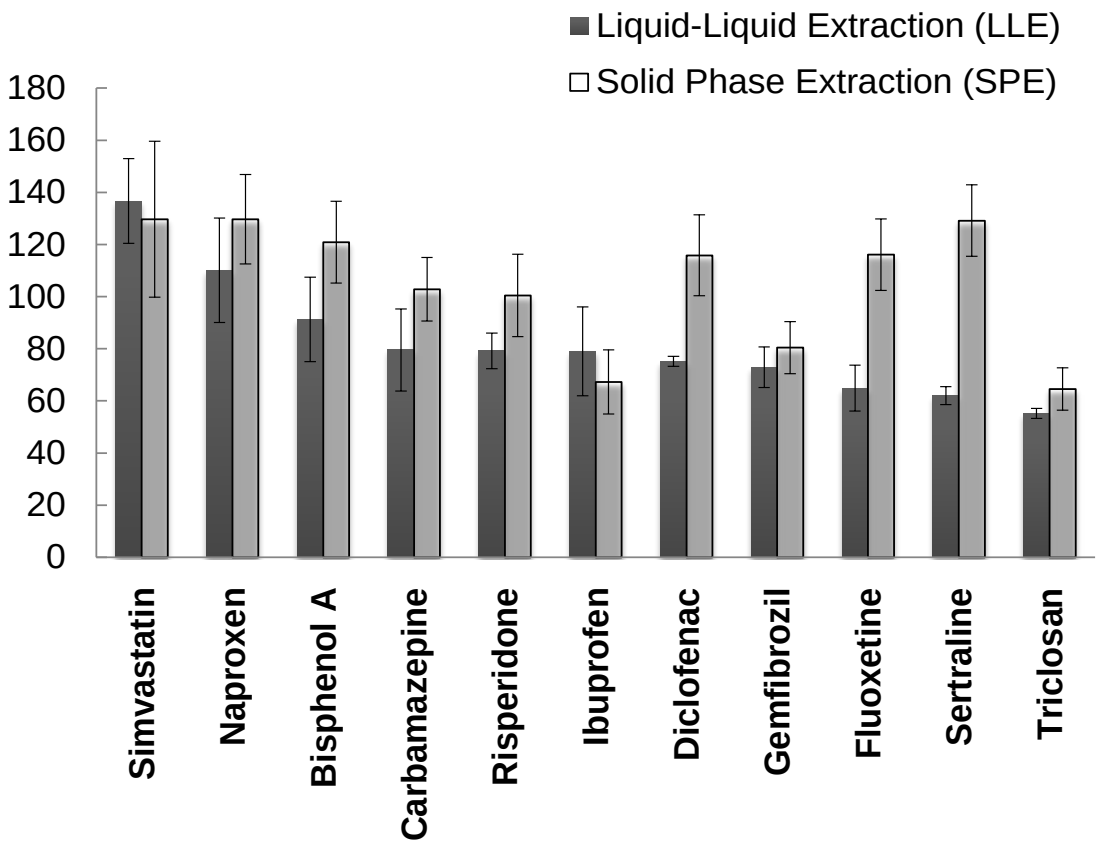


Figure 2.1 Absolute recovery (%) of individual PPCPs for extraction of 20 µL fish plasma micro-aliquot using LLE and SPE. Data are presented as mean recovery, with error bars representing standard deviation of triplicate samples (n = 3).

Table 2.2 MRM transitions and MS parameters for target pharmaceutical and personal care product (PPCP) compounds and isotope labeled internal standards.

Compound	Ion Transitions (m/z)[a]	Retention Time (min)	Dwell time (ms)	Polarity	Declustering Potential (V)	Entrance Potential (V)	Collision energy (eV)	Collision cell exit potential (V)
Naproxen	229.2 > 169.2	2.51	20	-	-50	-6	-41	-6
	229.2 > 170	2.51	20	-	-50	-6	-19	-9
Warfarin-d_5	312.1 > 160.9	2.59	20	-	-236	-12	-34	-9
TCPAA-$^{13}C_6$ [b]	259 > 201	2.76	20	-	-34	-10	-19	-7
Ibuprofen	205 > 159	3.39	20	-	-37	-3	-9	-5
Ibuprofen-d_3	208.2 > 163.8	3.37	20	-	-45	-7	-12	-12
Diclofenac	294 > 250	3.39	20	-	-34	-10	-15	-5
	294 > 34.9	3.39	20	-	-34	-10	-54	-4
Carbamazepine	237.1 > 194	3.61	20	+	187	7	27	11
Carbamazepine-d_{10}	247.1 > 204.1	3.61	20	+	156	9	27	12
Bisphenol A	227.1 > 133	3.86	20	-	-130	-7	-31	-8
Bisphenol A-d_6	233.1 > 138	3.86	20	-	-120	-11	-33	-7
Gemfibrozil	249. 1> 121	4.08	20	-	-60	-4	-20	-6
Gemfibrozil-d_6	255.3 > 121.1	4.08	20	-	-258	-10	-22	-6
Risperidone	411.5 > 190.8	4.27	20	+	238	5	28	14
	411.5 > 110	4.27	20	+	238	5	69	7
Proponolol-d_7	267.2 > 116	4.34	20	+	209	11	25	6
Triclosan	286.8 > 35	4.84	20	-	-80	-11	-39	-9
Triclosan-$^{13}C_{12}$	299 > 35	4.84	20	-	-57	-10	-39	-6
Fluoxetine	310.2 > 44.1	5.11	20	+	61	8	54	11
Fluoxetine-d_5	315.4 > 44	5.11	20	+	151	7	57	11
Simvastatin	399 > 115	5.16	20	-	-55	-3	-31	-2
Sertraline	306.2 > 159	5.37	20	+	45	5	36	9

	306.2 > 275.2	5.37	20	+	45	5	17	51

[a] For compounds with two ion transitions, the first MRM transition was used for quantification and the second for confirmation. Only one MRM transition was monitored for internal standards.

[b] $^{13}C_6$-2,4,5-trichlorophenoxyacetic acid used as injection internal standard for determining recovery of internal surrogate compounds spiked.

2.3.2 Matrix effects

LC-MS/MS is widely used for identification and quantification of PPCPs in environmental and biological samples. It is well-established that this analytical technique suffers from ion suppression or enhancement related to sample matrix interference. The average ME values (*n*=3) for target analytes are shown in Table 2.3. The average ME values of diclofenac, gemfibrozil, ibuprofen, naproxen, BPA were around 100%, indicating negligible matrix effects. The average ME values of simvastatin, fluoxetine and sertraline were 74.4, 78.9 and 65.4%, respectively, indicating moderate signal suppression. The average ME value was 28.21% for triclosan which indicates significant ion suppression. The average ME values of carbamazepine and risperidone were 156 and 136%, respectively, indicating signal enhancement. The relatively high mean ME for carbamazepine (156%) also exhibited high variability among triplicate samples (RSD= 42.84 %), indicating a potential ME range of 113-198%. It is important to note that ME values in this range are relatively common using LC-MS/MS. Moreover, the observed matrix effect for carbamazepine will likely have negligible effect on measurement accuracy, as this method utilizes deuterium labeled carbamazepine-d_{10} for isotope dilution quantification of this compound.

Triclosan and sertraline exhibited significant matrix effects, with a relatively high degree of signal suppression. Isotope dilution quantification of triclosan residues using $^{13}C_{12}$-triclosan provided effective resolution of this effect. We did not utilize an isotopically labeled sertraline internal surrogate compound in the present study. However, use of the structurally similar labeled internal surrogate, fluoxetine-d_5, an SSRI antidepressant, was sufficient, as the recovery and matrix effects were comparable. This was also the case for

the five other target analytes that were assigned an alternative labeled compound, including diclofenac (IS; ibuprofen-d_3), simvastatin (IS; $^{13}C_{12}$-triclosan), naproxen (IS; wafarin-d_5) and risperidone (IS; proponolol-d_7).

Table 2.3 Matrix effect (ME, %), method detection limits (MDLs) and method quantification limits (MQLs) for target PPCPs in fish plasma.

Compound	Mean Matrix Effect (ME, %) ± SD	MDL (pg/µL)	MQL (pg/µL)
Naproxen	100.3±6.3	0.69	2.30
Ibuprofen	118.9±2.7	0.63	2.10
Diclofenac	104.8 ±5.8	0.48	1.60
Carbamazepine	156.0±42.8	0.19	0.64
Bisphenol A	102.8±11.4	8.11	27.00
Gemfibrozil	105.1±6.2	0.09	0.30
Risperidone	136.3±13.1	0.015	0.05
Triclosan	28.2±4.9	0.39	1.31
Fluoxetine	78.9±4.9	1.30	4.33
Simvastatin	74.4±8.2	0.12	0.38
Sertraline	65.4±10.2	0.42	1.40

2.3.3 Detection and quantification limits

For LC-MS/MS analyses, analyte responses were consistently linear over the concentration range of 0.5 to 300 ng/mL, with correlation coefficients (r^2) ranging between 0.991 and 0.999. Relative responses were very reproducible over this concentration range, with relative standard deviations (RSD) between 2 and 20 %. Thus, mean RR values (n = 5) were used for quantification of target analytes.

For each target analyte, a method detection limit (MDL) was defined as a signal to-noise (S/N) ratio of 3:1 in sample matrix and the method quantification limit (MQL) as S/N of 10:1. Specifically, MDLs and MQLs were determined by monitoring the signal of target

analytes that yielded a signal-to-noise of 3 and 10 respectively in zebrafish plasma extract.

For some target analytes (BPA, triclosan, diclofenac, gemfibrozil, ibuprofen, naproxen and fluoxetine), trace residues were observed in procedural blanks. In such cases, a value equal to 3 times and 10 times the standard deviation of observed blank concentrations was used for MDL and MQL, respectively. A summary of the obtained MDLs and MQLs for the target PPCPs is shown in Table 2.3.

In general, the method performed very well, typically achieving detection of target analytes in the low to sub parts per billion (ppb) range. BPA exhibited comparatively higher detection limits, due to more pronounced background contamination in procedural blanks. For other compounds MDLs ranged between 0.015 and 1.30 pg/µL plasma. Similarly, MQLs of the target compounds were generally low, ranging between 0.05 and 4.33 pg/µL plasma. The developed LC-MS/MS based method for analysis of multi-class PPCPs achieved MDLs and MQLs comparable to previously reported methods, which typically utilized larger sample volume (0.5-2 mL) and more comprehensive extraction and cleanup steps [32,131,138]. For example, in the present study utilizing LLE of fish plasma micro-aliquots, MDLs for ibuprofen and triclosan, commonly targeted contaminants in biomonitoring studies, were 0.63 pg/µL and 0.39 pg/µL, respectively. These are equivalent to previously reported MDLs for ibuprofen (0.28 pg/µL) and triclosan (0.57 pg/µL) following extraction of large sample volumes and conventional SPE cleanup [131].

2.3.4 Quantitative determination of PPCP residues in fish plasma micro-aliquots

Measured concentrations of PPCPs in plasma of zebrafish are shown in Table 2.4. Ten

compounds were determined at concentrations higher than MDLs. Simvastatin residues were not detectable in these plasma samples, thus indicating concentrations below the MDLs (< 0.12 pg/μL). For the high dose group, all target analytes were detected well above the quantification limits. For the low dose group, PPCPs were typically detected at lower concentrations. For some PPCPs, concentrations in the low dose samples were below the MDLs (bisphenol A, fluoxetine, sertraline). However, several PPCPs were observed in plasma samples of fish from the unexposed control group. For example, residues of ibuprofen (0.94 ± 0.31 pg/μL), diclofenac (1.74 ±1.48 pg/μL), gemfibrozil (0.35 ± 0.31 pg/μL) and triclosan (0.98 ± 0.04 pg/μL) were quantifiable in plasma of control fish.

A common contaminant biomonitoring approach is to conduct in situ exposure experiments with wastewater effluent or receiving water samples. In particular, the recently proposed “Fish Plasma Model” requires accurate determination of accumulated PPCP residues in fish plasma following exposure to WWTP effluents [138,139]. Table 2.5 shows concentrations of several PPCPs monitored in fish plasma following in situ exposure to WWTP effluents [32,131]. Concentrations in fish plasma in those studies typically ranged between 0.1 to 100 pg/μL. The MDLs and/or MQLs achieved in the present study (also shown in Table 2.5) were in most cases lower than the reported concentrations. For example, the MQL of ibuprofen in the present study (2.1 pg/μL) is sufficiently low enough to quantify ibuprofen residues previously observed in fish plasma samples, which ranged between 2.5 and 102 pg/μL. It is important to note that PPCP concentrations in our low dose group exposure experimental system, were in many cases, lower than actual concentrations in WWTP effluent [32].

In addition to biomonitoring initiatives, the developed method may also be applicable for use in laboratory-based bioaccumulation and toxicological studies of PPCPs using small laboratory fishes. Pharmacokinetic and toxicology studies of contaminants of emerging concern in fish often require analysis of plasma [142-146]. Common test organisms used in such studies include zebrafish (*Danio rerio*), Japanese medaka (*Oryzias latipes*) and fathead minnows (*Pimephales promelas*). These species are relatively small in size (1-5 g) and exhibit low blood compartment volumes. The developed method can provide quantification of low levels of PPCP residues in as little as 20 μL of fish plasma.

Table 2.4 Measured concentrations (mean ± SD, pg/µL) of PPCPs in zebrafish plasma micro-aliquots for control, low-dose and high dose exposure groups.

	Plasma Concentration (pg/µL)						
	Control Group (*n*=20)	**Low Dose (n=3) (4 hours)**	**Low Dose (n=3) (24 hours)**	**Low Dose (n=3) (3 days)**	**High Dose (n=3) (4 hours)**	**High Dose (n=3) (24 hours)**	**High Dose (n=3) (3 days)**
Naproxen	< MDL	25.80 ± 2.37	50.30 ± 1.34	15.00 ± 0.78	131.00 ± 3.67	279.00 ± 17.40	82.70 ± 6.42
Ibuprofen	0.94 ± 0.31	13.00 ± 0.10	16.30 ± 0.65	9.09 ± 0.62	68.30 ± 9.57	77.40 ± 6.69	49.70 ± 4.14
Diclofenac	1.74 ±1.48	1.52 ± 0.38	2.68 ± 0.28	0.99 ±0.46	11.20 ± 0.67	25.20 ± 1.96	17.50 ± 1.79
Carbamazepine	0.40 ± 0.38	0.80 ± 0.04	2.30 ± 0.28	1.10 ± 0.17	7.65 ± 0.01	13.20 ±1.26	7.32 ± 0.75
Bisphenol A	< MDL	< MDL	< MDL	< MDL	17.60 ± 2.20	10.50 ± 1.60	10.40 ± 0.48
Gemfibrozil	0.35 ± 0.31	32.10 ± 1.90	46.60 ± 3.50	28.50 ±1.09	174.00 ± 13.40	235.00 ± 38.00	179.00 ± 8.60
Risperidone	< MDL	< MDL	0.07 ± 0.01	0.093 ± 0.003	0.21 ± 0.02	0.65 ± 0.01	0.88 ± 0.03
Triclosan	0.98 ± 0.04	0.90 ± 0.04	0.95 ± 0.25	0.93 ± 0.03	2.08 ± 0.43	3.21 ± 0.24	4.41 ± 0.37
Fluoxetine	<MDL	<MDL	<MDL	1.32 ± 0.14	2.20 ± 0.43	7.13 ± 0.93	6.90 ± 0.70
Simvastatin	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL
Sertraline	< MDL	< MDL	< MDL	0.44 ± 0.39	1.83 ± 0.73	2.20 ±0.50	2.26 ± 0.17

Table 2.5 Method detection limits (MDLs) and method quantification limits (MQLs) of PPCPs in fish plasma micro-aliquots (this study) compared with observed concentrations of those compounds previously reported in biomonitoring studies utilizing fish plasma.

	PPCP concentration in fish plasma (pg/μL)					
	MDL (this study)	**MQL (this study)**	**Umea, Sweden** [a]	**Stockholm, Sweden** [a]	**Gothenburg, Sweden** [a]	**Matsuyama, Japan** [b]
Naproxen	0.69	2.30	36	33	46	NA
Ibuprofen	0.63	2.10	13	5.5	102	2.5-3.8
Diclofenac	0.48	1.60	20	2.2	7.4	0.15-0.83
Carbamazepine	0.19	0.64	0.9	0.3	1	0.03-0.055
Risperidone	0.015	0.05	0.4	0.3	2.4	NA
Triclosan	0.39	1.31	NA	NA	NA	11-110
Sertraline	0.42	1.40	1.2	1.1	< 0.5	0.14-0.51

[a] Data are mean concentrations reported by Fick et al. [147].
[b] Data are Min-Max range of concentrations reported by Tanoue et al. [131].

2.4 Conclusions

We developed a method for fast, reliable analysis of multiple PPCP residues in fish plasma micro-aliquots using LC-MS/MS. The key advantages of the method include (i) low sample size requirement (20 μL plasma), (ii) a simple LLE sample preparation step using minimal solvent and (iii) use of a high efficiency HPLC column and fast polarity switching for rapid analyte separation and effective monitoring positive and negative ions in a single 9 min run. Quantification limits of the target PPCPs in fish plasma were in the low to sub pg/μL (ppb) range, which is sufficiently low for in situ contaminant biomonitoring and laboratory studies investigating pharmacokinetics and toxicology of these compounds.

CHAPTER 3 ASSESSING UPTAKE AND ELIMINATION KINETICS AND BIOCONCENTRATION FACTORS OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS IN ZEBRAFISH

3.1 Introduction

"Pharmaceuticals and personal care products (PPCPs) generally refer to any product used by individuals for personal health or cosmetic reasons or used by agribusiness to enhance growth or health of livestock" as defined by US EPA. PPCPs comprise a diverse collection of thousands of chemical substances, including prescription and over-the-counter therapeutic drugs, veterinary drugs, fragrances, and cosmetics [19]. The occurrence of PPCPs in the environment has received increasing concern in recent years.

The annual usage/consumption of PPCPs can be enormous. For example, it has been estimated that approximately 110 tonnes of antibiotics are used annually as feed additives in fish farms, as growth promoters in livestock production and as coccidiostatica in poultry production [148]. Consumption of some highly consumed pharmaceuticals in Denmark varied between 1 and 33 tonnes in 1995 [149,150]. Annual consumption of pharmaceuticals in Germany is around 2700 tonnes [72]. The total usage of antibacterial substances in Sweden is approximately 35 tonnes of active substance per year [149]. Triclosan, the antimicrobial agent used in many personal care products, is classified as a High Production Volume (HPV) chemical by US EPA, with annual production volumes exceeding a million pounds.

Discharge of PPCPs to the environment typically occurs via waste water associated with households, hospitals, manufacturing facilities and agriculture. PPCP residues have been detected in municipal wastewater treatment plant (WWTP) effluents [151-154], receiving waters [23,155-158], as well as tap water [159,160]. Levels in WWTP effluents and surface waters are typicall in the ng/L (ppt) to μg/L (ppb) range [122]. There is increasing concern regarding ecological risks of PPCPs, as many of these compounds are frequently detected in aquatic organisms in receiving waters, including various invertebrates and fish [29-32,127,130,132].

Nallani et al. [144] reported a very low bioconcentration factor (BCF) of ibuprofen (BCFs; 0.08–1.4), following a 28 day bioconcentration study in fathead minnow and channel catfish. However, some more hydrophobic pharmaceuticals may have a greater potential to bioaccumulate in organisms and food chains, especially for those compounds exhibiting slow in vivo metabolic transformation rates [161]. Fent et al. [72] investigated PPCPs for chronic lowest observed effect concentrations (LOEC) in laboratory organisms and found that they were about two orders of magnitude higher than maximal concentrations in sewage treatment plant (STP) effluents. The LOEC of diclofenac of fish was within the range of wastewater concentrations, whereas the LOEC of propranolol and fluoxetine of zooplankton and benthic organisms were close to maximal measured STP effluent concentrations. Information regarding bioaccumulation behavior and chronic low-level exposure of PPCPs in aquatic organisms is lacking.

The objective of the present study was to conduct a bioconcentration study to evaluate the uptake and elimination kinetics and steady-state BCFs of several PPCPs in zebrafish (*Danio rerio*). The exposure study was conducted using a flow-through system,

employing environmentally relevant exposure concentrations to simulate natural receiving waters. The study provides compound specific bioaccumulation kinetic parameters (uptake, depuration and fecal egestion rate constants, k_u, k_d, k_e, d^{-1}), kinetically derived BCFs (BCF_k) and observed steady-state BCFs (BCF_{ss}), which will be useful for future biomonitoring and risk assessment initiatives. The studied PPCPs vary widely in therapeutic class and physicochemical properties, thus relationships between chemical properties and bioaccumulation potential were investigated.

3.2 Materials and Methods

3.2.1 Standards and Reagents

Details are described previously in Chapter 2.

3.2.2 Experimental setup and collection of samples

Adult female zebrafish was purchased from a local fish farm in Singapore (Mainland tropical fish farm, Singapore) and were allowed to acclimate to laboratory conditions for two weeks prior to PPCPs exposure. These fish was maintained in dechlorinated water at a temperature of 25 ± 2°C with a 16:8 hour light/dark cycle. Bioconcentration experiments were carried out following procedures and protocols outlined in OECD guidelines for testing of chemicals [88]. This test consisted of two phases, namely the uptake and depuration phase. Water and exposed fish were sampled throughout the duration of the experiment to assess chemical uptake and elimination kinetics. The experiment consisted of continuous aqueous exposure of a mixture of multiple chemicals over a 6-d period, followed by a 7-d depuration phase. Separate groups of randomly

selected fish were exposed to the test chemicals including one group (280 fish) receiving a low dose (L) and another group (280 fish) receiving a high dose (H). A separate group of randomly selected fish (280 fish) kept in clean water served as the control group. The low dose group was at concentrations similar to environmental levels.

All flow-through experiments started at least two days before the introduction of the fish in order to allow the exposure tanks to equilibrate with the target test concentration. Chemical concentrations in water are determined every day to make sure the chemical concentration were stable during exposure period (Table 3.2). Time-weighted average measured test concentrations were used to calculate BCFs. Water samples were taken daily to determine the exposure concentrations (Table 3.2). During the exposures, the fish were fed with artemia twice daily. At six time-points during uptake phase (i.e., 4h, 8h, 1d, 2d, 3d, and 6d) and four time-points during the depuration phase (i.e., 7d, 8d, 10d and 13d) fish (three replicates of 5 pooled individuals) from the treatment groups and control group were sacrificed, at which time liver, ovary, muscle and plasma were collected and immediately frozen and stored at -20℃ for later processing.

3.2.3 Sample Extraction

Water samples (15 mL) collected from the flow-through system was mixed with 1.5 mL of 1% formic acid, and spiked with isotopically labeled surrogate standards prior to solid phase extraction (SPE). SPE was performed using Phenomenex X-33u 200 mg cartridges. The conditioning of the SPE cartridges was performed with 5 mL of methanol, followed by 5 mL of Milli-Q water at a flow rate of 5 mL/min through the cartridges using a vacuum system. Then the 15 mL water samples were passed through the cartridges at a

flow rate of 5 mL/min. The cartridges were then washed with 5 mL of 5% methanol in water and then eluted with 3 mL of acetone and 5 mL of methanol. The extract was then evaporated to dryness under gentle nitrogen. 120 μL of mixture of methanol : Milli-Q water (1:4) was added to rinse the tube before transferring to LC vials with 200 μl of glass insert. Finally, 25 ng of TCPAA-13C6 (injection internal standards, ISTD) was spiked into the extracts prior to analysis.

Details regarding extraction of plasma are described previously in Chapter 2.

Frozen tissues/organs (liver ~0.1g; muscle ~0.5g; ovary ~0.5g) were thawed and weighed directly into 15 mL of polypropylene centrifuge tubes. 0.5 mL of Milli-Q water was added into tube. Tissues were homogenized with a sonicator. Then 2 mL of 1 % of formic acid was added and labeled surrogate internal standards were spiked. The spiked tissue sample was extracted with 5 mL of methanol via sequential sonication for 30 min (twice) and centrifuged at 10000 rpm under 4^{o}C for 5 min. The supernatant of extracts was diluted to 100 mL with Milli-Q water for SPE and ISTD spiking.

Fecal matter were dried under 30 ^{o}C incubator and weighed. Feces samples were spiked with labeled surrogate internal standards then extracted with 5 mL of methanol via sequential (2 times) sonication for 15 min. Extracts were diluted to 100 mL with milli-Q water, followed by cleaned up by SPE, then ISTD spiking.

3.2.4 LC-ESI-MS/MS analysis

Details are described previously in Chapter 2.

3.2.5 Data analysis

Following Martin et al. [79] we utilized measured concentrations and fish growth measurements to determine growth corrected concentrations and corresponding bioaccumulation metrics of the studied PFCs. Specifically, the exponential growth model was used to predict the fish weight (FW),

$$FW = a \cdot \exp(g \cdot t) \quad (1)$$

where a is a constant, g is the growth rate, and t is time. Observed concentrations in zebrafish samples were corrected for growth by determining the increase in FW at each sampling interval, relative to t = 0, using Eq.1. The depuration rate constant (k_d) was determined by linear regression after fitting the growth corrected concentrations ($C_{fish(t)}$) during the depuration phase to the first-order decay model,

$$C_{fish(t)} = a \cdot \exp(-k_d \cdot t) \quad (2)$$

where a is a constant. The half-life was calculated as $\ln(2)/k_d$. Uptake rate constants (k_u) were determined by iterative nonlinear regression (Origin, Ver 9.0, Northhampton, MA, USA) after fitting the growth-corrected concentrations to the integrated form of the kinetic rate equation for constant aqueous exposure,

$$C_{fish(t)} = [(k_u)(C_w)/(k_d)] \cdot [1 - \exp(-k_d \cdot t)] \quad (3)$$

where C_w is the average exposure water concentration and k_d is a fixed parameter [79,162]. Kinetically-derived bioconcentration factors (BCF_k) were determined as the quotient of k_u/k_d. Additionally, a steady-steady state bioconcentration factor (BCF_{ss}) was

determined using the measured concentration in zebrafish at the end of the uptake phase divided by the constant concentration in water (i.e., $BCF_{ss} = C_{f(ss)}/C_w$). Also, fecal egestion rate constants (k_e) were determined by linear regression of the first-order decay model $C_{feces}(t) = a \cdot \exp(-k_e \cdot t)$, where a is a constant. Analysis of variance was calculated by Student's t-test. In all cases, the accepted level of significance (α) was 0.05.

3.3 Results and Discussion

3.3.1 Toxicological effects of PPCPs on zebrafish

The behavior of zebrafish during the exposure experiment was closely monitored to assess any contaminant related impacts. Primarily, mortality, swimming and feeding behavior, growth rate and liver somatic index (LSI) were assessed. For the high dose (H) group only 2 fish (0.56 %) died during the uptake phase and no fish died during depuration phase. No mortality was observed in the control (C) and low dose (L) groups. No statistically significant differences were observed in fish growth rate between the exposed fish and those in the control group ($p > 0.46$), (Table 3.1). All three groups of zebrafish exhibited normal growth throughout the experiment (Table 3.1). No obvious abnormality in fish swimming pattern was observed. Some difference in feeding behavior was observed between the H group and the L/C groups. Fish in the H group were comparatively slower in response to supplied feed. The fish in the H group generally exhibited a lower degree of feeding, as denoted by excess feed in the bottom of tank. No statistically significant difference was found between the liver somatic index (LSI) of exposed groups and control fish. However, the trend for LSI in H group fish was higher than other groups, indicating elevated LSI beyond day 6. The fish monitoring data

indicate that fish in the H group may have suffered sub-lethal effects due to continuous high PPCP exposure during the experiment. Karlsson et al. [163] showed that LSI in rats increased significantly when exposed to BPA, which was one of the test chemicals in the present study.

Table 3.1 Uptake and depuration phase duration, growth rate constant, and the associated coefficient of determination, mortality, and liver somatic index for exposed and control zebrafish used in bioconcentration experiments.

	Uptake period (d)	**Depuration period (d)**	**Mean initial fish mass (g)**	**Growth rate (g/d) (R^2)**	**Mortality (%)**	**Liver somatic index (%)**
Control	6	7	0.82	0.0188 (0.86)	0	2.75
Low Dose	6	7	0.81	0.0208 (0.96) (p = 0.46)	0	2.76 (p = 0.52)
High Dose	6	7	0.84	0.0158 (0.92) (p = 0.48)	0.56	3.25 (p = 0.83)

Table 3.2 Test compounds, physicochemical properties of selected PPCPs compounds, and mean aqueous exposure concentration (Mean ± SD, n=18).

Chemical	Class	Acidic/Basic	pK_a [a]	Log K_{ow} [a]	Log D_{ow} (pH=7) [a]	Waterborne Concentration (μg/L)	
						Low Dose	High Dose
Naproxen	Analgesic	acidic	4.2	3.24	0.76	3.89 ± 0.54	9.48 ± 0.46
Ibuprofen	Analgesic	acidic	4.4	3.56	1.09	1.03 ± 0.11	5.04 ± 0.71
Diclofenac	Analgesic	acidic	4	4.45	1.26	1.54 ± 0.51	8.00 ± 0.96
Gemfibrozil	Antihyperlipidemic	acidic	4.7	4.77	1.93	4.48 ± 0.43	12.5 ± 1.40
Risperidone	Antipsychotic	basic	8.24	3.59	1.04	0.027 ± 0.005	0.20 ± 0.04
Diphenhydramine	Antihistamine	basic	9	3.77	2.03	0.89 ± 0.076	4.35 ± 0.38
Fluoxetine	Antidepressant	basic	10	4.05	1.59	0.88 ± 0.05	3.60 ± 0.34
Sertraline	Antidepressant	basic	9.5	4.66	2.88	0.20 ± 0.02	0.76 ± 0.11
Carbamazepine	Antiepileptic	neutral	15.4	3.64	3.52	1.36 ± 0.070	5.35 ± 0.46
Bisphenol A	Plastic Monomer	neutral	9.5	4.25	4.25	1.94 ± 1.39	5.72 ± 2.8
Simvastatin	Antihyperlipidemic	neutral	13.5	4.68	4.17	< MDL	0.010 ± 0.008
Triclosan	Antiseptic	neutral	7.8	5.35	5.31	0.042 ± 0.01	0.234 ± 0.07

[a] Estimated values, obtained from SPARC.

3.3.2 Concentrations of PPCPs in water and zebrafish

During the uptake phase, measured PPCP water concentrations ranged between 0.027 ± 0.005 (risperidone) and 4.48 ± 0.43 μg/L (gemfibrozil) in the low exposure tank and between 0.010 ± 0.008 (simvastatin) and 12.5 ± 1.40 (gemfibrozil) in the high exposure tank (Table 3.2). Concentrations were stable throughout the exposure phase. Thus, the mean water concentrations were substituted as C_w (μg/L) in equation 2 for determination of k_u values. PPCPs were not detectable in control tanks or in exposure group tanks during the during depuration phase (> day 6).

Figure 3.1 shows the growth-corrected uptake and depuration phase concentrations of PPCPs in zebrafish liver, muscle, ovary and plasma. Most of test chemicals were detected in plasma and tissues except for diclofenac in ovary (L group), BPA in plasma (L group) and simvastatin in plasma (all groups). The observed concentrations were used to determine uptake rate constants (k_u), depuration rate constants (k_d), half-life and BCF_k values of the individual PPCPs by growth-corrected uptake and depuration phase concentrations using equation 2 and 3. Also, the measured chemical concentrations observed in fish tissues at the end of the uptake phase were used to determine the apparent BCF_{ss} ($BCF_{ss} = C_{fss}/C_w$).

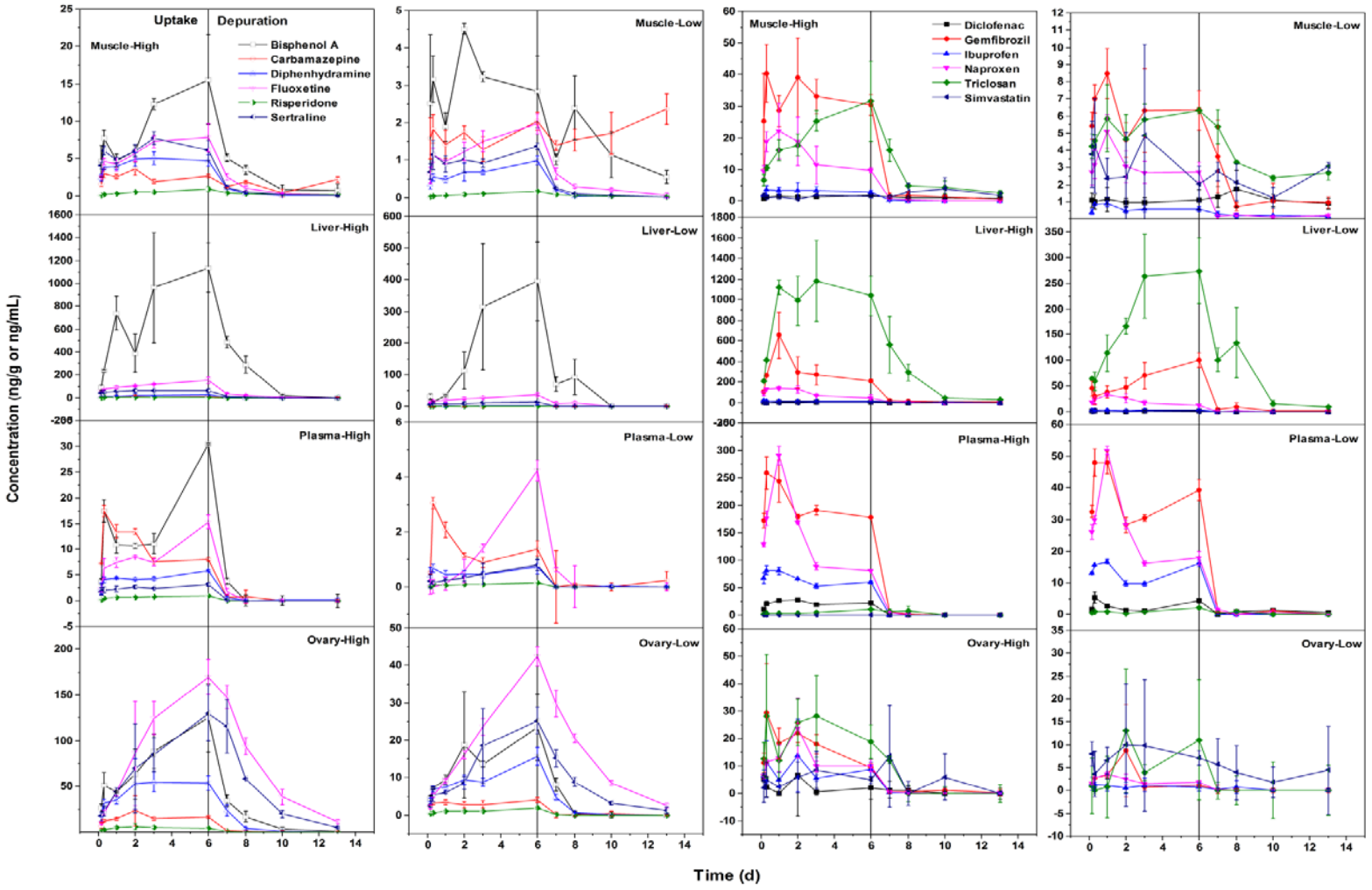


Figure 3.1 Growth-corrected uptake and depuration phase concentrations (n=3, mean ± SD) for PPCPs in muscle, liver, ovary and plasma.

Table 3.3 Rate of uptake (k_u ± standard error), rate of depuration (k_d ± standard error), half-life ($t_{1/2}$ ± standard error), kinetically derived bioconcentration factor (BCF_k, estimated by k_u/k_d) and steady-state bioconcentration factor (BCF_{ss}, estimated by C_f/C_w). Regression coefficients (r^2) are shown in parantheses.

Test Compound	K_u (L/kg/d)	K_d (1/d)	Half-life (d)	BCF_k (L/Kg)	BCF_{ss} (L/Kg)
		Plasma-Low			
Naproxen	108.02 ± 245.63 (0.35)	2.44 ± 0.28 (1.00)	0.28 ± 0.03	44.36 ± 101.00	4.36 ± 0.68
Ibuprofen	282.99 ± 758.38 (0.71)	3.82 ± 0.66 (1.00)	0.18 ± 0.03	74.18 ± 199.20	12.55 ± 4.65
Diclofenac	20.64 ± 46.26 (0.17)	1.75 ± 1.23 (0.68)	0.40 ± 0.28	11.83 ± 27.80	2.82 ± 0.94
Gemfibrozil	117.36 ± 120.67 (0.73)	3.62 ± 0.76 (1.00)	0.19 ± 0.04	32.43 ± 34.03	7.30 ± 1.48
Risperidone	9.36 ± 5.79 (0.63)	2.88 ± 0.21 (1.00)	0.24 ± 0.02	3.25 ± 2.03	3.74 ± 1.49
Diphenhydramine	6.35 ± 4.73 (0.72)	2.76 ± 0.88 (0.99)	0.25 ± 0.08	2.30 ± 1.87	0.59 ± 0.16
Fluoxetine	0.29 ± 0.05 (0.99)	1.90 ± 0.21 (0.99)	0.36 ± 0.04	0.15 ± 0.03	> 4.83 ± 0.29 [b]
Sertraline	1.11 ± 0.30 (0.87)	5.15 ± 0.70 (1.00)	0.13 ± 0.02	0.22 ± 0.07	> 3.95 ± 0.40 [b]
Carbamazepine	10.42 ± 16.79 (0.24)	2.50 ± 1.40 (0.93)	0.28 ± 0.15	4.16 ± 7.10	0.83 ± 0.26
Bisphenol A	ND	ND	ND	ND	ND
Simvastatin	ND	ND	ND	ND	ND
Triclosan	7.58 ± 5.60 (0.38)	1.20 ± 0.57 (0.83)	0.58 ± 0.27	6.31 ± 5.55	> 51.29 ± 12.35 [b]
		Plasma-High			
Naproxen	187.25 ± 315.20 (0.31)	2.81 ± 0.33 (1.00)	0.25 ± 0.03	66.66 ± 112.48	8.95 ± 0.72
Ibuprofen	357.18 ± 1269.44 (0.84)	3.76 ± 0.56 (1.00)	0.18 ± 0.03	94.94 ± 337.73	11.24 ± 1.83
Diclofenac	14.20 ± 4.46 (0.87)	3.58 ± 0.53 (1.00)	0.19 ± 0.03	3.96 ± 1.38	2.52 ± 0.39
Gemfibrozil	213.73 ± 156.29 (0.82)	3.40 ± 0.16 (1.00)	0.20 ± 0.01	62.94 ± 46.11	14.64 ± 1.75
Risperidone	8.06 ± 1.97 (0.92)	2.34 ± 0.11 (1.00)	0.30 ± 0.01	3.44 ± 0.86	3.99 ± 1.03
Diphenhydramine	5.27 ± 1.54 (0.88)	2.22 ± 0.20 (1.00)	0.31 ± 0.03	2.37 ± 0.73	1.06 ± 0.20
Fluoxetine	2.22 ± 0.95 (0.69)	2.15 ± 0.06 (1.00)	0.32 ± 0.01	1.03 ± 0.44	> 4.26 ± 0.41 [b]
Sertraline	20.93 ± 5.45 (0.91)	2.96 ± 0.01 (1.00)	0.23 ± 0.00	7.06 ± 1.84	3.67 ± 0.85
Carbamazepine	22.23 ± 25.25 (0.51)	2.44 ± 0.60 (0.99)	0.28 ± 0.07	9.13 ± 10.61	1.45 ± 0.14
Bisphenol A	1.20 ± 0.78 (0.39)	2.05 ± 0.08 (1.00)	0.34 ± 0.01	0.58 ± 0.38	> 5.32 ± 2.57 [b]

Simvastatin	ND	ND	ND	ND	ND
Triclosan	7.98 ± 2.54 (0.87)	0.45 ± 0.17 (0.84)	1.53 ± 0.56	17.61 ± 8.52	> 46.60 ± 14.12 [b]
		Liver-Low			
Naproxen	70.23 ± 88.04 (0.55)	2.74 ± 1.17 (0.97)	0.25 ± 0.11	25.66 ± 33.98	3.82 ± 0.98
Ibuprofen	FF	3.54 ± 0.95 (1.00)	0.20 ± 0.05	NA	2.69 ± 0.35
Diclofenac	FF	3.83 ± 0.64 (1.00)	0.18 ± 0.03	NA	1.11 ± 0.41
Gemfibrozil	9.91 ± 4.19 (0.62)	2.74 ± 0.86 (0.99)	0.25 ± 0.08	3.62 ± 1.91	> 22.49 ± 2.17 [b]
Risperidone	52.46 ± 21.89 (0.78)	1.91 ± 0.71 (0.94)	0.36 ± 0.13	27.42 ± 15.27	15.91 ± 5.59
Diphenhydramine	FF	2.55 ± 0.32 (1.00)	0.27 ± 0.03	NA	3.38 ± 1.71
Fluoxetine	79.09 ± 30.60 (0.80)	0.90 ± 0.26 (0.91)	0.77 ± 0.22	87.87 ± 42.20	> 41.61 ± 2.52 [b]
Sertraline	332.86 ± 129.27 (0.83)	1.59 ± 0.40 (0.96)	0.44 ± 0.11	209.35 ± 96.73	> 64.68 ± 10.67 [b]
Carbamazepine	2.33 ± 2.28 (0.46)	0.29 ± 0.07 (0.90)	2.38 ± 0.58	7.99 ± 8.05	1.24 ± 0.63
Bisphenol A	45.96 ± 13.96 (0.90)	1.33 ± 0.39 (0.93)	0.52 ± 0.15	34.64 ± 14.69	183.23 ± 134.46
Simvastatin	FF	FF	NA	NA	> 57.06 ± 47.32 [a]
Triclosan	3846.98 ± 724.94 (0.94)	0.56 ± 0.19 (0.86)	1.24 ± 0.42	6869.60 ± 2644.71	6464.25 ± 1564.73
		Liver-High			
Naproxen	166.14 ± 347.80 (0.47)	3.11 ± 0.45 (1.00)	0.22 ± 0.03	53.39 ± 112.02	6.12 ± 4.26
Ibuprofen	FF	3.70 ± 0.30 (1.00)	0.19 ± 0.02	NA	2.26 ± 0.37
Diclofenac	0.19 ± 0.05 (0.90)	3.66 ± 1.77 (0.99)	0.19 ± 0.09	0.05 ± 0.03	0.51 ± 0.07
Gemfibrozil	113.03 ± 108.89 (0.37)	2.20 ± 0.25 (1.00)	0.31 ± 0.04	51.28 ± 49.75	19.13 ± 3.99
Risperidone	36.66 ± 10.14 (0.90)	2.18 ± 0.23 (1.00)	0.32 ± 0.03	16.83 ± 4.97	14.01 ± 3.86
Diphenhydramine	21.19 ± 9.90 (0.73)	2.80 ± 0.12 (1.00)	0.25 ± 0.01	7.56 ± 3.55	4.67 ± 1.05
Fluoxetine	83.29 ± 28.19 (0.84)	1.29 ± 0.18 (0.98)	0.54 ± 0.07	64.76 ± 23.62	> 42.97 ± 4.11 [b]
Sertraline	522.43 ± 83.53 (0.97)	1.73 ± 0.22 (0.99)	0.40 ± 0.05	301.63 ± 61.27	79.37 ± 11.80
Carbamazepine	9.19 ± 6.19 (0.58)	3.92 ± 3.67 (0.98)	0.18 ± 0.17	2.35 ± 2.70	1.85 ± 0.71
Bisphenol A	99.52 ± 37.51 (0.79)	0.78 ± 0.06 (0.99)	0.89 ± 0.07	128.25 ± 49.42	183.46 ± 91.10
Simvastatin	8.26 ± 4.06 (0.94)	1.89 ± 1.04 (0.85)	0.37 ± 0.20	4.37 ± 3.24	> 151.44 ± 136.49 [b]

Triclosan	8608.20 ± 1920.59 (0.94)	0.65 ± 0.03 (1.00)	1.07 ± 0.05	13304.79 ± 3040.51	4732.92 ± 1499.46
		Muscle-Low			
Naproxen	8.20 ± 6.51 (0.65)	2.58 ± 1.02 (0.97)	0.27 ± 0.11	3.18 ± 2.81	0.73 ± 0.11
Ibuprofen	5.37 ± 3.99 (0.64)	0.32 ± 0.14 (0.62)	2.20 ± 0.96	17.05 ± 14.70	0.53 ± 0.08
Diclofenac	FF	FF	NA	NA	0.18 ± 0.09
Gemfibrozil	16.91 ± 12.26 (0.78)	0.70 ± 0.21 (0.87)	0.99 ± 0.29	24.09 ± 18.88	1.43 ± 0.38
Risperidone	2.28 ± 0.37 (0.95)	0.52 ± 0.12 (0.91)	1.34 ± 0.31	4.42 ± 1.25	> 6.50 ± 2.10
Diphenhydramine	2.30 ± 1.32 (0.74)	1.59 ± 0.10 (1.00)	0.44 ± 0.03	1.44 ± 0.83	1.12 ± 0.27
Fluoxetine	5.40 ± 2.52 (0.73)	1.02 ± 0.15 (0.98)	0.68 ± 0.10	5.29 ± 2.58	> 2.25 ± 0.40
Sertraline	40.92 ± 18.64 (0.81)	1.59 ± 0.18 (0.99)	0.44 ± 0.05	25.80 ± 12.13	5.32 ± 1.10
Carbamazepine	23.14 ± 32.17 (0.83)	FF	NA	NA	1.21 ± 0.21
Bisphenol A	16.37 ± 14.26 (0.64)	0.19 ± 0.13 (0.41)	3.57 ± 2.36	84.38 ± 92.19	1.57 ± 1.13
Simvastatin	FF	FF	NA	NA	> 329.26 ± 115.50 [a]
Triclosan	945.59 ± 251.57 (0.92)	0.18 ± 0.06 (0.75)	3.83 ± 1.23	5224.26 ± 2175.83	136.06 ± 36.73
		Muscle-High			
Naproxen	13.17 ± 10.83 (0.56)	2.27 ± 0.21 (1.00)	0.31 ± 0.03	5.80 ± 4.79	1.12 ± 0.13
Ibuprofen	4.67 ± 1.33 (0.91)	2.10 ± 0.47 (0.98)	0.33 ± 0.07	2.22 ± 0.81	0.65 ± 0.11
Diclofenac	0.25 ± 0.15 (0.66)	0.52 ± 0.05 (0.99)	1.33 ± 0.13	0.47 ± 0.29	0.09 ± 0.03
Gemfibrozil	28.13 ± 14.17 (0.85)	2.78 ± 0.77 (0.99)	0.25 ± 0.07	10.11 ± 5.81	2.62 ± 0.47
Risperidone	1.70 ± 0.38 (0.91)	0.52 ± 0.13 (0.89)	1.33 ± 0.34	3.27 ± 1.11	> 5.01 ± 0.92 [b]
Diphenhydramine	4.96 ± 0.81 (0.96)	1.43 ± 0.07 (1.00)	0.49 ± 0.02	3.47 ± 0.59	1.12 ± 0.10
Fluoxetine	4.51 ± 1.65 (0.82)	1.06 ± 0.06 (1.00)	0.65 ± 0.04	4.25 ± 1.57	2.10 ± 0.23
Sertraline	57.48 ± 21.85 (0.85)	1.59 ± 0.14 (1.00)	0.44 ± 0.04	36.17 ± 14.11	8.72 ± 1.81
Carbamazepine	3.68 ± 1.98 (0.74)	FF	NA	NA	0.43 ± 0.10
Bisphenol A	1.13 ± 0.52 (0.58)	0.92 ± 0.14 (0.97)	0.75 ± 0.11	1.22 ± 0.59	2.43 ± 1.24
Simvastatin	FF	FF	NA	NA	143.03 ± 123.52

Triclosan	87.38 ± 21.21 (0.88)	0.73 ± 0.14 (0.95)	0.95 ± 0.18	119.54 ± 36.45	121.80 ± 41.32
		Ovary-Low			
Naproxen	FF	2.83 ± 0.70 (0.99)	0.24 ± 0.06	NA	0.42 ± 0.09
Ibuprofen	FF	FF	NA	NA	0.90 ± 0.32
Diclofenac	FF	FF	NA	NA	NA
Gemfibrozil	FF	1.88 ± 2.23 (0.67)	0.37 ± 0.44	NA	0.20 ± 0.05
Risperidone	0.19 ± 0.07 (0.71)	1.90 ± 0.06 (1.00)	0.36 ± 0.01	0.10 ± 0.04	53.10 ± 21.62
Diphenhydramine	3.02 ± 1.72 (0.40)	1.26 ± 0.08 (1.00)	0.55 ± 0.04	2.40 ± 1.37	13.76 ± 5.61
Fluoxetine	1.14 ± 0.08 (0.99)	0.38 ± 0.01 (1.00)	1.81 ± 0.06	2.99 ± 0.24	> 48.41 ± 2.93 [b]
Sertraline	0.88 ± 0.20 (0.92)	0.51 ± 0.01 (1.00)	1.35 ± 0.04	1.71 ± 0.40	> 126.36 ± 12.64 [b]
Carbamazepine	2.93 ± 1.17 (0.31)	3.24 ± 1.77 (0.98)	0.21 ± 0.12	0.90 ± 0.61	2.55 ± 0.65
Bisphenol A	1.87 ± 0.66 (0.75)	1.22 ± 0.19 (0.98)	0.57 ± 0.09	1.54 ± 0.59	> 12.09 ± 8.65 [b]
Simvastatin	FF	0.14 ± 0.09 (0.24)	5.06 ± 3.36	NA	> 896.99 ± 164.42 [a]
Triclosan	0.56 ± 0.48 (0.44)	5.14 ± 65.49 (0.68)	0.13 ± 1.72	0.11 ± 1.39	225.91 ± 127.33
		Ovary-High			
Naproxen	FF	2.73 ± 0.39 (1.00)	0.25 ± 0.04	NA	1.06 ± 0.05
Ibuprofen	FF	3.53 ± 1.11 (1.00)	0.20 ± 0.06	NA	1.43 ± 0.48
Diclofenac	0.25 ± 0.15 (0.66)	0.41 ± 0.14 (0.86)	1.67 ± 0.57	0.59 ± 0.41	0.17 ± 0.11
Gemfibrozil	FF	2.74 ± 1.20 (0.97)	0.25 ± 0.11	NA	1.35 ± 0.45
Risperidone	1.78 ± 0.42 (0.85)	1.73 ± 0.09 (1.00)	0.40 ± 0.02	1.03 ± 0.25	28.16 ± 6.78
Diphenhydramine	13.98 ± 3.69 (0.80)	0.96 ± 0.12 (0.98)	0.73 ± 0.09	14.64 ± 4.31	12.35 ± 1.09
Fluoxetine	7.17 ± 0.46 (0.99)	0.33 ± 0.05 (0.96)	2.09 ± 0.34	21.65 ± 3.73	> 47.17 ± 4.51 [b]
Sertraline	5.94 ± 0.74 (0.97)	0.39 ± 0.09 (0.93)	1.77 ± 0.39	15.18 ± 3.88	> 171.23 ± 25.29 [b]
Carbamazepine	7.30 ± 2.98 (0.56)	2.12 ± 0.24 (1.00)	0.33 ± 0.04	3.45 ± 1.46	3.00 ± 0.30
Bisphenol A	7.03 ± 2.45 (0.68)	1.18 ± 0.10 (0.99)	0.59 ± 0.05	5.94 ± 2.13	> 21.85 ± 10.54 [b]
Simvastatin	FF	FF	NA	NA	641.32 ± 544.36
Triclosan	FF	1.07 ± 0.87 (0.66)	0.65 ± 0.52	NA	103.60 ± 37.57

[a] Calculated by concentration at steady state divided by MDL (0.010 ng/mL) as the chemical concentration in water is lower than

MDL
[b] Compounds have not approached the steady state, calculated by the concentration at day 6 divided by water concentration
FF: Fitting failed
NA: Not available
ND: Not detected in tissue

3.3.2.1 Uptake kinetics

Uptake rates (k_u) of the various PPCPs in different tissues ranged widely, between 0.19 to 8608.20 L/kg/d (Table 3.3). For the majority of the studied PPCPs uptake was very fast. Some compounds only took one day to approach the steady state, (e.g. ibuprofen and sertraline in liver). Other compound like triclosan uptake was slower. Also, uptake rates of triclosan differed substantially between the different tissues. For example, k_u for triclosan in liver, muscle and ovary was 3850, 946 and 0.56 L/kg/d, respectively.

The nonlinear regression equation (Eq. 3) was not suitable for determination of uptake rates (k_u) in some cases. Nonlinear regression model worked well for determination of k_u for triclosan, BPA and simvastatin. However, determination of k_u using the nonlinear regression model was problematic for other compounds such as carbamazepine, naproxen and diclofenac. For those compounds, the model fitting failed, with relatively low r^2 values. For example, during the uptake phase, concentrations of naproxen and gemfibrozil in liver and diclofenac, gemfibrozil, ibuprofen, naproxen and carbamazepine in plasma were unstable, exhibiting a trend whereby concentrations increased then declined prior to the apparent steady state concentration. The observed delayed maxima may be due to in vivo biotransformation of those compounds [164].

The observed uptake rates were comparable to previously reported values. For example, Wang and Gardinali [165] investigated uptake of PPCPs in mosquito fish (*Gambusia holbrooki*) and observed uptakes rates in fish tissues equal to 0.2, 5.1 and 15.4 L/kg/d for carbamazepine, diphenhydramine and ibuprofen respectively.

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The pH-partition hypothesis [166] may help to explain the rapid uptake of the PPCPs. Due to the polar ionogenic organic chemicals (IOCs) exhibit a relatively high degree of ionization, they cross biological membranes at a faster rate [98]. Karlsson has reported that uptake of diclofenac and fluoxetine is highly sensitive to changes in pH with bioconcentration factors varying by over two orders of magnitude (diclofenac) and four orders of magnitude (fluoxetine) across three pH units in worms [167].

3.3.2.2 Elimination kinetics

Observed depuration rate constants (k_d) for the various PPCPs ranged between 0.14 and 5.14 d^{-1}, with corresponding half-lives ($t_{1/2}$) in the range of 0.13 to 5.06 days (Table 3.3). The depuration half-lives tended to be lower in liver compared to ovary and muscle, likely due to higher rates of metabolic transformation and clearance in liver.

The observed depuration rates and half-lives of the studied PPCPs are comparable to those previously reported. For example, $t_{1/2}$ values of 1.3, 1.4 and 3.0 days were reported for ibuprofen, diphenhydramine and carbamazepine in mosquito fish [165]. These depuration rates for PPCPs are much faster than those for more persistent IOCs such as perfluoroalkyl acids, which exhibit $t_{1/2}$'s on the order of weeks [79,84]. This is likely due to higher rates of metabolic transformation of PPCPs compared to the relatively high recalcitrance of the latter compounds [79].

Fecal elimination can play an important role in contaminant elimination in fish. Collected zebrafish fecal material during the depuration phase of the present study, residues of PPCPs were observed at concentrations typically greater than internal fish concentrations. Figure 3.2 shows the concentrations of various PPCPs in fecal material during the depuration phase. Fecal elimination is shown to be relatively fast, which is consistent with the observations for the depuration rates for tissues. Table 3.4 shows the observed fecal egestion rate constants (k_e, d^{-1}) for the various PPCPs in zebrafish during the depuration phase. Compounds such as ibuprofen, diclofenac, risperidone and carbamazepine exhibited k_e values of approximately 0.8 to 1.2 d^{-1}, with clearance occurring in approximately 1-2 days. k_e values for triclosan and BPA were substantially lower (0.06-0.47 d^{-1}), with clearance taking approximately 3-4 days. The fecal egestion rate constants were lower than the depuration rate constants in the tissues, indicating the presence of other elimination processes such as respiratory elimination and biotransformation.

Table 3.4 Rate of fecal egestion (k_e ± standard error) in depuration phase. Corresponding regression coefficient (r^2) values are shown in parentheses.

Test Compound	Low Dose Group k_e (d^{-1})	High Dose Group k_e (d^{-1})
Naproxen	0.969 ± 0.053 (0.55)	0.831 ± 0.038 (0.69)
Ibuprofen	1.203 ± 0.031 (0.79)	1.247 ± 0.071 (0.27)
Diclofenac	1.039 ± 0.035 (0.76)	0.744 ± 0.122 (0.02)
Gemfibrozil	0.656 ± 0.032 (0.80)	0.536 ± 0.061 (0.37)
Risperidone	1.194 ± 0.029 (0.81)	0.956 ± 0.045 (0.58)
Diphenhydramine	0.928 ± 0.036 (0.81)	0.759 ± 0.017 (0.93)
Fluoxetine	0.659 ± 0.046 (0.70)	0.465 ± 0.031 (0.77)
Sertraline	0.738 ± 0.034 (0.79)	0.559 ± 0.021 (0.90)
Carbamazepine	1.118 ± 0.042 (0.70)	0.972 ± 0.017 (0.94)
Bisphenol A	0.465 ± 0.033 (0.78)	0.234 ± 0.034 (0.66)
Simvastatin	-	-
Triclosan	0.165 ± 0.074 (0.30)	0.059 ± 0.067 (0.15)

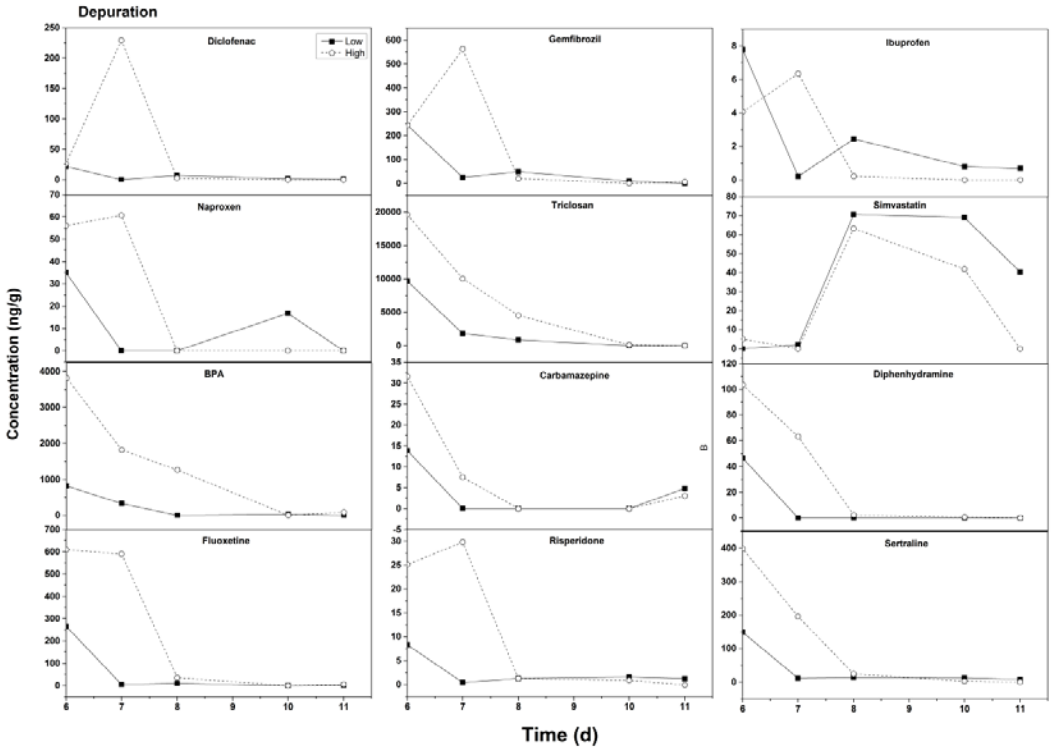


Figure 3.2 Concentration of PPCPs in fish fecal material collected during the depuration phase.

3.3.2.3. Tissue-specific and whole-body BCFs

BCFs for the various PPCPs in plasma, liver, muscle and ovary for both the low exposure and high exposure group are shown in Table 3.3. No significant differences were observed between BCFs in the low and high exposure groups, indicating no concentration dependent bioaccumulation behaviour. Steady-state BCF_{ss} ranged from 0.09 to 6464 L/Kg (Table 3.3).

Estimated whole-body BCF_{ss} of the various PPCPs ranged between 0.2 and 465 (see Table 3.5). Ibuprofen, naproxen, diclofenac, risperidone, gemfibrozil, sertraline and diphenhydramine exhibited very low BCF_{ss} values, typically between approximately 0.1 and 200, indicating low bioconcentration potential Also, the data show that BCF_{ss} values of these PPCPs were similar for plasma, liver, muscle, ovary. Conversely, triclosan, BPA and simvastatin exhibited much higher BCF_{ss}. In addition, the BCFs of triclosan, BPA and simvastatin were varied among different compartments, with BCFs ordered liver > ovary > muscle > plasma. The BCF_{ss} of triclosan in liver was approximately 50 times greater than those in muscle and plasma. For example, BCF_{ss} of triclosan in liver and muscle in the low exposure group was 6464 and 136, respectively. This is likely attributable to a combination of higher sorptive capacity of liver and ovary tissue and the hydrophobic nature of those PPCPs.

Table 3.5 Steady-state bioconcentration factor of whole body of fish (BCF_{ss} ± standard deviation). The whole body burden was calculated by the sum of tissue burden of plasma, liver, muscle and ovary time wet weight of plasma, liver, muscle and ovary, respectively. The whole body BCF was calculated by the whole body burden divided by the sum of wet weight of plasma, liver, muscle and ovary. The average weight of plasma, liver, muscle and ovary per fish are 0.0045g, 0.2g, 0.25g and 0.15g, respectively in this study.

Test Compound	**Whole body BCF_{ss} (L/Kg)**	
	Low	**High**
Naproxen	0.80 ± 4.11	1.41 ± 8.11
Ibuprofen	0.88 ± 9.11	1.11 ± 4.17
Diclofenac	0.18 ± 7.46	0.16 ± 2.23
Gemfibrozil	2.05 ± 3.22	3.07 ± 9.37
Risperidone	23.38 ± 5.21	13.60 ± 2.58
Diphenhydramine	5.69 ± 1.96	5.25 ± 9.46
Fluoxetine	20.44 ± 1.24	19.97 ± 1.91
Sertraline	50.87 ± 5.15	69.41 ± 17.26
Carbamazepine	1.68 ± 5.14	1.41 ± 7.13
Bisphenol A	13.83 ± 9.91	17.85 ± 8.62
Simvastatin	> 513.55 ± 37.99	317.99 ± 256.11
Triclosan	465.06 ± 113.43	331.81 ± 191.18

Figure 3.3 illustrates the observed relationship between apparent steady state BCFs (log BCF_{ss}) with and model predicted BCF (log BCF_k) of various PPCPs in different tissues. The data are in general agreement with model predicted BCF's. However, BCF_{ss} values for some compounds in muscle-Low group were substantially higher (10-100 times higher) than calculated BCF_k values. On the contrary, BCF_{ss} values for some compounds in ovary-Low group were significantly lower (10-100 times) than calculated BCF_k values. In addition, in many cases, the non-linear model did not fit the uptake curves very well, thus no estimate of BCF_k was possible. For those compounds, we relied on the apparent steady state BCF_{ss} to assess bioconcentration potential.

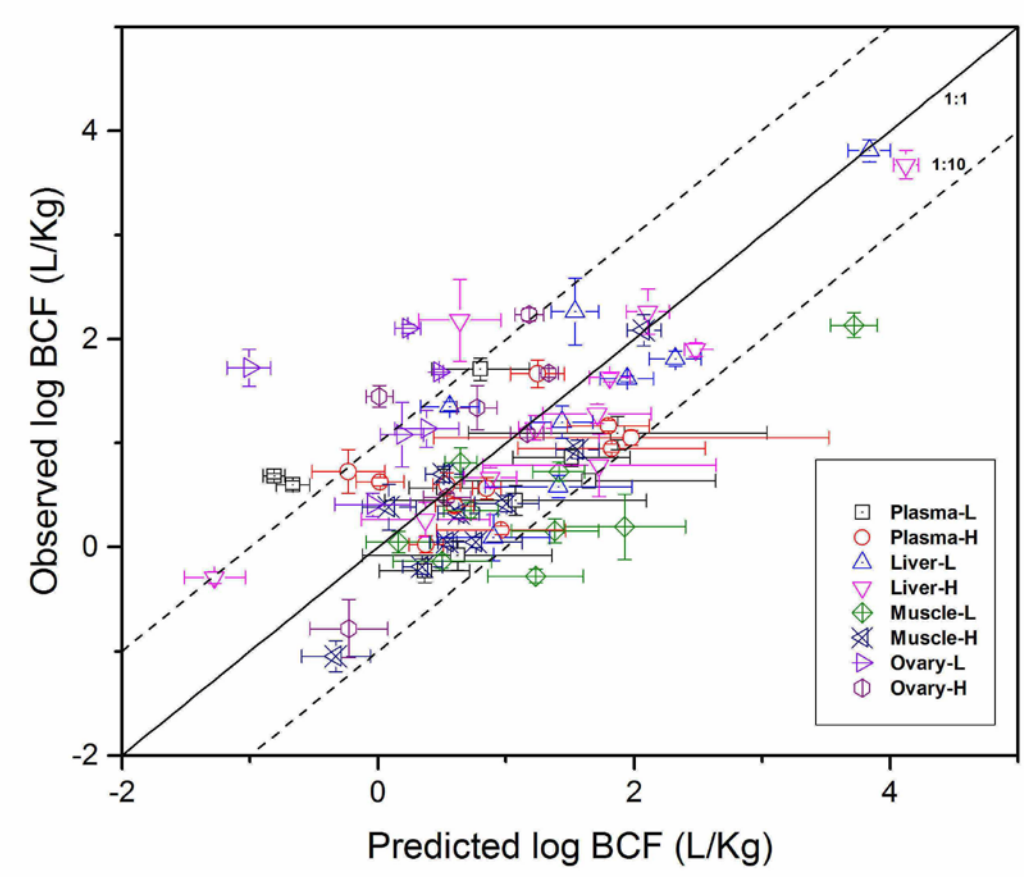


Figure 3.3 Observed versus predicted Log BCF_s (i.e., calculated by first-order model) in different tissues. The solid lines represent perfect model agreement 1:1. Dashed lines represent 1log unit interval of predicted concentrations. Long Dashed dot lines represent 2 log units interval of predicted concentrations.

3.3.3 Influence of octanol-water distribution coefficient (D_{ow})

Figure 3.4 and Figure 3.5, respectively illustrate the relationship between observed $t_{1/2}$ and log BCF_{ss} versus the octanol-water distribution coefficient (D_{ow}) for PPCPs in liver, ovary and muscle. The majority of PPCPs are classified as IOCs, including weak acids and weak bases. Log D_{ow} values (calculated at pH 7) for the studied PPCPs varies between 0.76 (ibuprofen) to 5.3 (triclosan). Due to the fact the internal pH of fish tissues is close to neutrality [168,169], pH 7 was used for log D_{ow} values. Figure 3.4 shows

positive linear relationships between $t_{1/2}$ and log D_{ow} for liver, muscle and plasma Positive linear relationships were observed between BCF_{ss} and log D_{ow} for liver, ovary, muscle and whole fish, but not for plasma. The data also indicate that that BCF_{ss}-log D_{ow} relationship was much stronger than BCF_{ss}-log K_{ow}, demonstrating the importance of distinguishing fraction of ionic vs. neutral form of these IOCs.

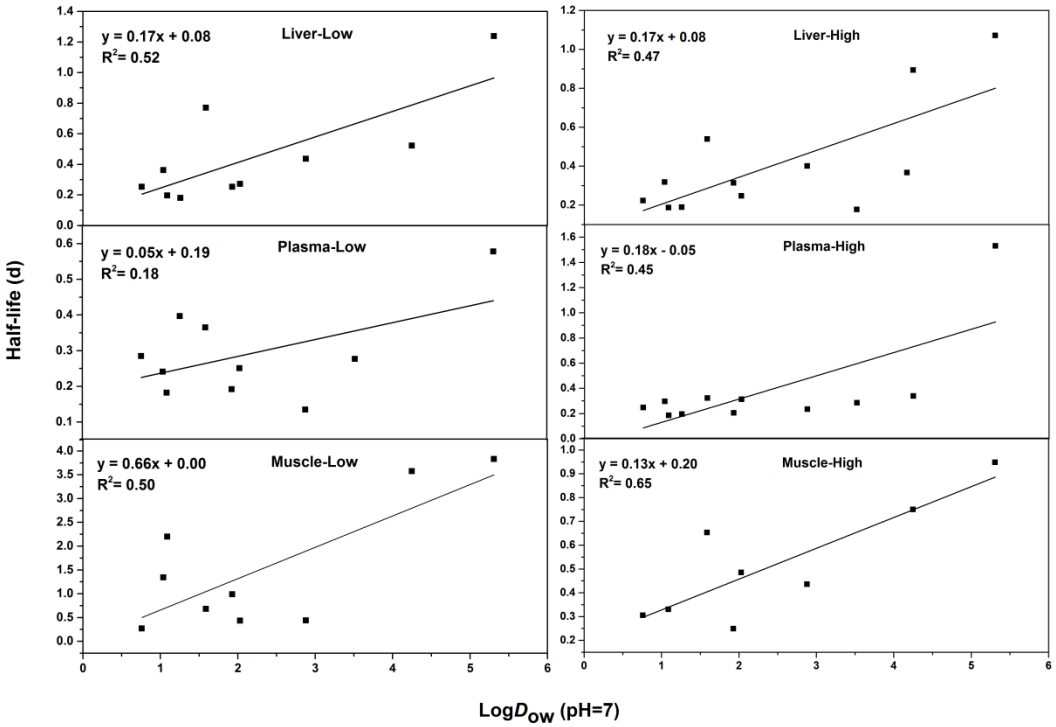


Figure 3.4 Relationship between half-life and Log D_{ow} for PPCPs in liver, plasma and muscle.

Bioconcentration potential has been well predicted by the K_{ow} and one-compartment first order model for neutral compounds, but for IOCs, K_{ow} is not appropriate for predicting bioconcentration potential. Armitage et al have proposed to consider the pH dependent distribution coefficient, log D_{ow} for bioconcentration assessments of IOCs [98]. The data from the present study indicate that log D_{ow} provides reasonable prediction of

bioconcentration potential of PPCPs.

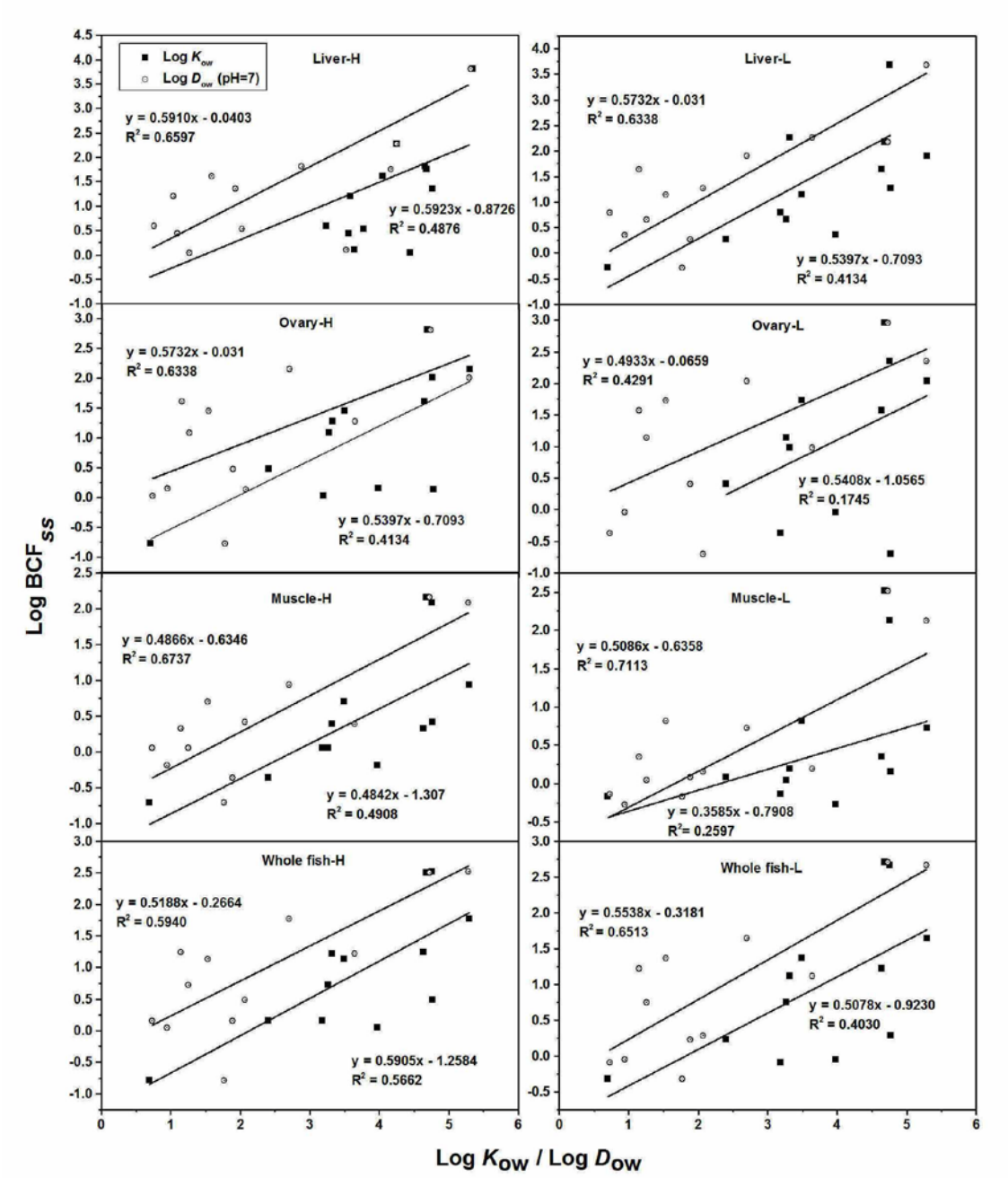


Figure 3.5 Log BCF_{ss} relationships with Log K_{ow} and Log D_{ow} for liver, ovary, muscle and whole fish.

3.4 Conclusions

The present study investigated the uptake and elimination kinetics and tissue-specific

bioconcentration of twelve pharmaceuticals and personal care products (PPCPs). The study provides measures of key bioaccumulation metrics, including uptake rates (k_u), depuration rate constants (k_d), half-life and kinetically derived and apparent steady-state bioconcentration factors (BCF_k and BCF_{ss}). The results showed rapid uptake and depuration in zebrafish for many of the PPCPs. BCFs in fish tissues were low for most of PPCPs tested in this study. Half-lives ($t_{1/2}$) of the studied PPCPs ranged between 0.13 to 5.06 days. The whole body BCF_{ss} at steady state ranged between 0.2 and 465. The highest tissue-specific BCF_{ss} was observed for triclosan in liver (6,464 ± 1,565). The BCF_{ss} of triclosan was tissue dependent, with BCF values in liver (6,464 ± 1,565) exceeding those in muscle (136 ± 37) by approximately 50 times, which indicates the importance of conducting tissue-specific analysis in order to avoid under or over prediction of bioaccumulation potential and exposure. Relatively strong positive relationships were observed between BCF_{ss} and log D_{ow} ($r^2 > 0.6$). While the study demonstrates that chemical D_{ow} is a reasonably good predictor of PPCP bioconcentration, further studies of uptake, elimination and metabolism of IOCs are needed to better understand their bioaccumulation behaviour and exposure risks in aquatic organisms.

CHAPTER 4 CHEMICAL AND BIOLOGICAL FACTORS GOVERNING BIOACCUMULATION AND EXPOSURE RISKS OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS IN FISH

4.1 Introduction

Ecological impacts related to the discharge and distribution of pharmaceuticals and personal care products (PPCPs) in the environment is of increasing. PPCP residues have been detected in wastewater [151-154], surface water [23,155-158], as well as finished drinking water [159,160]. In general, PPCPs in WWTP effluents and surface waters are present at ng/ to μg/L (ppt-ppb) range [122]. PPCP residues have also been detected in a variety of aquatic organisms [28], including marine mussels [29,127,130] and fish tissues [30-32,132]. Currently, there is limited information regarding the fate, bioaccumulation behavior and exposure risks of PPCs in aquatic ecosystems.

The potential risks of PPCPs in aquatic ecosystems is likely more associated to chronic low-dose exposure and sub-lethal type effects, rather than high-dose exposure and acute toxicity. Acute toxicity data of about 100 Pharmaceuticals were compiled in a table by Webb [170]. The effect concentrations of these pharmaceuticals ranged from 0.1 to 10,000 mg/L. Aquatic species are continuously exposed over long periods of time or even over their entire life cycle, however there is currently a lack of chronic toxicity data for PPCPs in aquatic organisms [72].

Previous studies regarding the bioaccumulation of PPCPs in fish have reported relatively low bioaccumulation potential of PPCPs, denoted by a bioconcentration factor (BCF) or bioaccumulation factor (BAF), which was attributed to the polar nature and susceptibility to metabolic transformation of these compounds [138,144,171,172]. It is well understood that bioaccumulation of nonpolar compounds is driven by equilibrium partitioning with organism lipids [173]. In contrast, bioaccumulation of PPCPs, which are mostly ionogenic organic compounds (IOCs), is likely more related to ambient pH and dissociation [172]. Ionization reduces uptake, bioaccumulation, and toxicity of acidic and basic compounds, due to the loss of pure lipophilic characteristics [98,100].

The purpose of the present study is to examine chemical and biological factors governing the bioaccumulation, tissue distribution and maternal transfer of PPCPs in zebrafish (*Danio rerio*). The study aims to provide new information regarding the role of phospholipids and proteins on bioaccumulation potential in fish. Specifically, the study involves (i) determination of empirical lipid-water, phospholipid-water and protein-water distribution coefficients of several PPCPs in zebrafish plasma and tissues (D_{lw}, D_{lipw}, D_{pw}), (ii) comparison of observed steady state bioconcentration factors (BCF_{ss}) of individual PPCPs with predicted BCF using a recently proposed mechanistic bioaccumulation model for ionogenic organic contaminants. The relationship between physicochemical properties, plasma and tissue protein and lipid composition and bioaccumulation behavior is explored and evaluated. The findings may benefit future efforts to assess the bioaccumulation, tissue distribution and chronic exposure risks of PPCPs in fish.

4.2 Theoretical Section

The current state of knowledge regarding ionogenic organic contaminants such as PPCPs indicates bioaccumulation behavior is likely related to partitioning of these relatively polar compounds in phospholipids and/or binding with proteins and organic anion transporters [98,105,108,109,114,174,175]. The key parameters for these proposed mechanisms are the compounds liposome-water partition coefficient (D_{lipw}) and protein-water distribution coefficient (D_{pw}). pH corrected liposome-water distribution coefficients (D_{lipw}) are required for assessing partitioning and steady state distribution as liposomes are more characteristic of biomembranes, which are mainly composed of phospholipids, compared to octanol [106,172,176,177]. Liposome-water partitioning coefficient (Log K_{lipw}) values were determined using Equation (1) for non-polar compounds (i.e. carbamazepine and simvastatin) or equation (2) for polar compounds (i.e. ibuprofen et al) [116,177]:

$$\text{Log } K_{lipw} = 1.05 \times \text{Log } K_{ow} - 0.32 \qquad (1)$$

$$\text{Log } K_{lipw} = 0.90 \times \text{Log } K_{ow} + 0.52 \qquad (2)$$

D_{lipw} values at pH 7 were determined using Equation 3,

$$D_{lipw}\,(\text{pH } 7) = \chi_N \times K_{lipw,\,N} + \chi_I \times K_{lipw,\,N} \times 0.1 \qquad (3)$$

where χ_N is the fraction of chemical in neutral form and χ_I is the fraction of chemical in charged form at pH 7 as calculated using the Henderson-Hasselbalch equation.. $K_{lipw,\,N}$ was K_{lipw} of neutral species.

Protein-water partitioning distribution coefficient (Log K_{pw}) was estimated using equation 3 [107]:

$$\text{Log } K_{pw} = 0.57 \times \text{Log } K_{ow} + 0.69 \qquad (4)$$

Armitage et al. recently proposed a mechanistic bioaccumulation model for IOCs in fish [98]. The model incorporates neutral lipids, phospholipids and non-lipid organic matter (i.e., proteins) and their relative sorptive capacity for IOCs. The sorption capacity of the biota compared to water (D_{BW}, in L/kg) is from Armitage mass balance BCF model for IOCs. The model has modified the Schmitt's [97] approach and based on following considering : (1) separate phospholipids from neutral (storage) lipids, and (2) use octanol-water and membrane-water distribution ratios to account for sorptive capacity of the neutral and charged form of the chemical [98]. The modified equation is shown below,

$$D_{BW} = f_{SL}D_{OW} + f_{PL}D_{MW} + f_{NLOM}\rho_{NLOM}D_{OW} + f_W \qquad (5)$$

where f_{SL} and f_{PL} are the volume fractions of neutral lipids and phospholipids, respectively. Phospholipids, neutral lipids and protein were determinded in the different tissues of zebrafish (Table 4.1). f_{NLOM} and f_W are the volume fractions of unlipid organic matter (protein in this case) and water. The f_W was assumed to 92% [178] in the plasma and 80% in the other tissues [98]. ρ_{NLOM} =0.05, which is the protein contribution factor to sorptive capacity. Octanol-water distribution ratio (D_{OW}) and membrane-water distribution ratio (D_{MW}) are estimated from the respective partition coefficients for the neutral and charged form, as below

$$D_{OW}=\chi_N K_{OW,N} + \chi_I K_{OW,I} \text{ and } D_{MW}= \chi_N K_{MW,N} + \chi_I K_{MW,I} \quad (6)$$

The apparent octanol-water and membrane-water partition coefficients for the charged form are calculated from the partition coefficients of the neutral form,

$$\log K_{OW,I} = \log K_{OW,N} - \Delta_{OW} \text{ and}$$

$$\log K_{MW,I} = \log K_{MW,N} - \Delta_{MW} \quad (7)$$

where Δ_{OW} and Δ_{MW} are scaling factors relating K_{OW} and K_{MW}. According to Armitage et al. [98], Δ_{OW}= -3.1 and Δ_{MW}= -2.0 for carboxylic acid/ sulfonic acids /other acids and Δ_{OW}= -3.1 and Δ_{MW}= -0.3 for primary amine; Δ_{OW}= -3.1 and Δ_{MW}= -0.5 for secondary amine; Δ_{OW}= -3.1 and Δ_{MW}= -1.25 for tertiary amine and Δ_{OW}= -3.1 and Δ_{MW}= -1.25 for other bases. The $\log K_{OW,N}$ was obtained from EPISuite V4.1 and other references (see Table 1.3). $K_{MW,N}$ can be estimated from the equation below [179],

$$\log K_{MW,N} = 1.01 \log K_{OW,N} + 0.12 \quad (8)$$

4.3 Experimental Section

4.3.1 Bioaccumulation experiments

Details regarding the experimental setup for flow-through bioaccumulation studies and chemical analysis are described previously in Chapter 3.

4.3.2 Determination of protein, phospholipid and neutral lipid content in plasma and tissues

Total protein of fish tissues were extracted with protein extraction buffer (BioRad) and

determined by Nanodrop 2000 spectrophotometer (Thermo scientific, USA). Phospholipid was determined by phospholipid assay kit (Sigma, USA). Due to the phospholipid assay kit only quantifies phosphatidylcholine which contains typically on 50% of total phospholipids in zebrafish [180], total phospholipid content were determined as the observed phosphatidylcholine concentration multiplied by a factor of two. Triglyceride was determined by triglyceride quantification kit (BioVision) and cholesterol was determined by cholesterol quantification kit (Sigma). Total neutral lipids were determined as total triglycerides + total cholesterol.

4.3.3 Data analysis

Apparent D_{pw}, D_{lipw} and D_{lw} for the various PPCPs in different tissues and whole fish were determined using the protein, phospholipid or neutral lipid corrected concentration of PPCPs compared to the water concentration of PPCPs (e.g., D_{pw} = C_p ng/kg protein divided by C_w ng/L).

To explore the observed concentration and BCF data for PPCPs, Principal Component Analysis (PCA) (XLSTAT, Addinsoft™) was used to examine the influence of co-variables on observed tissue concentrations and BCFs of PPCPs in zebrafish. Compiled PCA results included eigenvectors, squared cosines of the variables, Pearson's correlation coefficients (r) and contribution of the variables (%). Two separate PCAs were performed using (i) absolute chemical concentration in a given tissue as the observational response variable and tissue constituent properties (neutral lipid, phospholipid and protein content) as covariables and (ii) steady state bioconcentration factor (BCF_{ss}) as observational response, with physical-chemical properties, pK_a, log D_{ow}, log D_{lipw}, log D_{pw} as

covariables.

4.3.4 Tissue components determination

Total protein of fish tissues were extracted with protein extraction buffer (BioRad) and determined by Nanodrop 2000 spectrophotometer (Thermo scientific, USA). Phospholipid was determined by phospholipid assay kit (Sigma, USA). Due to the phospholipid assay kit only quantifies phosphatidylcholine which contains about 50% of total phospholipids in zebrafish [180], phospholipids contents were calculated by phosphatidylcholine concentration divided by 50%. Triglyceride was determined by triglyceride quantification kit (BioVision) and cholesterol was determined by cholesterol quantification kit (Sigma).

4.4 Results and Discussion

4.4.1 Protein and lipid measurements

Table 4.1 summarizes the measured protein, phospholipid, cholesterol and triglyceride concentrations (mg/g). Protein concentrations were highest in liver (409 ± 73.9) and ovary (235 ± 26.8). Phospholipids in plasma (7.66 ± 1.68) were higher than other tissues, which were approximately 0.5 to 0.9 mg/g. Cholesterol ranged from 1.60 ± 0.28 in liver to 5.40 ± 0.65 in plasma. Triglycerides ranged between 7.57 ± 1.84 in plasma to 28.4 ± 7.18 in muscle. Total neutral lipids (cholesterol + triglycerides) was therefore highest in muscle tissue (30.0 ±7.19), followed by liver (24.9 ± 6.08), ovary (18.7± 1.62) and plasma (13.0 ±1.95).

Table 4.1 Protein, phospholipid and neutral lipid components in the tissues. Neutral lipids components were calculated by the sum of cholesterol and triglyceride. The whole components were calculated by the sum of mass of protein etc. in the plasma, liver, muscle and ovary divided by the sum of wet weight of plasma, liver, muscle and ovary. The average weight of plasma, liver, muscle and ovary per fish are 0.0045g, 0.02g, 0.25g and 0.15g, respectively in this study.

	Tissue Components (mg/g)				
	Protein	**Phospholipid**	**Cholesterol**	**Triglyceride**	**Neutral lipids (Cholesterol + Triglycerides)**
Plasma	56.31±5.63	7.66±1.68	5.40±0.65	7.57±1.84	12.97±1.95
Liver	409.74±73.94	0.50±0.14	1.60±0.28	23.26±6.07	24.86±6.08
Muscle	79.33±10.90	0.78±0.18	1.57±0.37	28.39±7.18	29.96±7.19
Ovary	234.57±26.83	0.88±0.16	5.21±0.61	13.52±1.50	18.73±1.62
whole fish	149.51±25.75	0.88±0.10	2.90±0.23	22.67±3.60	25.57±3.61

4.4.2 Observed bioaccumulation patterns of PPCPs

The PCA results are shown graphically in Figure 4.1. For PCA with absolute concentrations in zebrafish tissue, PC1 and PC2 explain 71.5% of the variability in the data. PC1 (explaining 40.6 % variability) was related to correlation of carbamazepine, risperidone, diphenhydramine, triclosan, BPA and fluoxetine, liver and ovary samples and sample protein content. PC2 (explaining 30.8 % of the variability) was the result of correlation between concentrations of diclofenac, ibuprofen, naproxen and gemfibrozil with plasma samples and phospholipid content, coupled with the association of simvastatin concentrations with liver and ovary samples and neutral lipids (Figure 4.1 A).

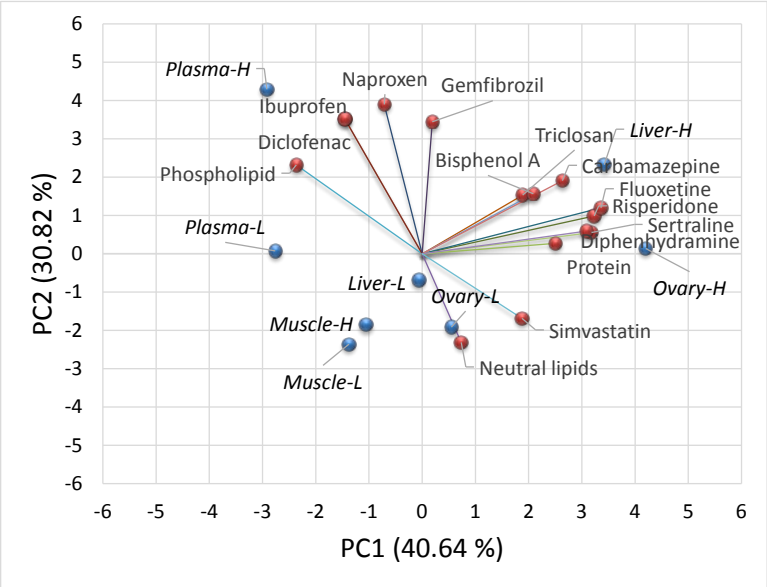
Figure 4.1B illustrates the PCA results when using BCF_{ss} as the response variable, against physicochemical properties. PC 1 explains the majority of the variability (62.1 %) and is driven by close association between triclosan, BPA, sertraline, simvastatin and chemical Log D_{ow}, Log D_{lipw}, demonstrated by strong eigenvectors (> 0.4) and factor loadings (>

0.9) for these co-variables.

The PCA results are consistent with the observed pattern of the various PPCPs in different tissues. Figure 4.2 illustrates plots of the relative distribution (%) of individual PPCPs in plasma, liver, muscle and ovary for the low and high exposure group of zebrafish. As shown in the PCA results, diclofenac, ibuprofen, naproxen and gemfibrozil are shown to have high distribution in plasma, while gemfibrozil, BPA and triclosan are highest in liver tissue. Also, Figure 4.2 shows that fluoxetine, sertraline, carbamazepine, risperidone, diphenhydramine and simvastatin concentrations tend to be highest in ovary.

The PPCPs in Figure 4.2 are ordered lowest to highest hydrophobicity from left to right on the x-axis, ranging from Narpoxen (log D_{ow} = 0.76) to triclosan (log D_{ow} = 5.31). The compounds with log D_{ow} < 1 are shown to be more distributed in plasma, while compounds with log D_{ow} between 1 and 3 are distributed more in ovary, with the exception of gemfibrozil and diclofenac. These two compounds and other PPCPs with logDow>3 (carbamazepine, simvastatin, BPA and triclosan) are distributed more in liver. In addition, the tissue distribution has the similar pattern with D_{pw} distribution.

(A)



(B)

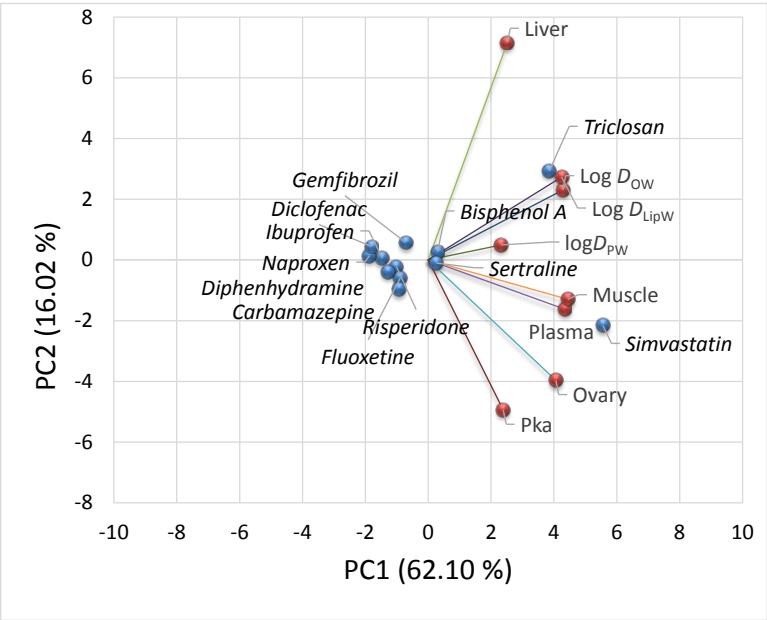


Figure 4.1 Biplots showing loadings (red) and scores (blue) of principal components (PC1 and PC2) for PCA conducted using (A) absolute concentrations or (B) BCF_{ss} of individual PPCPs in different tissues as the observational response variable. Covariables included tissue, neutral lipid, phospholipid and protein content of the tissues, as well as different physicochemical properties (D_{ow}, D_{lipw}, D_{pw}).

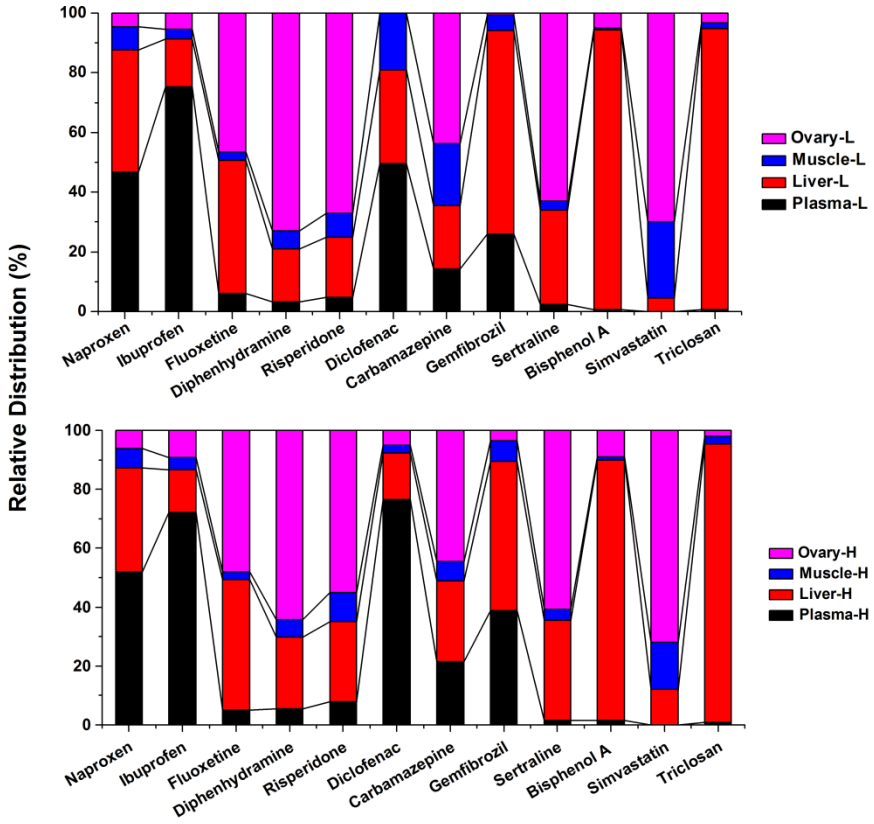


Figure 4.2 Relative distribution (%) of PPCPs in the different tissues of zebrafish for L and H groups.

In the present study, phospholipids are relatively high in plasma (7.66 mg/g) while protein is high in liver tissue (409 mg/g). Ovary has moderate levels of protein (235 mg/g) and phospholipids (0.9 mg/g). In general, acidic PPCPs (e.g., naproxen, ibuprofen) were shown to be more associated with plasma, while basic PPCPs, as well as simvastatin and carbamazepine (neutral compounds) were mostly distributed to ovary. BPA and triclosan (phenolic compounds) were more distributed in liver, which has high protein (409 mg/g) and phospholipids (0.50 mg/g).

4.4.3 Maternal transfer and distribution in eggs

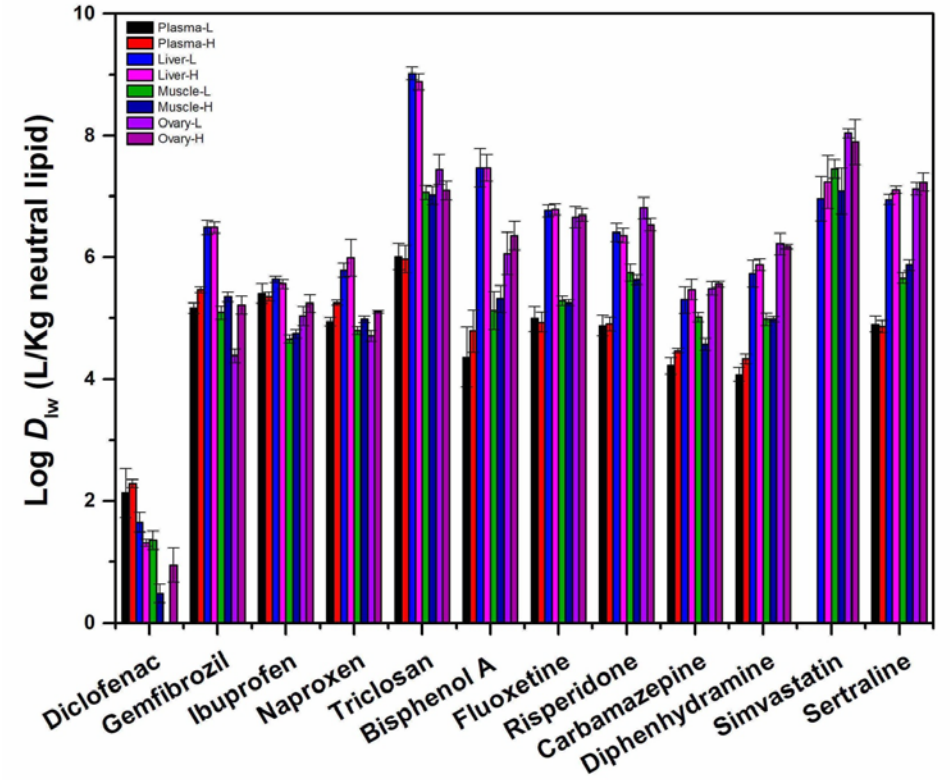
The observed data indicate some PPCPs exhibit a high degree of maternal transfer and accumulation in eggs of zebrafish. In particular, diphenhydramine, risperidone, fluoxetine, sertraline, simvastatin and carbamazepine exhibit high burdens in eggs. Potential reproductive dysfunction of these compounds may be of particular concern for wild fish, considering likely continuous chronic exposure conditions. Organisms are usually more sensitive to contaminants during embryonic development, especially for egg-laying organisms such as fish and birds [21,181]. Maternal transfer is the predominant exposure route for accumulation of contaminants in embryos, which could result in reproductive and early developmental toxicity [21,182-184]. It has been reported that reproductive effects showed in fathead minnows when exposured of low concentrations of EE2. For example, male fish exposed to EE2 at 4 ng/L leaded to secondary sexual characteristics failure and the sex ratio change [6]. A recent study showed chronic histopathological effects in rainbow trout after 28 days of exposure of diclofenac at the concentration ranged from 1 to 500 μg/L [171]. Thus, the degree of accumulation and distribution of PPCPs in eggs may be critical for assessing reproductive effects. More studies are needed to further assess the potential risks of these PPCPs, namely basic pharmaceuticals (e.g., diphenhydramine, fluoxetine, risperidone) that exhibit a high degree of maternal transfer in fish.

4.4.4 Apparent D_{lw}, D_{lipw}, D_{pw} of PPCPs in zebrafish plasma and tissues

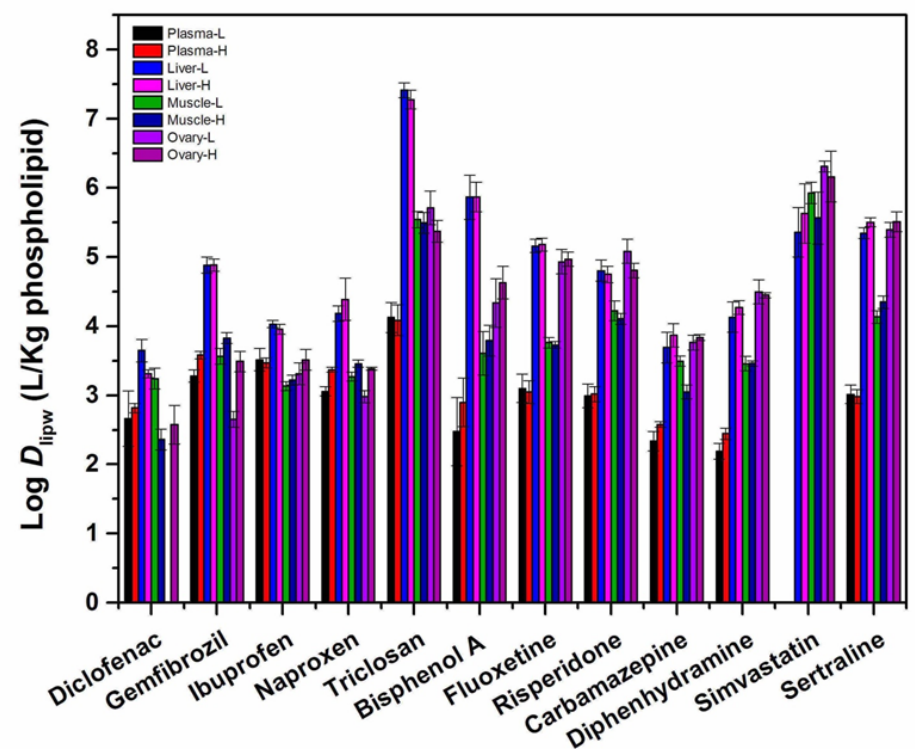
Figure 4.3 A-C illustrates the observed distribution coefficients for the various PPCPs studied, including protein-water (D_{pw}), phospholipid-water (D_{lipw}) and neutral lipid-water

(D_{lw}) in plasma, liver, muscle and ovary for low and high exposure groups. The results indicate that distribution coefficients for the various constituents vary widely among the various PPCPs studied. Not surprisingly, triclosan, BPA, sertraline and simvastatin and carbamazepine, the most hydrophobic of the compounds studied (log D_{ow} 3-5), exhibited the highest partitioning to organic phase (i.e., lipids and proteins) within plasma and liver. However, the D_{lw} values for most of the compounds greatly exceeded the compounds D_{ow} value, indicating that triglycerides and cholesterol do not represent the total amount of lipid like material in zebrafish tissues. In many cases, a given distribution coefficient exhibited significant differences among tissues. For example, log D_{lw} for triclosan varied between approximately 5.9, 7.1 and 9.2 for plasma, muscle and liver.

(A)



(B)



(C)

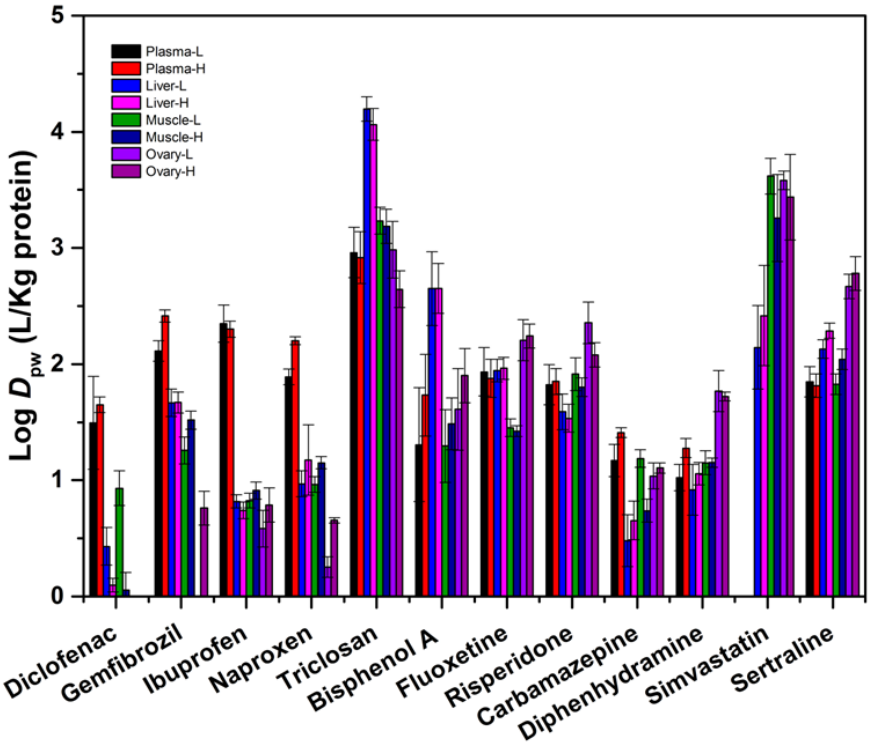


Figure 4.3 Observed steady state distribution coefficients, including (A) lipid-water

distribution coefficient (D_{ow} ± SD), (B) membrane-water distribution coefficient (D_{lipw} ± SD), (C) protein-water distribution coefficient (D_{pw} ± SD).

Protein-water distribution coefficients (log D_{pw}) values ranged between approximately 0.1 and 4.5 for the various compounds. For a given compound, D_{pw} values varied in different tissues, indicating the proteins in different tissues are not equal regarding to protein binding capacity. It is consistent with the findings suggesting IOCs associate strongly with proteins such as serum albumin, liver fatty acid binding protein (LFABP), and organic anion transporter (OAT) proteins mainly in the kidney [114]. Although zebrafish genome does not have albumin, other proteins in the plasma may perform the same function as albumin [185]. Armitage et al. [175] argued that bulk protein fraction in tissues cannot explain the observed tissue distribution of ionogenic perfluoroalkyl acids in organisms, due to the fact that different tissues with similar bulk protein (e.g, liver and muscle) exhibit substantially different concentrations. Essentially, protein-corrected tissue concentrations and hence D_{pw} values tend to differ among tissues with similar protein content.

This assertion seems to hold for the observed distribution of ionogenic PPCPs in zebrafish. For example, protein-corrected concentrations and D_{pw} values of diclofenac, gemfibrozil, ibuprofen, naproxen and carbamazepine in plasma were much higher than those in other tissues. Similarly, protein-corrected concentrations and D_{pw} values of fluoxetine, risperidone, diphenhydramine, sertraline and simvastatin in ovary were much higher than those in other tissues and those of triclosan and BPA in liver were elevated above other compartments. The observed protein corrected concentrations and D_{pw} values of the various PPCPs in zebrafish plasma and tissues indicate that acidic compounds

distributed preferentially in plasma protein, while basic compounds distributed more in ovary protein and neutral compounds were more associated with liver protein.

Figure 4.4 illustrates the apparent D_{lw}, D_{lipw} and D_{pw} for whole-fish, based on observed lipid, phospholipid and protein corrected concentrations, versus theoretical values of those coefficients compiled from the literature and physical-chemical property databases. Apparent D_{lw} are typically > 10 times above lipid-water distribution, based on D_{ow}. Relationships observed for D_{lipw} and D_{pw} are also not well represented. The data suggest that it is not one single constituent is responsible for the sorptive capacity and distribution of these IOCs in zebrafish, but more likely a combined contribution of all constituents (neutral lipids + phospholipids + proteins).

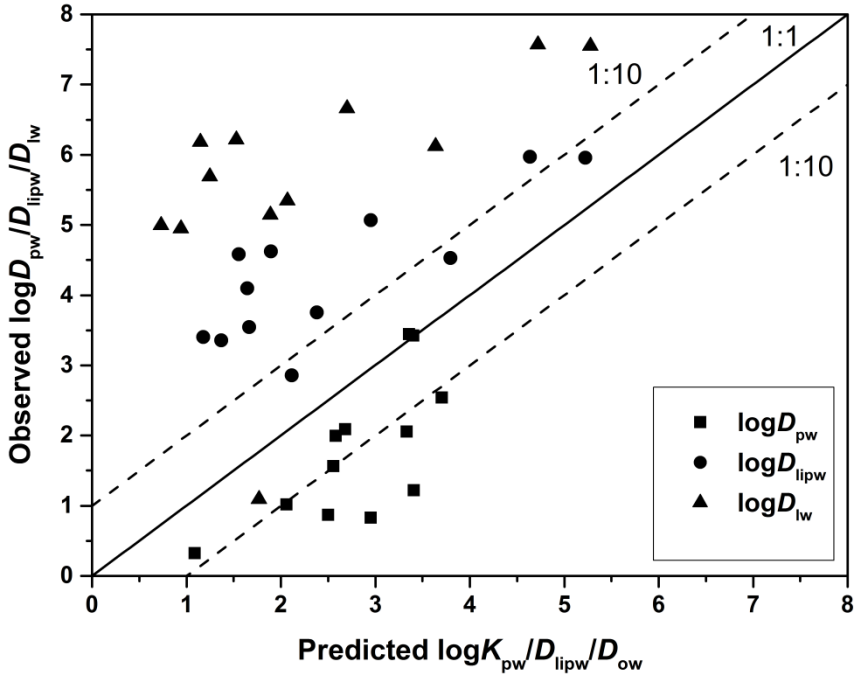


Figure 4.4 Plot observed logD_{pw} / D_{lipw} / D_{lw} in the whole fish versus predicted logK_{pw} / D_{lipw} / D_{ow}. The solid lines represent perfect model agreement 1:1. Dashed lines represent 1log unit interval of predicted concentrations.

4.4.5 Predicted distribution of PPCPs using a mechanistic bioaccumulation model for IOCs

The recently proposed mechanistic model representing bioaccumulation of ionogenic organic contaminants (IOCs) in fish incorporates the contribution of neutral lipids, phospholipids and proteins (denoted as non-lipid organic matter or NLOM in their modelling framework). Figure 4.5 illustrates the predicted distribution (% of total) of individual PPCPs in neutral lipids, phospholipids, proteins and water, following parameterization of the Armitage et al. bioaccumulation with measured protein and lipid content for zebrafish. The model predicted distribution demonstrates the importance of the sorptive capacity of phospholipids for these IOCs, which contribute between 15~90% to the overall sorptive capacity, depending on compartment. However, the distribution varies substantially among the different compounds, with acid, low D_{ow} pharmaceuticals (ibuprofen, naproxen) highly soluble in the aqueous phase of plasma and tissues (> 60%). Also, more hydrophobic PPCPs (e.g., triclosan, BPA, sertraline) are mainly associated with neutral lipids and protein. Figure 4.5 highlights the relative importance of phospholipids in plasma for all the PPCPs, due to the relatively high D_{lipw} values of these compounds and phospholipid content in plasma. Consequently, the D_{lipw} of the compound will likely be important for determining the extent of uptake and steady-state concentrations in plasma.

Figure 4.6 shows a comparison of observed steady state bioconcentration factors (BCF_{ss}, L/kg) versus the model predicted BCF_{ss}, which is assumed equivalent to the biota-water distribution coefficient (D_{BW}, L/kg) proposed in the Armitage et al. model for IOCs bioaccumulation potential. For the majority of compounds, the predicted BCF is

comparable with the observed BCF. Many of the predictions were close to the 1:1 observed vs. predicted line (perfect model agreement), while others were slightly different, generally within a factor of 10. However, in some cases, the model over-predicted the observed BCF. For example, model predicted BCFs of diclofenac and gemfibrozil were 10-20 times higher than the observed BCF_{ss}.

Overall, the relatively good model agreement suggests there is a combined contribution of lipids, protein and phospholipids and water to the sorptive capacity of PPCPs in fish tissues. The hypotheses that protein binding or sorption of phospholipid alone are responsible for distribution of PPCPs does not seem plausible, as removing any of the compartment constituents from the model would greatly underestimate the observed bioaccumulation potential.

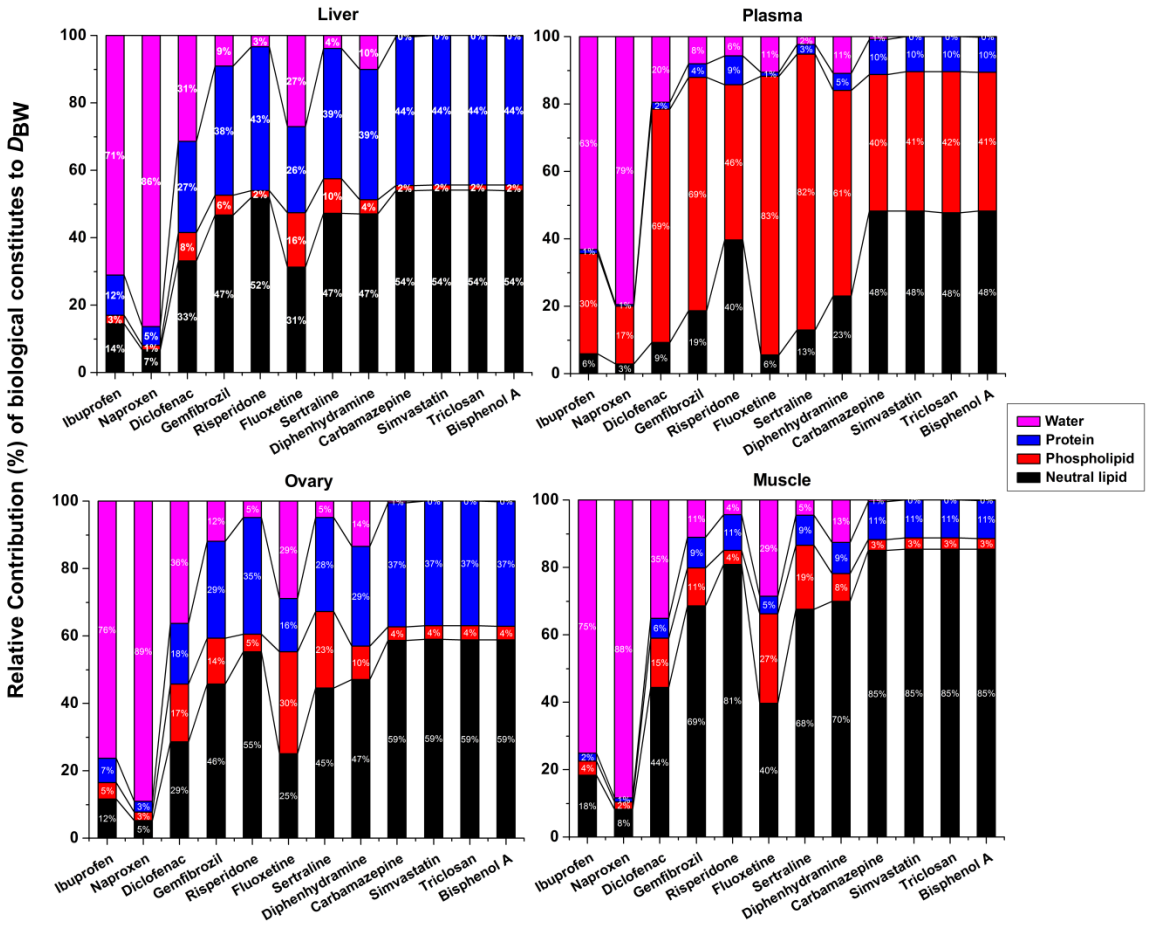


Figure 4.5 Predicted partitioning and distribution (% of total) of individual PPCPs in neutral lipids, phospholipids, proteins and water in zebrafish using the Armitage et al. bioaccumulation model for ionogenic organic contaminants, parameterized using lipid and protein content in zebrafish plasma and tissues.

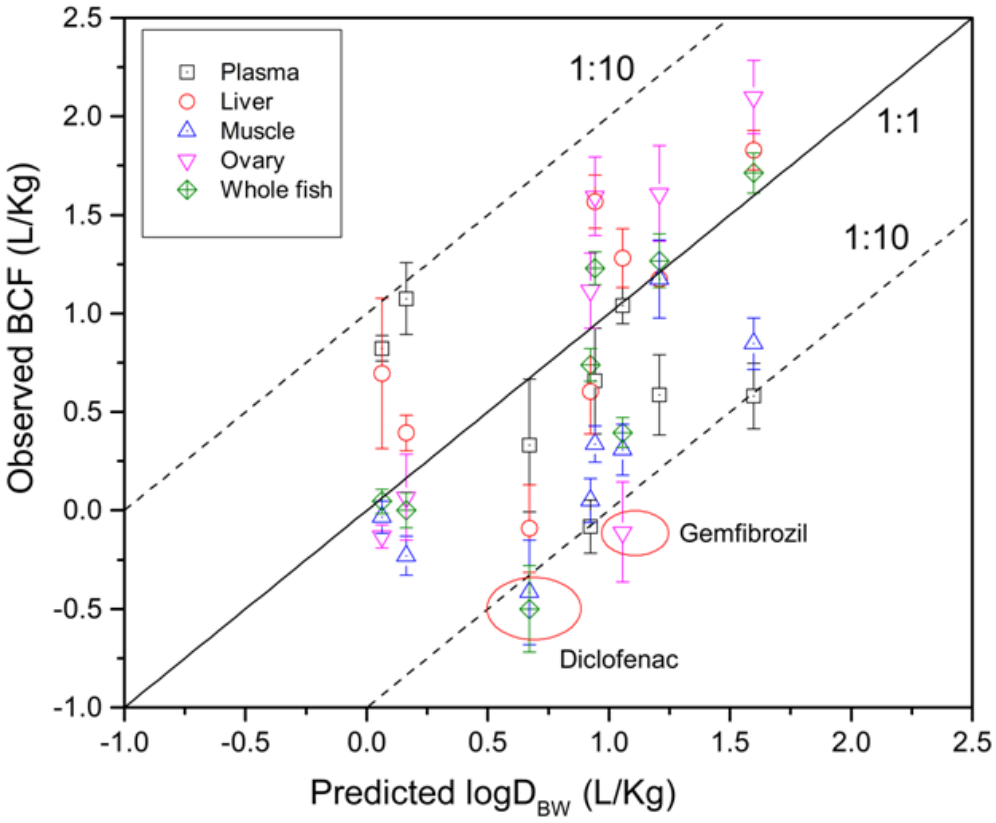


Figure 4.6 Modeled predicted log D_{BW} (L/kg) vs. observed log BCF (log BCF ± SD, L/kg, n=6) in zebrafish plasma, tissues and whole-fish. Model predictions were generated by parameterizing the steady state biota-water distribution equation in the Armitage et al. bioaccumulation model for IOCs.

4.5 Conclusions

Observed steady-state bioconcenration factors (BCF_{ss}) of various pharmaceuticals and personal care products (PPCPs) in zebrafish plasma and tissues exhibited a general increasing trend with increasing lipid-water distribution coefficient (log D_{lw}), membrane-water distribution coefficient (log D_{lipw}) and protein-water (log D_{pw}). Several basic pharmaceuticals (i.e., diphenhydramine, fluoxetine, risperidone) that exhibit a high degree of maternal transfer and preferential accumulation in zebrafish eggs, indicating possible reproductive dysfunction related exposure risks of these compounds in wild fish. Apparent values of D_{pw} / D_{lipw} / D_{lw} of individual PPCPs varied among different tissues

and were generally much higher than theoretical values reported in the literature and physical-chemical property databases. The data suggest that not one single constituent is responsible for the sorptive capacity and distribution of these IOCs in zebrafish, but rather a combined contribution of all constituents (neutral lipids + phospholipids + proteins). Application of a recently developed mechanistic model for bioaccumulation of ionogenic organic contaminants (IOCs) in fish, which incorporates this combined sorptive capacity of biological constituents, demonstrated reasonably accurate prediction of observed steady-state bioconcentration factors (BCF_{ss}) of PPCPs in zebrafish plasma and tissues. While further investigation into the mechanisms governing the bioaccumulation and tissue distribution of ionogenic compounds such as PPCPs, the findings suggest that the IOC bioaccumulation model presented by Armitage et al model may be useful tool for forecasting tissue-specific or whole-body bioaccumulation factors (BAFs) in aquatic ecosystems.

CHAPTER 5 ASSESSING BIOACCUMULATION POTENTIAL OF MONO- AND DI-PERFLUOROALKYL PHOSPHONIC ACIDS (PFPAs) IN ZEBRAFISH (*DANIO RERIO*): COMPARISON TO PERFLUOROCARBOXYLIC ACIDS (PFCAs) AND PERFLUOROSULFONIC ACIDS (PFSAs)

5.1 Introduction

Perfluorinated chemicals (PFCs) are typically amphiphilic and exhibit both lipophilic properties as well as a hydrophilic component, due to the presence of ionizable (anionic) functional groups. These compounds are recalcitrant in the environment and organisms, due in part to the high bond energy (about 110 kcal/mol) of the C-F covalent bond [13,186-188]. Numerous studies have documented the occurrence e and bioaccumulation of perfluorocarboxylic acids (PFCAs) and perfluorosulfonic acids (PFSAs) residues in tissues and biofluids of wildlife and humans worldwide [189-191].

Perfluorooctanoic acid (PFOA, $C_7F_{15}COOH$) and perfluorooctane sulfonic acid (PFOS, $C_8F_{17}SO_3^-$) are two extensively studied PFCs [18,34,192]. In addition to these eight carbon chain PFCs, several studies have been conducted to assess the bioaccumulation behavior of longer chain PFCAs, ranging from C_9 (Perfluoro-n-nonanoic acid, PFNA) to C_{14} (Perfluoro-n-tetradecanoic acid, PFTeA). Bioaccumulation potential of PFCAs and PFSAs has been shown to be highly related to carbon chain length. Bioconcentration factors (BCFs) are relatively low for C_8-C_{11} PFAs (4.0-4,900 L/kg), whereas BCFs of long-chain PFCs (C_{12}-C_{14} PFCAs) are higher (18,000-40,000 L/kg) [79,174].

Compared with PFCAs and PFSAs, studies regarding the bioaccumulation potential of other emerging PFCs of concern, including fluorotelomer acids (FTAs), fluorotelomer sulfonates (FTSs), perfluorosulfonamides (FSOAs) and perfluoroalkyl phosphonic acids (PFPAs), are limited. The latter PFC class includes mono- and di-substituted perfluorinated phosphonic acids (mono-PFPAs and di-PFPAs), high production volume fluorinated surfactants, which have recently been detected at appreciable concentrations in wastewater effluent and surface water [164,193-195]. Based on molecular structure, the calculated octanol-water partition coefficient (log K_{ow}) values of PFPAs ranges between 3.5 and 6.0 for mono-PFPAs and between 7.0 and 11.0 for di-PFPAs (Table 1.5). D'eon et al.[164] investigated uptake and elimination kinetics of mono- and di-PFPAs in Sprague-Dawley rats. To our knowledge there are currently no reports of bioaccumulation potential of mono- and di-substituted PFPAs in fish.

The primary objective of the present study is to examine the uptake and elimination kinetics and tissue-specific bioaccumulation potential of mono- and di-PFPAs in adult female zebrafish (*Danio rerio*). PFCAs and PFSAs were also studied for comparison. The study provides compound specific bioaccumulation kinetic parameters, including uptake, depuration and fecal egestion rate constants (k_u, k_d, k_e), kinetically derived bioconcentration factors (BCF_k) and observed steady-state bioconcentration factors (BCF_{ss}). The studied PFCs vary widely in physicochemical properties, thus relationships between chemical properties and bioaccumulation potential are evaluated and discussed.

5.2 Materials and Methods

5.2.1 Standards and Reagents

Standards of PFOS, PFDA, PFDoA, PFTeDA were purchased from Alfa Aesar (Heysham, Lancs). PFNA, PFTrDA and PFUnDA were purchased from Aldrich (St. Louis, USA). PFOA and PFBA were purchased from Merck (Hohenbrunn, Germany). PFHxA was purchased from TCI-EP (Tokyo, Japan). Other PFCs and isotopically labeled PFCs including PFDPA (C_{10}-mono-PFPA), PFOPA (C_8 mono-PFPA), PFHxPA (C_6 mono-PFPA), Cl-PFHxPA, $^{13}C_8$-PFOA, $^{13}C_4$-PFOA, $^{13}C_4$-PFOS, $^{13}C_2$-PFDA, $^{13}C_2$-PFDoA, $^{13}C_5$-PFNA were purchased from Wellington Laboratories Inc. (Ontario, Canada). Masurf-780, a commercial mixture containing mono- and di-PFPAs, was obtained from Mason Chemical Company. All standards were prepared in methanol and stored at -20^{o}C. HPLC grade methanol was purchased from Fisher Scientific Inc. (Loughborough, UK).

5.2.2 Experimental setup and collection of samples

Adult female zebrafish were purchased from a local fish farm in Singapore (Mainland tropical fish farm, Singapore) and were allowed to acclimate to laboratory conditions for two weeks prior to PFCs exposure. These fish were maintained in dechlorinated water at a temperature of 25 ± 2°C with a 16 : 8 hour light/dark cycle. Bioconcentration experiments were carried out following procedures and protocols outlined in OECD guideline for testing of chemicals [88]. Water and exposed fish were sampled throughout the duration of the experiment to assess chemical uptake and elimination kinetics. The experiment consisted of 24 d uptake phase involving continuous aqueous exposure of a mixture of multiple chemicals, followed by a 24 d depuration phase in clean water.

Separate groups of randomly selected fish were exposed to the test chemicals including a low dose exposure group (300 fish) and a high dose exposure group (300 fish). A separate group (300 fish) kept in clean water served as the control group.

The flow through system was run at least two days before the introduction of the fish to enable stable concentrations of the test chemicals. Water samples were acquired every other day to monitor the stability of contaminant concentrations during the uptake phase. During the experiments fish were fed artemia twice daily. At seven time-points during uptake phase (i.e., 1d, 5d, 10d, 15d, 20d, 22d and 24d) and four time-points during the depuration phase (i.e., 25d, 32d, 40d and 48d) fish (three replicates of 5 pooled individuals) from the treatment groups and control group were sacrificed. Samples of liver, muscle, ovary and plasma were collected and stored at -20°C.

5.2.3 Sample Extraction

For analysis of PFCs in water and zebrafish samples, we followed the approach of Taniyasu et al.[196], which involves simultaneous extraction and analysis of a wide range of perfluorinated compounds in water and biota. For water samples, 50 mL samples were spiked with isotopically labeled surrogate standards prior to solid phase extraction (SPE) with Phenomenex X-AW 33u polymeric weak anion cartridges (200 mg, 6 mL), coupled to a vacuum manifold. SPE cartridges were conditioned with 4 mL of 0.01 N KOH in methanol, followed by 4 mL of methanol and 4 mL of Milli-Q water, with a flow rate of 5 mL/min. Water samples were loaded on the cartridges at a flow rate of 1 drop/s. The cartridges were washed with 4 mL of 25 mM sodium acetate solution, then eluted with 3 mL of 0.01 N KOH in methanol. Extracts were evaporated to dryness under a gentle

nitrogen stream. 200 μL of methanol : Milli-Q water (2 : 3, v/v) was used to rinse and transfer concentrated extracts to polypropylene LC vials. Lastly, 2.45 ng of $^{13}C_8$-PFOA (injection internal standard), used to quantify recovery of internal surrogate compounds, was spiked prior to LC-ESI-MS/MS analysis.

For analysis of tissues, subsamples (liver ~0.1 g; muscle ~0.5 g; ovary ~0.5 g; fecal matter ~0.2 g) were thawed and weighed directly into 15 mL of polypropylene centrifuge tubes. 0.5 mL of Milli-Q water was added, then samples were homogenized via sonication. 5 mL of 0.01 N KOH in methanol was added, along with internal surrogate compounds. Samples were then extracted via sonication for 30 min, then centrifuged at 10000 rpm at 4^{o}C for 5 min. Supernatant was transferred to another 15 mL tube. This process was repeated a second time. Duplicate extracts (10 mL) were combined. A 1 mL aliquot of this extract was diluted with 50mL of Milli-Q water, then extracted via SPE (as above) and spiked with recovery standard ($^{13}C_8$-PFOA, 2.45 ng).

For plasma samples, 20 μL of thawed plasma (obtained from 5 pooled fish) was added to a 2 mL centrifuge tube and spiked with isotopically labeled internal surrogate compounds. 0.5 mL of 0.01 N KOH in methanol was added. Samples were extracted via sonication for 30 min, followed by centrifugation at 12000 rpm at 4^{o}C for 5 min. Supernatant was transferred to another 2 mL tube. This was repeated a second time. The duplicate extracts were combined, evaporated under a gentle nitrogen stream to near dryness and spiked with recovery standard ($^{13}C_8$-PFOA, 2.45 ng).

Separation of target PFCs was performed with a DIONEX Ultimate 3000 UPLC system, using an Agilent Zorbax Eclipse plus C18 column (2.1×50 mm, 3.5 μm). The column was

maintained at a temperature of 40°C. The sample volume injected was 10 μL. The mobile phase used for separation of PFCAs and PFSAs consisted of a binary mixture of solvents A (10 mM ammonium acetate in Milli-Q water) and B (10 mM ammonium acetate in methanol), at a flow rate of 0.3 mL/min. The gradient elution was as follows: initial conditions, 30% B; 0.5-1 min, increase linearly to 50% B; 1-5 min, increase linearly to 100% B; 5-7 min, 100% B; 7-7.2 min, return to initial conditions; 7.2-10 min, 30% B. Separation conditions for PFPAs were as follows: solvent A (10 mM of ammonium acetate in Milli-Q water and spiked with 3 mL of 30% NH_4OH per liter water; solvent B (10 mM of ammonium acetate in methanol and spiked with 3 mL of 30% NH_4OH per liter methanol), at a flow rate of 0.5 mL/min. The gradient elution was as follows: initial conditions, 50% B; 0.5-3 min, increase linearly to 70% B; 3-6.5 min, increase linearly to 90% B; 6.5-8 min, 100% B; 8-8.2 min, return to initial conditions; 8.2-11 min, 50% B.

5.2.4 LC-ESI-MS/MS analysis

The HPLC instrument was coupled to a QTRAP 5500 hybrid triple quadrupole-linear ion trap mass spectrometer (AB SCIEX, Foster City, CA, USA) equipped with a turbo Ion Spray source. Source-dependent parameters were: curtain gas, 10 psi; nitrogen collision gas, high; source temperature was 550 ◦C; ion spray voltage was -4500 V; ion source gases GS1 and GS2 were set at 30 psi. Other compound specific parameters are summarized in Table 5.3.

Identification and quantification of all target analytes and internal surrogate compounds were conducted via tandem mass spectrometry. Ions were acquired in multiple reaction monitoring (MRM) mode, with a dwell time of 25 ms. Optimum precursor and product

ions and MS operating conditions were determined by infusion with a syringe pump. A summary of the monitored MRM transitions and corresponding MS operating parameters is shown in Table 5.3.

An isotope dilution calibration approach was utilized for quantification of target PFCs in sample extracts. Specifically, a series of five calibration standard solutions (CS1-CS5) were prepared, with varied concentrations of the target PFCs (range : ~0.1-100 pg/μL) and a fixed concentration of the internal surrogate compounds. The relative response (RR) for each target analyte was computed for the five calibration solutions. Mean RR values were used for quantification only if the relative standard deviation (RSD) of the five observed RR values was below 20%, thereby indicating good linearity [22]. Because native di-PFPAs standards were not commercially available at the time of this study, we employed a semi-quantification approach similar to D'eon et al. [164], which assumes the monitored mono-PFPAs (which are commercially available and hence used for calibration) produce a similar LC-ESI-MS/MS peak response as the di-PFPAs. While this semi-quantification approach may have limitations for trace determination in environmental samples, the accuracy of the generated concentration data is sufficient for the purposes of this bioaccumulation experiment, which is primarily assessing relative concentrations over time and between tissues.

Relative and absolute recoveries of target analytes and internal surrogates were determined and assessed by spike-recovery trials. Procedural blanks (Milli-Q water) were analyzed to check for any background contamination. Method detection limits (MDLs) were determined by monitoring the signal of target analytes that yielded a signal-to-noise of 3 in extracts. If necessary, PFC residues observed in procedural blanks were subtracted

from the final sample concentration.

5.2.5 Data analysis

Details regarding the data analysis are described previously in Chapter 3.

5.3 Results and Discussion

5.3.1 Toxicological effects of PFCs on zebrafish

Mortality, swimming and feeding behavior, growth rate and liver somatic index (LSI) were closely monitored to assess any toxicological effects throughout the experiments (Table 5.1). No significant fish deaths were observed (1-2 fish deaths per group). No significant differences in growth were observed. The weight of fish from all three groups (control, low and high exposure) increased exponentially, indicating normal growth throughout the experiments. No obvious differences in behavior or swimming pattern were observed. No significant differences were observed between the LSI of treatment and control fish. The results suggest that exposure concentrations used in the study did not interfere with the normal growth pattern and general health condition of zebrafish, which is consistent with previous low-level chronic exposure studies of PFCs in fish [197].

Table 5.1 Uptake and depuration phase duration, growth rate constant, mortality and liver somatic index for exposed and control zebrafish during the bioconcentration experiments.

	Uptake period (d)	**Depuration period (d)**	**Mean initial fish mass (g)**	**Growth rate (g/d) (R^2)**	**Mortality (%)**	**Liver somatic index (%)**
Control	24	24	0.8308	0.0089 (0.84)	0.33	2.56
Low Dose	24	24	0.8032	0.0099 (0.83) (p=0.88)	0.66	2.55 (p=0.95)
High Dose	24	24	0.8090	0.0119 (0.84) (p=0.50)	0.32	2.68 (p=0.51)

5.3.2 Concentrations of PFPAs, PFCAs and PFSAs in water and zebrafish

During the 24 d uptake phase, measured water concentrations of individual target compounds (mean ± SD) ranged between $2.1 \times 10^{-5} \pm 3.4 \times 10^{-5}$ μg/L (6/10 di-PFPA) to 4.5 ± 0.6 μg/L (C_6 mono-PFHxPA) in the low exposure tank and between 0.0018±0.01 μg/L (6/8 di-PFPA) and 17.8 ± 1.3 μg/L (PFBS) in the high exposure tank (Table 5.2). Concentrations were relatively constant throughout the exposure phase. PFCs were not detectable in control tanks or in exposure group tanks during the depuration phase. The growth-corrected uptake and depuration phase concentrations of PFCs in plasma, liver, muscle and ovary of exposed zebrafish are shown in Figure 5.1. The test compounds were detected in all tissues, with the exception of PFBS in muscle tissue of fish in the low-dose exposure group.

Table 5.2 Mean aqueous exposure concentration of selected PFCs (n=36)

Test Compound	Waterborne Concentration (ng/mL) ± SD	
	Low Dose	High Dose
PFBS	2.153±0.281	17.802±1.279
PFHxS	0.687±0.062	8.593±1.532
PFOS	1.115±0.307	15.180±5.066
PFBA	1.205±0.020	6.710±0.422
PFHxA	1.223±0.084	7.023±0.207
PFOA	0.858±0.221	6.644±1.202
PFNA	0.685±0.170	3.627±0.806
PFDA	1.574±0.192	3.470±0.608
PFUnDA	2.030±0.610	14.812±3.833
PFDoDA	1.788±0.670	11.603±5.436
PFTrDA	0.730±0.253	8.874±3.470
PFTeDA	0.150±0.033	3.003±0.832
PFHxPA	4.516±0.606	4.393±0.264
PFOPA	1.605±0.194	6.458±0.475
PFDPA	0.656±0.209	4.179±0.634
6/6 di-PFPA	0.148±0.037	1.577±0.499
6/8 di-PFPA	0.00042±0.00059	0.018±0.011
8/8 di-PFPA	0.00003±0.000045	0.001±0.000
6/10 di-PFPA	0.000021±0.000034	0.001±0.000
8/10 di-PFPA	< MDL	0.001±0.000
6/12 di-PFPA	0.000024±0.000027	0.001±0.000

Table 5.3 MRM transitions and MS parameters of target analytes and internal standards.

Name	Precursor ion	Product ion	Retention Time (min)	Dwell time (ms)	Declustering Potential (V)	Entrance Potential (V)	Collision energy (eV)	Collision cell exit potential (V)
PFBS [a]	299	80	3.22	25	-244	-9	-65	-4
PFBS	299	98.9		25	-244	-9	-37	-5
PFHxS	398.8	79.8	4.16	25	-288	-10	-73	-4
PFOS	498.9	79.9	4.83	25	-275	-8	-100	-4
PFOS	498.9	98.9		25	-275	-8	-86	-5
PFBA	212.9	168.9	1.87	25	-82	-11	-12	-3
PFHxA	312.9	268.9	3.69	25	-110	-14	-12	-5
PFOA	412.9	368.9	4.52	25	-140	-14	-14	-16
PFNA	462.9	418.9	4.86	25	-71	-9	-15	-8
PFDA	512.9	468.9	5.12	25	-71	-5	-15	-6
PFUnDA	562.9	518.8	5.36	25	-155	-11	-16	-10
PFDoDA	612.9	568.9	5.57	25	-59	-10	-18	-7
PFTrDA	662.9	618.9	5.74	25	-115	-11	-18	-8
PFTrDA	662.9	169.1		25	-115	-11	-35	-8
PFTeDA	712.9	668.8	5.89	25	-77	-4	-18	-8
PFTeDA	712.9	168.9		25	-77	-4	-37	-9
^{13}C4-MPFBA	217	171.9	1.82	25	-54	-4	-13	-22
^{13}C2-MPFHxA	315	270.1	3.69	25	-65	-4	-13	-33
^{13}C4-MPFOA	417	371.9	4.52	25	-135	-11	-13	-19
^{13}C5-MPFNA	468	422.9	4.84	25	-79	-4	-15	-24
^{13}C2-MPFDA	515	469.9	5.12	25	-78	-4	-13	-9
^{13}C2-MPFUnDA	565	519.9	5.35	25	-105	-4	-16	-26
^{13}C2-MPFDoA	615	570	5.57	25	-75	-6	-19	-11
^{18}O2-MPFHxS	403	103	4.15	25	-145	-6	-44	-3
^{13}C4-MPFOS	503	80	4.83	25	-185	-6	-90	-4
^{13}C8-MPFOA [b]	421	375.9	4.51	25	-73	-5	-16	-11
PFDPA	598.9	78.9	4.17	25	-174	-10	-40	-8
PFOPA	499	78.9	2.74	25	-166	-10	-40	-4
PFHxPA	399	78.9	0.90	25	-150	-10	-40	-4
6/6 di-PFPA	701	401	5.89	25	-165	-10	-40	-4

6/8 di-PFPA	801	501	6.58	25	-165	-10	-40	-4
8/8 di-PFPA	901	501	7.17	25	-165	-10	-40	-4
6/10 di-PFPA	901	601	7.19	25	-165	-10	-40	-4
8/10 di-PFPA	1001	601	7.82	25	-165	-10	-40	-4
6/12 di-PFPA	1001	701	7.95	25	-165	-10	-40	-4
Cl-PFHxPA [c]	415	78.9	1.02	25	-165	-9	-79	-4

[a] For compounds with two ion transitions, the first MRM transition was used for quantification and the second for confirmation. Only one MRM transition was monitored for internal standards.
[b] $^{13}C_8$-MPFOA, used as injection internal standard.
[c] Cl-PFHxPA, used as internal surrogate standard for mono- and di-PFPAs.

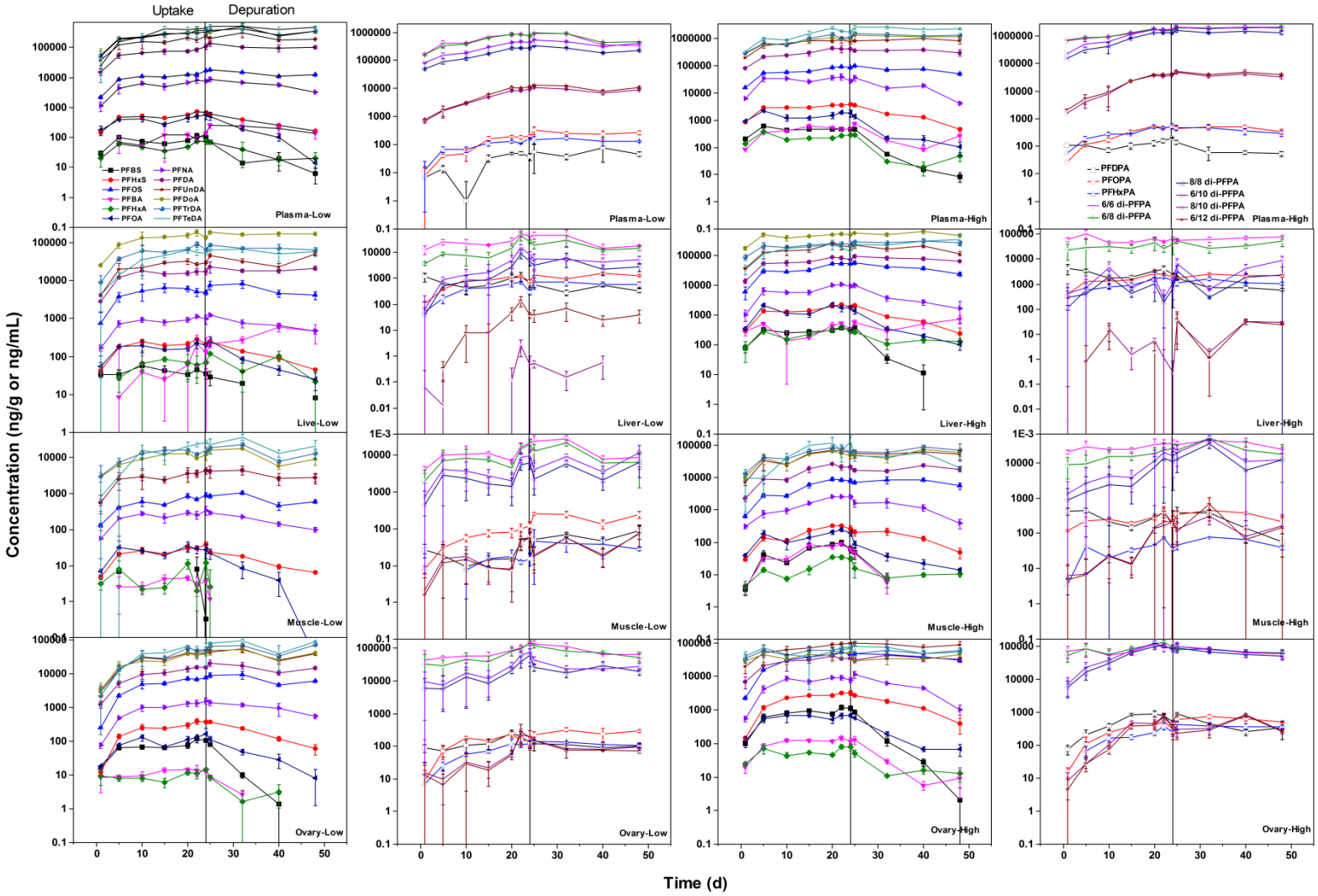


Figure 5.1 Growth-corrected uptake and depuration phase concentrations (n=3, mean ± SD) for PFCs in muscle, liver, ovary and plasma

5.3.2.1 Uptake kinetics

Uptake rates (k_u) of the various PFCs in different tissues ranged by several orders of magnitude, between approximately 0.2 and 1.2×10^9 L/kg/d (Table 5.4). The nonlinear regression (Eq. 3 in Capter 3) was not suitable for determination of k_u in some cases. In general, observed uptake rates of PFCAs and PFSAs were consistent with previously reported k_u values in fish [79]. For many of the PFCs, several days were required before an apparent steady state was achieved. In many cases, k_u differed substantially between the different tissues. For example, k_u values for PFOPA (mono C_8-PFPA) in plasma, liver, muscle and ovary of fish in the low dose group were 5.3 ± 1.6, 82.6 ± 29.9, 3.8 ± 0.7 and 16.9 ± 7.5 L/kg/d, respectively, with highest rates of uptake occurring in liver. For PFOS, k_u values (L/kg/d) for plasma (1917.0 ± 546.6) and liver (1194.0 ± 331.3) were significantly ($p < 0.05$) higher than those in muscle (78.9 ± 25.1) and ovary (494.9 ± 63.4). The observed uptake rates increase with increasing of chain length, which is consistent with previous studies [79].

Previous studies of neutral organic contaminants have demonstrated that k_u is related to gill uptake efficiency (E_W), which is in turn, correlated with chemical K_{ow}. Specifically, k_u and E_W generally increase with increasing hydrophobicity (log K_{ow}'s between 2 to 5), but tend to drop for very hydrophobic compounds (log K_{ow}'s > 7) [198,199]. Erickson et al.[103,104] have previously investigated the governing mechanisms related to uptake and elimination kinetics of ionizable organic chemicals (IOCs) at fish gills. In contrast to neutral hydrophobic compounds, uptake of IOCs can be affected by ambient pH. Further, the acidic pH at gill surfaces may affect the relative amounts of neutral and ionized forms. In terms of chemical flux, ionized molecules may contribute to uptake and elimination by

providing high diffusion gradients of neutral molecules across membranes, as well as permeate membrane barriers. PFCAs, PFSAs and di-PFPAs are anions at neutral pH (pH 7), while mono-PFPAs are dianionic. However, these amphiphilic compounds exhibit relatively long perfluorocarbon tails (typically perfluorinated carbons > 6), which undoubtedly influences the transport kinetics of these compounds across fish gills. In particular, anionic di-PFPAs (> 12 perfluorocarbons) exhibit a high degree of hydrophobicity, with estimated D_{ow} values ranging between 6.05 and 10.2 (Table 1.5).

Table 5.4 Rate of uptake (k_u ± standard error, (r^2)), rate of depuration (k_d ± standard error (r^2)), half-life ($t_{1/2}$ ± standard error), kinetically-derived bioconcentration factor (BCF_k, k_u/k_d) and steady-state bioconcentration factor (BCF_{ss}, C_f/C_w) of PFCs in zebrafish plasma, liver, muscle and ovary for the low dose (L) and high dose (H) treatment groups.

Test Compound	k_u (L/kg/d)				k_d (1/d)				Half-life ($t_{1/2}$, d)				BCF_k (tissues, L/Kg) (plasma, dimensionless)				BCF_{ss} (tissues, L/Kg) (plasma, dimensionless)			
Plasma-L																				
PFBS	23.4	±	14.5	(0.75)	0.194	±	0.003	(0.94)	3.57	±	0.06		120.4	±	74.5		40.0	±	11.4	
PFHxS	186.1	±	60.7	(0.88)	0.108	±	0.003	(0.81)	6.42	±	0.18		1722.9	±	564.0		877.9	±	197.0	
PFOS	1917.0	±	546.6	(0.90)			FF			NA				NA			1.3×10^4	±	4461.2	
PFBA	10.1	±	3.8	(0.85)	0.023	±	0.007	(0.81)	30.14	±	9.17		438.7	±	214.0		99.0	±	16.4	
PFHxA	26.6	±	18.9	(0.69)	0.072	±	0.010	(0.96)	9.63	±	1.34		369.5	±	268.1		60.4	±	4.3	
PFOA	252.5	±	142.4	(0.77)	0.130	±	0.011	(0.99)	5.33	±	0.45		1942.1	±	1107.9		623.3	±	172.7	
PFNA	1653.4	±	459.8	(0.91)	0.032	±	0.007	(0.87)	21.66	±	4.74		5.2×10^4	±	1.8×10^4		1.2×10^4	±	2988.8	
PFDA	8315.2	±	2209.1	(0.91)	0.009	±	0.006	(0.20)	77.02	±	51.34		9.2×10^5	±	6.6×10^5		6.2×10^4	±	1.4×10^4	
PFUnDA	1.4×10^4	±	3673.1	(0.91)			FF			NA				NA			1.1×10^5	±	3.5×10^4	
PFDoA	2.5×10^4	±	4593.4	(0.96)			FF			NA				NA			2.1×10^5	±	7.8×10^4	
PFTrDA	6.3×10^4	±	8109.8	(0.98)			FF			NA				NA			4.5×10^5	±	1.6×10^5	
PFTeDA	2.5×10^5	±	4.3×10^4	(0.97)			FF			NA				NA			2.9×10^6	±	6.8×10^5	
PFHxPA	2.4	±	0.7	(0.91)	0.009	±	0.004	(0.49)	77.02	±	34.23		267.0	±	141.1		30.2	±	9.4	
PFOPA	5.3	±	1.6	(0.94)	0.011	±	0.009	(0.13)	63.01	±	51.56		478.5	±	416.3		121.8	±	15.8	
PFDPA	5.9	±	2.1	(0.92)			FF			NA				NA			67.7	±	25.1	
6/6 di-PFPA	5.4×10^5	±	1.3×10^5	(0.93)	0.046	±	0.017	(0.74)	15.07	±	5.57		1.2×10^7	±	5.2×10^6		5.6×10^6	±	1.5×10^6	
6/8 di-PFPA	1.6×10^8	±	3.6×10^7	(0.94)	0.039	±	0.013	(0.75)	17.77	±	5.92		4.1×10^9	±	1.7×10^9		1.9×10^9	±	2.7×10^9	
8/8 di-PFPA	4.8×10^8	±	1.1×10^8	(0.94)	0.021	±	0.010	(0.52)	33.01	±	15.72		2.3×10^{10}	±	1.2×10^{10}		9.2×10^9	±	1.4×10^{10}	
6/10 di-PFPA	1.2×10^9	±	2.7×10^8	(0.93)	0.019	±	0.010	(0.45)	36.48	±	19.20		6.3×10^{10}	±	3.6×10^{10}		2.2×10^{10}	±	3.5×10^{10}	
8/10 di-PFPA	1.9×10^7	±	3.2×10^6	(0.98)	0.014	±	0.009	(0.26)	49.51	±	31.83		1.4×10^9	±	9.1×10^8		$> 6.0\times10^8$	±	4.4×10^7 [a]	
6/12 di-PFPA	1.3×10^7	±	2.8×10^6	(0.97)	0.013	±	0.011	(0.11)	53.32	±	45.12		9.9×10^8	±	8.7×10^8		4.5×10^8	±	5.0×10^8	
Plasma-H																				
PFBS	19.6	±	0.7	(0.88)	0.129	±	0.004	(0.92)	5.37	±	0.17		151.8	±	7.3		25.7	±	1.9	
PFHxS	123.3	±	31.1	(0.94)	0.047	±	0.003	(0.86)	14.75	±	0.94		2623.5	±	683.0		382.7	±	84.7	
PFOS	774.3	±	180.2	(0.93)			FF			NA				NA			5871.0	±	1972.7	
PFBA	17.2	±	4.4	(0.93)	0.083	±	0.055	(0.44)	8.35	±	5.53		206.8	±	147.1		71.9	±	7.4	
PFHxA	29.1	±	13.6	(0.83)	0.214	±	0.083	(0.92)	3.24	±	1.26		136.0	±	82.6		41.0	±	2.2	
PFOA	237.6	±	153.9	(0.69)	0.251	±	0.055	(0.98)	2.76	±	0.61		946.5	±	647.3		279.2	±	50.9	
PFNA	3543.3	±	1483.2	(0.87)	0.060	±	0.024	(0.72)	11.55	±	4.62		5.9×10^4	±	3.4×10^4		9405.8	±	3039.1	
PFDA	1.2×10^4	±	2819.7	(0.93)	0.008	±	0.004	(0.41)	86.64	±	43.32		1.5×10^6	±	8.2×10^5		1.2×10^5	±	2.1×10^4	
PFUnDA	1.2×10^4	±	1489.5	(0.98)			FF			NA				NA			5.7×10^4	±	1.5×10^4	
PFDoA	1.3×10^4	±	3682.0	(0.89)	0.006	±	0.003	(0.56)	115.52	±	57.76		2.2×10^6	±	1.3×10^6		8.1×10^4	±	3.8×10^4	

PFTrDA	1.6×10^{4}	±	6101.2	(0.82)	0.007	±	0.003	(0.64)	99.02	±	42.44	2.3×10^{6}	±	1.3×10^{6}	1.2×10^{5}	±	4.9×10^{4}
PFTeDA	6.2×10^{4}	±	2.4×10^{4}	(0.83)	0.009	±	0.004	(0.51)	77.02	±	34.23	6.9×10^{6}	±	4.1×10^{6}	6.2×10^{5}	±	1.7×10^{5}
PFHxPA	7.1	±	1.7	(0.94)	0.023	±	0.005	(0.88)	30.14	±	6.55	307.0	±	99.4	115.0	±	15.2
PFOPA	3.4	±	0.8	(0.96)			FF			NA			NA		79.0	±	7.7
PFDPA	4.2	±	2.9	(0.32)	0.080	±	0.029	(0.77)	8.66	±	3.14	52.6	±	40.9	43.3	±	9.4
6/6 di-PFPA	1.2×10^{5}	±	4.3×10^{4}	(0.81)			FF			NA			NA		1.1×10^{6}	±	3.6×10^{5}
6/8 di-PFPA	1.2×10^{7}	±	4.4×10^{6}	(0.79)			FF			NA			NA		9.2×10^{7}	±	5.6×10^{7}
8/8 di-PFPA	5.5×10^{7}	±	1.3×10^{7}	(0.94)			FF			NA			NA		1.3×10^{9}	±	2.8×10^{8}
6/10 di-PFPA	8.6×10^{7}	±	2.0×10^{7}	(0.94)			FF			NA			NA		1.7×10^{9}	±	2.8×10^{8}
8/10 di-PFPA	1.0×10^{6}	±	2.9×10^{5}	(0.95)	0.013	±	0.010	(0.17)	53.32	±	41.01	7.9×10^{7}	±	6.5×10^{7}	3.7×10^{7}	±	1.6×10^{7}
6/12 di-PFPA	1.1×10^{6}	±	2.9×10^{5}	(0.96)			FF			NA			NA		4.0×10^{7}	±	1.5×10^{7}
Liver-L																	
PFBS	32.1	±	20.6	(0.75)	0.233	±	0.006	(0.69)	2.97	±	0.08	137.6	±	88.6	17.6	±	3.5
PFHxS	94.5	±	3.4	(0.90)	0.151	±	0.006	(0.59)	4.59	±	0.18	625.8	±	33.4	365.0	±	75.8
PFOS	1194.0	±	331.3	(0.92)			FF			NA			NA		4410.5	±	1220.8
PFBA	1.0	±	1.4	(0.65)			FF			NA			NA		140.2	±	47.3
PFHxA	8.9	±	3.2	(0.88)			FF			NA			NA		53.8	±	7.1
PFOA	94.4	±	35.0	(0.89)	0.107	±	0.032	(0.89)	6.48	±	1.94	882.3	±	420.2	246.6	±	65.4
PFNA	351.1	±	86.7	(0.94)	0.036	±	0.010	(0.80)	19.25	±	5.35	9751.7	±	3624.8	1525.6	±	421.3
PFDA	2774.6	±	518.5	(0.97)			FF			NA			NA		1.1×10^{4}	±	1338.5
PFUnDA	2740.5	±	505.3	(0.96)			FF			NA			NA		1.4×10^{4}	±	4239.3
PFDoA	1.4×10^{4}	±	2945.1	(0.95)	0.004	±	0.003	(0.12)	173.29	±	129.97	3.6×10^{6}	±	2.8×10^{6}	8.9×10^{4}	±	3.8×10^{4}
PFTrDA	1.3×10^{4}	±	3560.3	(0.92)	0.014	±	0.004	(0.81)	49.51	±	14.15	9.6×10^{5}	±	3.7×10^{5}	1.1×10^{5}	±	4.3×10^{4}
PFTeDA	3.0×10^{4}	±	6537.3	(0.95)			FF			NA			NA		3.6×10^{5}	±	8.1×10^{4}
PFHxPA	13.2	±	7.5	(0.71)	0.011	±	0.003	(0.77)	63.01	±	17.19	1202.0	±	758.4	111.3	±	66.4
PFOPA	82.6	±	29.9	(0.86)			FF			NA			NA		605.9	±	187.2
PFDPA	151.8	±	103.1	(0.53)	0.022	±	0.017	(0.17)	31.51	±	24.35	6901.4	±	7099.0	1426.1	±	736.6
6/6 di-PFPA	2.1×10^{4}	±	1.3×10^{4}	(0.56)	0.049	±	0.021	(0.67)	14.15	±	6.06	4.3×10^{5}	±	3.3×10^{5}	3.2×10^{5}	±	9.9×10^{4}
6/8 di-PFPA	9.5×10^{5}	±	1.1×10^{6}	(0.61)			FF			NA			NA		8.3×10^{7}	±	1.2×10^{8}
8/8 di-PFPA	1.7×10^{6}	±	2.8×10^{6}	(0.58)			FF			NA			NA		2.2×10^{8}	±	3.6×10^{8}
6/10 di-PFPA	4.2×10^{6}	±	6.5×10^{6}	(0.61)	0.017	±	0.011	(0.24)	40.77	±	26.38	2.5×10^{8}	±	4.1×10^{8}	5.5×10^{8}	±	9.2×10^{8}
8/10 di-PFPA			FF				FF			NA			NA		$>1.0\times10^{5}$	±	1.0×10^{5}
6/12 di-PFPA	4.2×10^{4}	±	8.9×10^{4}	(0.37)			FF			NA			NA		3.8×10^{6}	±	5.2×10^{6}
Liver-H																	
PFBS	7.7	±	2.9	(0.89)	0.139	±	0.005	(0.89)	4.99	±	0.18	55.3	±	21.0	18.7	±	2.0
PFHxS	33.0	±	10.3	(0.88)	0.068	±	0.002	(0.94)	10.19	±	0.30	485.3	±	152.6	246.5	±	54.5
PFOS	301.1	±	89.4	(0.90)	0.180	±	0.003	(0.96)	3.85	±	0.06	1673.0	±	497.6	3555.4	±	1188.8
PFBA	95.9	±	148.0	(0.33)			FF			NA			NA		63.8	±	26.6
PFHxA	9.5	±	5.5	(0.68)	0.047	±	0.019	(0.62)	14.75	±	5.96	202.3	±	142.5	49.9	±	10.8
PFOA	133.4	±	111.3	(0.64)	0.185	±	0.026	(0.99)	3.75	±	0.53	721.3	±	610.4	266.3	±	48.4
PFNA	309.0	±	111.7	(0.85)	0.093	±	0.018	(0.94)	7.45	±	1.44	3322.7	±	1362.3	2789.6	±	648.3

PFDA	4022.4	±	1067.0	(0.92)	0.014	±	0.002	(0.94)	49.51	±	7.07	2.9×10^{5}	±	8.7×10^{4}	2.3×10^{4}	±	4413.5
PFUnDA	2053.8	±	631.1	(0.88)	0.015	±	0.012	(0.20)	46.21	±	36.97	1.4×10^{5}	±	1.2×10^{5}	1.4×10^{4}	±	3668.1
PFDoA	2.2×10^{4}	±	6187.3	(0.94)			FF			NA			NA		4.6×10^{4}	±	2.1×10^{4}
PFTrDA	1.6×10^{4}	±	3338.8	(0.93)			FF			NA			NA		2.8×10^{4}	±	1.1×10^{4}
PFTeDA	1.4×10^{4}	±	5359.8	(0.85)			FF			NA			NA		5.5×10^{4}	±	2.0×10^{4}
PFHxPA	21.5	±	7.2	(0.90)			FF			NA			NA		380.9	±	89.2
PFOPA	36.7	±	14.0	(0.83)			FF			NA			NA		338.5	±	47.8
PFDPA			FF		0.095	±	0.033	(0.81)	7.30	±	2.53		NA		771.7	±	248.3
6/6 di-PFPA	1.4×10^{5}	±	4.4×10^{5}	(0.48)			FF			NA			NA		3.7×10^{4}	±	1.4×10^{4}
6/8 di-PFPA	2.2×10^{6}	±	1.3×10^{6}	(0.73)			FF			NA			NA		1.8×10^{6}	±	1.2×10^{6}
8/8 di-PFPA			FF				FF			NA			NA		4.4×10^{5}	±	3.6×10^{5}
6/10 di-PFPA			FF				FF			NA			NA		6.8×10^{5}	±	5.8×10^{5}
8/10 di-PFPA			FF				FF			NA			NA		732.5	±	694.7
6/12 di-PFPA			ND				ND			ND			ND			ND	
Muscle-L																	
PFBS			ND				ND			ND			ND			ND	
PFHxS	7.0	±	2.3	(0.87)	0.233	±	0.007	(0.57)	2.97	±	0.09	30.0	±	10.1	49.0	±	8.4
PFOS	78.9	±	25.1	(0.88)			FF			NA			NA		732.6	±	244.9
PFBA	0.5	±	0.2	(0.89)	1.140	±	0.061	(1.00)	0.61	±	0.03	0.5	±	0.2	2.8	±	0.3
PFHxA			FF		1.527	±	0.011	(1.00)	0.45	±	0.00		NA		5.6	±	5.6
PFOA	16.2	±	9.1	(0.80)	0.142	±	0.017	(0.99)	4.88	±	0.58	113.9	±	65.4	32.8	±	8.5
PFNA	100.9	±	33.0	(0.89)	0.051	±	0.004	(0.98)	13.59	±	1.07	1977.5	±	665.4	434.9	±	142.3
PFDA	353.4	±	119.6	(0.79)			FF			NA			NA		2547.8	±	470.1
PFUnDA	425.5	±	86.9	(0.95)			FF			NA			NA		4342.5	±	1341.0
PFDoA	1176.6	±	300.2	(0.92)			FF			NA			NA		5948.2	±	2343.4
PFTrDA	3648.1	±	862.1	(0.93)			FF			NA			NA		1.9×10^{4}	±	7396.9
PFTeDA	7285.0	±	2252.0	(0.93)			FF			NA			NA		1.7×10^{5}	±	4.1×10^{4}
PFHxPA	0.4	±	0.3	(0.62)	0.020	±	0.004	(0.92)	34.66	±	6.93	21.9	±	17.0	3.0	±	0.6
PFOPA	3.8	±	0.7	(0.97)			FF			NA			NA		67.2	±	12.3
PFDPA	0.2	±	0.2	(0.45)			FF			NA			NA		78.6	±	31.6
6/6 di-PFPA	1.2×10^{4}	±	7717.6	(0.58)	0.049	±	0.029	(0.51)	14.15	±	8.37	2.5×10^{5}	±	2.2×10^{5}	1.3×10^{5}	±	3.9×10^{4}
6/8 di-PFPA	1.7×10^{6}	±	9.8×10^{5}	(0.57)	0.038	±	0.027	(0.33)	18.24	±	12.96	4.5×10^{7}	±	4.1×10^{7}	4.1×10^{7}	±	5.9×10^{7}
8/8 di-PFPA	2.4×10^{6}	±	3.1×10^{6}	(0.38)			FF			NA			NA		2.0×10^{8}	±	3.0×10^{8}
6/10 di-PFPA	5.1×10^{6}	±	6.6×10^{6}	(0.40)	0.049	±	0.029	(0.51)	14.15	±	8.37	1.0×10^{8}	±	1.5×10^{8}	4.3×10^{8}	±	7.1×10^{8}
8/10 di-PFPA	6108.2	±	1.2×10^{4}	(0.62)			FF			NA			NA		$>3.8\times10^{6}$	±	1.6×10^{5}
6/12 di-PFPA	3727.2	±	7017.9	(0.67)			FF			NA			NA		2.2×10^{6}	±	2.5×10^{6}
Muscle-H																	
PFBS	0.3	±	0.2	(0.79)	0.212	±	0.001	(1.00)	3.27	±	0.02	1.5	±	0.7	4.6	±	1.1
PFHxS	2.4	±	0.0	(0.88)	0.152	±	0.008	(0.46)	4.56	±	0.24	16.1	±	0.9	36.0	±	7.8
PFOS	27.4	±	8.8	(0.92)			FF			NA			NA		536.7	±	180.7
PFBA	1.0	±	0.4	(0.84)	0.284	±	0.014	(1.00)	2.44	±	0.12	3.5	±	1.5	11.1	±	3.1

PFHxA	0.2	±	0.1	(0.83)	0.064	±	0.042	(0.35)	10.83	±	7.11	2.5	±	2.1	4.7	±	0.3
PFOA	6.9	±	4.3	(0.68)	0.775	±	0.348	(0.88)	0.89	±	0.40	9.0	±	6.8	32.9	±	8.2
PFNA	33.4	±	7.0	(0.96)	0.050	±	0.019	(0.73)	13.86	±	5.27	668.0	±	290.3	700.5	±	155.7
PFDA	462.5	±	159.8	(0.88)			FF			NA			NA		6113.5	±	1070.7
PFUnDA	385.6	±	129.9	(0.88)			FF			NA			NA		4294.4	±	1200.0
PFDoA	677.4	±	294.3	(0.79)			FF			NA			NA		4543.5	±	2220.6
PFTrDA	964.2	±	308.1	(0.88)			FF			NA			NA		8074.0	±	3362.4
PFTeDA	1803.5	±	949.6	(0.81)	0.054	±	0.042	(0.27)	12.84	±	9.98	3.3×10^{4}	±	3.1×10^{4}	3.3×10^{4}	±	1.7×10^{4}
PFHxPA	1.3	±	1.0	(0.56)	0.039	±	0.014	(0.80)	17.77	±	6.38	34.5	±	27.6	12.5	±	6.0
PFOPA	20.0	±	15.1	(0.64)			FF			NA			NA		46.1	±	22.7
PFDPA			FF		0.059	±	0.027	(0.70)	11.75	±	5.38		NA		68.6	±	38.9
6/6 di-PFPA	2.2×10^{4}	±	1.0×10^{4}	(0.82)	0.032	±	0.016	(0.64)	21.66	±	10.83	6.9×10^{5}	±	4.7×10^{5}	2.2×10^{4}	±	7000.0
6/8 di-PFPA	1.2×10^{5}	±	4.2×10^{4}	(0.81)	0.078	±	0.019	(0.92)	8.89	±	2.16	1.5×10^{6}	±	6.5×10^{6}	1.3×10^{6}	±	8.0×10^{5}
8/8 di-PFPA	1.1×10^{5}	±	8.7×10^{4}	(0.85)			FF			NA			NA		1.2×10^{7}	±	3.2×10^{6}
6/10 di-PFPA	2.2×10^{5}	±	1.4×10^{5}	(0.85)	0.032	±	0.016	(0.64)	21.66	±	10.83	6.9×10^{6}	±	5.6×10^{6}	1.7×10^{7}	±	3.7×10^{6}
8/10 di-PFPA	1069.2	±	824.7	(0.91)	0.078	±	0.019	(0.92)	8.89	±	2.16	1.4×10^{4}	±	1.1×10^{4}	2.1×10^{5}	±	8.8×10^{4}
6/12 di-PFPA	767.2	±	480.2	(0.95)			FF			NA			NA		1.9×10^{5}	±	7.9×10^{4}
Ovary-L																	
PFBS	5.6	±	2.0	(0.84)	0.191	±	0.002	(0.98)	3.63	±	0.04	29.3	±	10.7	52.2	±	7.6
PFHxS	45.9	±	9.8	(0.95)	0.131	±	0.004	(0.78)	5.29	±	0.16	350.1	±	75.7	554.5	±	54.7
PFOS	494.9	±	63.4	(0.98)			FF			NA			NA		6681.3	±	1932.9
PFBA	11.9	±	6.2	(0.78)	0.233	±	0.049	(0.98)	2.97	±	0.63	51.0	±	28.5	11.4	±	0.4
PFHxA	21.8	±	35.1	(0.59)	0.492	±	0.274	(0.88)	1.41	±	0.78	44.3	±	75.4	10.4	±	2.1
PFOA	22.7	±	10.8	(0.76)	0.138	±	0.034	(0.94)	5.02	±	1.24	164.8	±	88.1	175.5	±	54.2
PFNA	163.5	±	23.0	(0.98)	0.036	±	0.005	(0.94)	19.25	±	2.67	4541.5	±	898.3	2149.4	±	581.7
PFDA	721.6	±	62.0	(0.99)	0.015	±	0.011	(0.18)	46.21	±	33.89	4.8×10^{4}	±	3.6×10^{4}	1.0×10^{4}	±	1233.0
PFUnDA	1745.7	±	324.0	(0.96)			FF			NA			NA		2.0×10^{4}	±	6188.5
PFDoA	1698.3	±	381.7	(0.94)			FF			NA			NA		2.1×10^{4}	±	7983.8
PFTrDA	4392.1	±	1300.1	(0.92)			FF			NA			NA		7.0×10^{4}	±	2.4×10^{4}
PFTeDA	3.3×10^{4}	±	1.2×10^{4}	(0.88)			FF			NA			NA		3.1×10^{5}	±	7.2×10^{4}
PFHxPA	1.2	±	0.2	(0.98)	0.012	±	0.001	(0.96)	57.76	±	4.81	104.0	±	18.2	25.5	±	3.5
PFOPA	16.9	±	7.5	(0.82)			FF			NA			NA		92.8	±	11.3
PFDPA	30.6	±	19.8	(0.53)	0.011	±	0.004	(0.69)	63.01	±	22.91	2778.8	±	2062.0	246.2	±	111.1
6/6 di-PFPA	3.7×10^{4}	±	2.0×10^{4}	(0.70)	0.038	±	0.006	(0.93)	18.24	±	2.88	9.7×10^{5}	±	5.4×10^{5}	8.3×10^{5}	±	3.0×10^{5}
6/8 di-PFPA	4.5×10^{6}	±	2.7×10^{6}	(0.82)	0.041	±	0.009	(0.88)	16.91	±	3.71	1.1×10^{8}	±	7.1×10^{7}	2.9×10^{8}	±	4.1×10^{8}
8/8 di-PFPA	2.7×10^{6}	±	2.5×10^{6}	(0.91)			FF			NA			NA		1.7×10^{9}	±	2.6×10^{9}
6/10 di-PFPA	9.6×10^{6}	±	7.8×10^{6}	(0.90)	0.061	±	0.034	(0.46)	11.36	±	6.33	1.6×10^{8}	±	1.5×10^{8}	3.3×10^{9}	±	5.4×10^{9}
8/10 di-PFPA	3.5×10^{4}	±	4.9×10^{4}	(0.80)	0.041	±	0.017	(0.59)	16.91	±	7.01	8.6×10^{5}	±	1.2×10^{6}	$>1.4\times10^{7}$	±	1.2×10^{6}
6/12 di-PFPA	3.0×10^{4}	±	5.8×10^{4}	(0.64)	0.051	±	0.018	(0.70)	13.59	±	4.80	5.9×10^{5}	±	1.2×10^{6}	1.0×10^{7}	±	1.1×10^{7}
Ovary-H																	
PFBS	8.6	±	2.5	(0.90)	0.099	±	0.006	(0.85)	7.00	±	0.42	86.6	±	25.8	66.3	±	5.5

PFHxS	38.0	±	5.3	(0.98)	0.520	±	0.002	(0.93)	1.33	±	0.01	73.0	±	10.3	381.2	±	68.1
PFOS	272.3	±	35.7	(0.98)			FF			NA			NA		3208.6	±	1123.0
PFBA	4.2	±	0.8	(0.96)	0.163	±	0.043	(0.95)	4.25	±	1.12	25.8	±	8.5	19.8	±	3.3
PFHxA	5.7	±	4.1	(0.68)	0.225	±	0.117	(0.84)	3.08	±	1.60	25.3	±	22.3	11.5	±	0.4
PFOA	31.4	±	7.6	(0.95)	0.148	±	0.016	(0.99)	4.68	±	0.51	212.1	±	56.3	103.6	±	18.8
PFNA	387.1	±	103.9	(0.92)	0.061	±	0.023	(0.76)	11.36	±	4.28	6346.6	±	2936.8	2355.2	±	589.0
PFDA	1778.3	±	302.3	(0.96)			FF			NA			NA		9835.0	±	1730.1
PFUnDA	750.6	±	128.2	(0.96)			FF			NA			NA		6437.4	±	1673.4
PFDoA	3954.3	±	1608.4	(0.85)			FF			NA			NA		4456.2	±	2088.1
PFTrDA	5933.3	±	2677.9	(0.83)			FF			NA			NA		8159.1	±	3195.5
PFTeDA	2.4×10^{4}	±	1.4×10^{4}	(0.72)	0.021	±	0.008	(0.63)	33.01	±	12.57	1.1×10^{6}	±	8.1×10^{5}	2.2×10^{4}	±	6742.8
PFHxPA	3.8	±	1.7	(0.83)	0.007	±	0.002	(0.84)	99.02	±	28.29	536.4	±	292.3	73.0	±	25.4
PFOPA	5.4	±	2.3	(0.85)			FF			NA			NA		67.3	±	27.0
PFDPA	18.3	±	9.3	(0.77)	0.064	±	0.022	(0.77)	10.83	±	3.72	285.4	±	174.7	156.7	±	51.6
6/6 di-PFPA	9.9×10^{4}	±	8.4×10^{4}	(0.65)	0.026	±	0.004	(0.94)	26.66	±	4.10	3.8×10^{6}	±	3.3×10^{6}	6.1×10^{4}	±	2.1×10^{4}
6/8 di-PFPA	4.0×10^{6}	±	2.8×10^{6}	(0.66)	0.015	±	0.005	(0.72)	46.21	±	15.40	2.6×10^{8}	±	2.1×10^{8}	6.5×10^{6}	±	4.1×10^{6}
8/8 di-PFPA	4.4×10^{6}	±	1.8×10^{6}	(0.87)	0.027	±	0.005	(0.93)	25.67	±	4.75	1.6×10^{8}	±	7.2×10^{7}	9.4×10^{7}	±	2.7×10^{7}
6/10 di-PFPA	5.8×10^{6}	±	2.4×10^{6}	(0.84)	0.023	±	0.003	(0.96)	30.14	±	3.93	2.5×10^{8}	±	1.1×10^{8}	9.3×10^{7}	±	1.5×10^{7}
8/10 di-PFPA	2.3×10^{4}	±	2.0×10^{4}	(0.63)			FF			NA			NA		5.9×10^{5}	±	3.9×10^{5}
6/12 di-PFPA	1.8×10^{4}	±	1.6×10^{4}	(0.62)			FF			NA			NA		5.4×10^{5}	±	3.6×10^{5}

[a] Calculated by concentration at steady state divided by MDL as water concentration determined lower than MDL

FF: Fitting failed

NA: Not available

ND: Not detected in the tissue (<MDL)

5.3.2.2 Elimination kinetics

The observed rate of depuration and half-lives of individual PFCs is shown in Table 5.4. Half-lives ranged between approximately 0.5 to 173 days for the various PFCs, which is consistent with previous reports for PFCAs and PFSAs [79]. The fastest elimination was observed for short-chain PFCAs and PFSAs (PFBS, PFHxS, PFBA, PFHxA). The half-life for PFBA and PFHxA in plasma in the high dose group was 8.35 ± 5.53 d and 3.24 ±1.26 d, respectively. Conversely, $t_{1/2}$ for PFDA and PFDoA in muscle of those fish was 86.64 ± 43.32 and 115.52 ± 57.76 days. Comparatively, mono- and di-PFPAs exhibited relatively long half-lives, in many cases longer than PFOS and long-chain PFCAs. For example, the plasma clearance half-lives for mono-PFHxPA (C_6), mono-PFOPA (C_8), PFOA (C_8) and PFNA (C_9) in the low dose group were 77.0 ± 34.2, 63.0 ± 51.6, 5.33 ± 0.45 and 21.66 ± 4.74 d, respectively. The half-life for di-PFPAs in the same tissue were comparable, ranging between 15.07 ± 5.57 (6/6 di-PFPA) to 53.32 ± 45.12 days. The observed half-lives of mono- and di-PFPAs in zebrafish in the present study were much longer than those reported previously for rats, which were 0.96 ± 0.11 to 2.8 ± 0.5 d for the mono-PFPAs and 1.8 ± 0.1 to 9.3 ± 1.5 d for the di-PFPAs [164]. The data suggest short chain PFCAs and PFSAs exhibit a high degree of respiratory elimination via gills, which is anticipated for these more water soluble compounds [55,79,84]. Long-chain PFCAs, PFSAs, mono- and di-PFPAs exhibited the longest half-lives, on the order of several days to several weeks.

All the PFCs were detected in zebrafish fecal material collected during the depuration phase (> day 24), (Figure 5.2). The calculated fecal egestion rate constant values (k_e) for the low dose group was generally greater than those for the high dose group (Table 5.5).

At day 47, the low dose group almost finished clearance through feces, whereas the high dose group still exhibited relatively high PFC concentrations in feces. The fecal egestion rate constants were lower than the depuration rate constants in tissues, suggesting that other clearance processes such as respiratory exchange are more dominant elimination routes compared to fecal egestion.

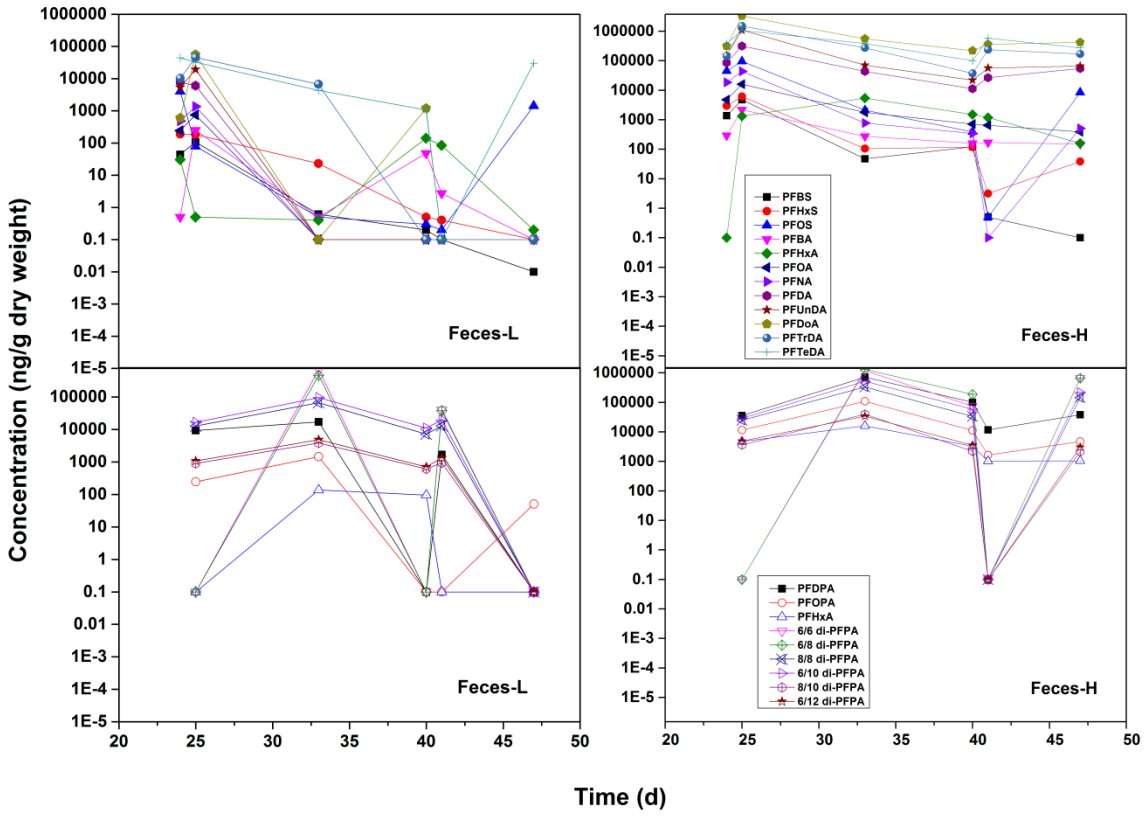


Figure 5.2 Concentration (ng/g dry weight) of PFCs in the feces collected during the depuration phase (> day 24).

Table 5.5 Rate of fecal egestion k_e (mean ± standard error) in depuration phase. Coefficient of determination (r^2) is shown in parentheses.

Test Compound	k_e (1/d) Low Group	k_e (1/d) High Group
PFBS	0.623 ± 0.013 (0.99)	0.565 ± 0.181 (0.44)
PFHxS	0.269 ± 0.014 (0.99)	0.502 ± 0.084 (0.99)
PFOS	-	0.472 ± 0.299 (0.99)
PFBA	0.625 ± 2.241 (0.93)	0.225 ± 0.039 (0.98)
PFHxA	-	-
PFOA	0.685 ± 0.079 (0.99)	0.258 ± 0.024 (0.99)
PFNA	0.685 ± 0.079 (0.99)	0.501 ± 0.059 (0.99)
PFDA	0.685 ± 0.079 (0.99)	0.217 ± 0.066 (0.94)
PFUnDA	0.685 ± 0.079 (0.99)	0.323 ± 0.075 (0.99)
PFDoA	0.680 ± 0.396 (0.99)	0.188 ± 0.039 (0.96)
PFTrDA	0.251 ± 0.016 (0.99)	0.181 ± 0.041 (0.95)
PFTeDA	-	0.078 ± 0.032 (0.60)
PFHxPA	-	-
PFOPA	-	-
PFDPA	-	-
6/6 di-PFPA	-	-
6/8 di-PFPA	-	-
8/8 di-PFPA	-	-
6/10 di-PFPA	-	-
8/10 di-PFPA	-	-
6/12 di-PFPA	-	-

5.3.2.3 Observed BCFs in plasma, muscle, liver, ovary and whole body

Tissue-specific and whole-body BCF_{ss} values for individual PFCs are summarized in Table 5.6. BCF_{ss} values, which represent apparent steady-state concentration ratios between biota and water, are in general agreement with the corresponding BCF_k values, determined as k_u/k_d. However, BCF_{ss} values for longer chain PFCAs and the mono- and di-PFPAs were substantially higher (10-100 times higher) than the estimated BCF_k values (Figure 5.3). BCF_{ss} values of individual PFCs ranged widely, between 2.8 and 2.2×10^{10}. For PFCAs, the BCF_{ss} values of PFBA, PFHxA and PFOA were low compared to PFCAs

with chain length > 8 (i.e., PFNA, PFDA, PFUnDA, PFDoA, PFTrDA and PFTeDA). Similarly, BCF_{ss} for PFHxS, PFBS (chain length ≤ 6) were low compared to those for PFOS (C_8), which were typically 10 fold higher. For PFPAs, the log BCF_{ss} values for di-PFPAs, having chain length ≥ 12, were 4 ~ 5 log unit greater than those mono-PFPA (C_6, C_8, C_{10}). The data indicate that for the same carbon chain length, the bioaccumulation potential is ordered PFSAs > PFCAs > mono-PFPAs. Further, the data from the present study indicate that di-PFPAs are more bioaccumulative than PFCAs with the same chain length. Similarly, Martin et al. [79] observed a BCF of PFOS was 2 ~ 3 log unit higher than that of the corresponding C_8 PFAs (PFOA). Differences in observed bioaccumulation potential between PFC classes have previously been attributed to different ionic interactions among functional groups [113].

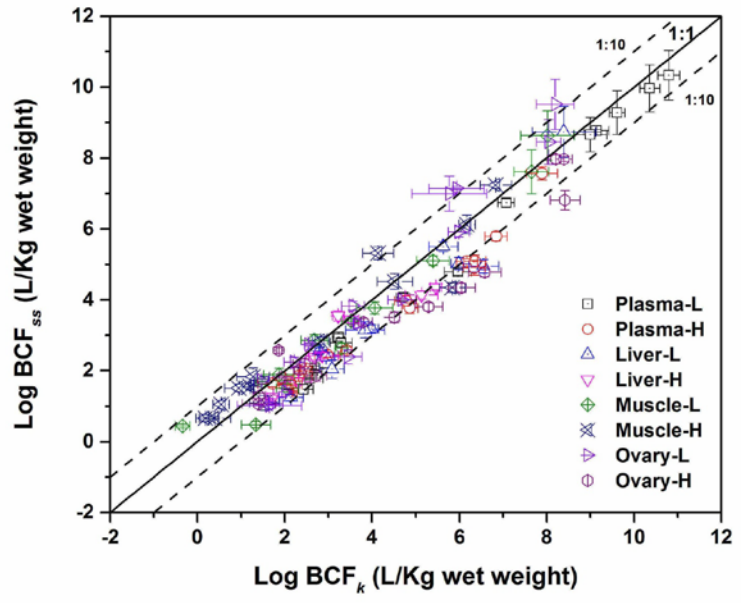


Figure 5.3 Observed (BCF ± Standard deviation) versus predicted Log BCF_k (i.e., calculated by first-order model, BCF ± Standard error) in different tissues. The solid lines represent perfect model agreement 1:1. Dashed lines represent 1log unit interval of predicted concentrations. X axis error bar represents the standard error of predicted log BCF_k and y axis error bar represents the standard deviation of observed log BCF.

Table 5.6 Observed steady-state bioconcentration factor (BCF_{ss} ± standard deviation) of individual perfluorinated compounds in zebrafish plasma, liver, muscle, ovary and whole body.

	BCF_{ss} Plasma (L/Kg)		**BCF_{ss} Liver (L/Kg)**		**BCF_{ss} Muscle (L/Kg)**		**BCF_{ss} Ovary (L/Kg)**		**BCF_{ss} Whole body (L/Kg) [a]**	
	Low Exposure Group (L)	**High Exposure Group (H)**	**Low Exposure Group (L)**	**High Exposure Group (H)**	**Low Exposure Group (L)**	**High Exposure Group (H)**	**Low Exposure Group (L)**	**High Exposure Group (H)**	**Low Exposure Group (L)**	**High Exposure Group (H)**
PFBS	40.0 ± 11.4	25.7 ± 1.9	17.6 ± 3.5	18.7 ± 2.0	-	4.6 ± 1.1	52.2 ± 7.6	66.3 ± 5.5	19.7 ± 2.6	27.3 ± 2.0
PFHxS	877.9 ± 197.0	382.7 ± 84.7	365.0 ± 75.8	246.5 ± 54.5	49.0 ± 8.4	36.0 ± 7.8	554.5 ± 54.7	381.2 ± 68.1	251.3 ± 23.2	171.6 ± 30.6
PFOS	1.3×10^4 ± 4461	5871±1972	4410.5 ± 1220	3555 ± 1188	732.6 ± 244.9	536.7 ± 180.7	6681± 1932	3208 ± 1123	3138 ± 868	1679 ± 562
PFBA	99.0 ± 16.4	71.9 ± 7.4	140.2 ± 47.3	63.8 ± 26.6	2.8 ± 0.3	11.1 ± 3.1	11.4 ± 0.4	19.8 ± 3.3	13.3 ± 1.0	17.3 ± 1.5
PFHxA	60.4 ± 4.3	41.0 ± 2.2	53.8 ± 7.1	49.9 ± 10.8	5.6 ± 5.6	4.7 ± 0.3	10.4 ± 2.1	11.5 ± 0.4	10.2 ± 1.6	9.6 ± 0.4
PFOA	623.3 ± 172.7	279.2 ± 50.9	65.4	266.3 ± 48.4	32.8 ± 8.5	32.9 ± 8.2	175.5 ± 54.2	103.6 ± 18.8	99.6 ± 26.0	71.5 ± 13.0
PFNA	1.2×10^4 ± 2.9×10^3	9405 ± 3039	1525 ± 421.3	2789 ± 648.3	434.9 ± 142.3	700.5 ± 155.7	2149 ± 581.7	2355± 589.0	1211 ± 303.7	1475.9 ± 330.7
PFDA	6.2×10^4 ± 1.4×10^4	1.2×10^5 ± 2.1×10^4	1.1×10^4 ± 1338	2.3×10^4 ± 4413	2547 ± 470.1	6113 ± 1070	1.0×10^4 ± 1233	9835± 1730	6218 ± 764.0	9436 ± 1653
PFUnDA	1.1×10^5 ± 3.5×10^4	5.7×10^4 ± 1.5×10^4	1.4×10^4 ± 4.2×10^3	1.4×10^4 ± 3.6×10^3	4342 ± 1341	4294 ± 1200	2.0×10^4 ± 6.2×10^3	6437 ± 1673	1.2×10^4 ± 3.5×10^3	6065± 1,570
PFDoA	2.1×10^5 ± 7.8×10^4	8.1×10^4 ± 3.8×10^4	8.9×10^4 ± 3.8×10^4	4.6×10^4 ± 2.1×10^4	5948 ± 2343	4543 ± 2220	2.1×10^4 ± 7983	4456.± 2088	1.7×10^4 ± 6.5×10^3	7261 ± 3406
PFTrDA	4.5×10^5 ± 1.6×10^5	1.2×10^5 ± 4.9×10^4	1.1×10^5 ± 4.3×10^4	2.8×10^4 ± 1.1×10^4	1.9×10^4 ± 7396	8074 ± 3362	7.0×10^4 ± 2.4×10^4	8159 ± 3195	4.6×10^4 ± 1.6×10^4	1.0×10^4 ± 4.0×10^3

PFTeDA	2.9×10^{6} ± 6.8×10^{5}	6.2×10^{5} ± 1.7×10^{5}	3.6×10^{5} ± 8.1×10^{4}	5.5×10^{4} ± 2.0×10^{4}	1.7×10^{5} ± 4.1×10^{4}	3.3×10^{4} ± 1.7×10^{4}	3.1×10^{5} ± 7.2×10^{4}	2.2×10^{4} ± 6742.8	2.6×10^{5} ± 5.7×10^{4}	3.7×10^{4} ± 1.1×10^{4}
PFHxPA	30.2 ± 9.4	115.0 ± 15.2	111.3 ± 66.4	380.9 ± 89.2	3.0 ± 0.6	12.5 ± 6.0	25.5 ± 3.5	73.0 ± 25.4	16.3 ± 2.6	52.3 ± 5.4
PFOPA	121.8 ± 15.8	79.0 ± 7.7	605.9 ± 187.2	338.5 ± 47.8	67.2 ± 12.3	46.1 ± 22.7	92.8 ± 11.3	67.3 ± 27.0	102.2 ± 13.0	67.7 ± 8.5
PFDPA	67.7 ± 25.1	43.3 ± 9.4	1426.1 ± 736.6	771.7 ± 248.3	78.6 ± 31.6	68.6 ± 38.9	246.2 ± 111.1	156.7 ± 51.6	201.1 ± 66.4	132.6 ± 23.6
6/6 di-PFPA	5.6×10^{6} ± 1.5×10^{6}	1.1×10^{6} ± 3.6×10^{5}	3.2×10^{5} ± 9.9×10^{4}	3.7×10^{4} ± 1.4×10^{4}	1.3×10^{5} ± 3.9×10^{4}	2.2×10^{4} ± 7000.0	8.3×10^{5} ± 3.0×10^{5}	6.1×10^{4} ± 2.1×10^{4}	4.4×10^{5} ± 1.2 × 10^{5}	4.8×10^{4} ± 1.5×10^{4}
6/8 di-PFPA	1.9×10^{9} ± 2.7×10^{9}	9.2×10^{7} ± 5.6×10^{7}	8.3×10^{7} ± 1.2×10^{8}	1.8×10^{6} ± 1.2×10^{6}	4.1×10^{7} ± 5.9×10^{7}	1.3×10^{6} ± 8.0×10^{5}	2.9×10^{8} ± 4.1×10^{8}	6.5×10^{6} ± 4.1×10^{6}	1.5×10^{8} ± 2.1×10^{8}	4.1×10^{6} ± 2.5×10^{6}
8/8 di-PFPA	9.2×10^{9} ± 1.4×10^{10}	1.3×10^{9} ± 2.8×10^{8}	2.2×10^{8} ± 3.6×10^{8}	4.4×10^{5} ± 3.6×10^{5}	2.0×10^{8} ± 3.0×10^{8}	1.2×10^{7} ± 3.2×10^{6}	1.7×10^{9} ± 2.6×10^{9}	9.4×10^{7} ± 2.7×10^{7}	8.2×10^{8} ± 1.3 × 10^{9}	5.4×10^{7} ± 1.2×10^{7}
6/10 di-PFPA	2.2×10^{10} ± 3.5×10^{10}	1.7×10^{9} ± 2.8×10^{8}	5.5×10^{8} ± 9.2×10^{8}	6.8×10^{5} ± 5.8×10^{5}	4.3×10^{8} ± 7.1×10^{8}	1.7×10^{7} ± 3.7×10^{6}	3.3×10^{9} ± 5.4×10^{9}	9.3×10^{7} ± 1.5×10^{7}	1.7×10^{9} ± 2.7×10^{9}	6.2×10^{7} ± 9.9×10^{6}
8/10 di-PFPA	> 6.0×10^{8} ± 4.4×10^{7}	3.7×10^{7} ± 1.6×10^{7}	>1.0×10^{5} ± 1.0×10^{5}	732.5 ± 694.7	>3.8×10^{6} ± 1.6×10^{5}	2.1×10^{5} ± 8.8×10^{4}	>1.4×10^{7} ± 1.2×10^{6}	5.9×10^{5} ± 3.9×10^{5}	>1.4×10^{7} ± 2.7×10^{5}	7.2×10^{5} ± 3.1×10^{5}
6/12 di-PFPA	4.5×10^{8} ± 5.0×10^{8}	4.0×10^{7} ± 1.5×10^{7}	3.8×10^{6} ± 5.2×10^{6}	-	2.2×10^{6} ± 2.5×10^{6}	1.9×10^{5} ± 7.9×10^{4}	1.0×10^{7} ± 1.1×10^{7}	5.4×10^{5} ± 3.6×10^{5}	9.8 × 10^{6} ± 1.1 × 10^{7}	7.3×10^{5} ± 2.8×10^{5}

[a] The whole body burden was calculated by the sum of tissue burden of plasma, liver, muscle and ovary time wet weight of plasma, liver, muscle and ovary, respectively. The whole body BCF was calculated by the whole body burden divided by the sum of wet weight of plasma, liver, muscle and ovary. The average weight of plasma, liver, muscle and ovary per fish are 0.0045g, 0.02g, 0.25g and 0.15g, respectively in this study, i.e. whole body BCF_{ss} = (C_{plasma}*0.0045 + C_{liver}*0.02 + C_{muscle}*0.25 + C_{ovary}*0.15) / (0.0045 + 0.02 + 0.25 + 0.15).

5.3.2.4 Concentration dependence

In the present study water concentrations in low and high dose groups differed by a factor of 5 to 50. In some cases, the observed BCF for a given compound was concentration dependent, as BCF values in the low dose group was significantly higher ($p < 0.05$) than those for the high dose group. BCFs in low dose group exceeded those in high group for long-chain PFCAs (PFDoA, PFTrDA, PFTeDA) and di-PFPAs in liver. For example, BCF_{ss} of PFTeDA in liver of low dose group fish was approximately 7 times higher than those in the high dose group. The difference between low and high dose group was even more pronounced for di-PFPAs, as BCF_{ss} were 10-800 times higher in low dose group compared to the high dose group. However, in other cases, BCF values did not significantly differ ($p > 0.05$) between the two aqueous exposure concentrations, which typically differed by a factor of 5 to 50 for the various PFCs. Liu et al. [105] recently demonstrated that exposure concentration greatly affected the BCF of PFCAs and PFSAs in bivalves exposed to 1 μg/L (low dosage) and 10 μg/L (high dosage). They observed that the sensitivity of the BCF divergence between low dose and high dose groups was positively related to carbon chain length and binding affinity of the compounds. The authors consequently proposed that PFC bioaccumulation may best be described by a nonlinear adsorption mechanism, rather than equilibrium partitioning.

5.3.2.5 Influence of physical-chemical properties

BCF values of individual PFCs exhibited a strong relationship with carbon chain length (Figure 5.4) and hydrophobicity (Figure 5.5). Specifically, positive linear relationships were observed between BCF_{ss} versus carbon chain length, log K_{ow} and log D_{ow}, with

correlation coefficients (r^2) ranging between 0.70 and 0.99. The BCF_{ss}-log D_{ow} and BCF_{ss}-log K_{ow} relationships exhibited comparable correlation coefficients ($r^2 > 0.9$). Martin et al. [79] observed similar relationships with carbon chain length and hydrophobicity for PFCAs and PFSAs.

The data in Figure 5.4 and Figure 5.5 illustrate that di-PFPAs are substantially more bioaccumulative than the mono-PFPAs. Whole-body BCF_{ss} of di-PFPAs (C_{12}-C_{18}) ranged between 5×10^4 and 2×10^9, while mono-PFPA BCF_{ss} values ranged only between 16 and 200. The observed differences in bioaccumulation behavior of mono-PFPAs and di-PFPAs is likely due to the fact that mono-PFPAs are di-anionic under standard environmental and physiological conditions (e.g., pH 7), thus increasing the water solubility of those compounds.

Figure 5.5 shows that BCF_{ss} drop slightly for the very hydrophobic compounds, 8/10 di-PFPA and 6/12 di-PFPA, which are very high molecular weight, hydrophobic compounds. Quantitative structure activity relationships (QSARs) for bioconcentration factor determination, developed primarily from empirical data for neutral hydrophobic compounds, predict a drop in BCFs decreases for high molecular weight, high K_{ow} compounds, typically exhibited for compounds with log $K_{ow} > 6$ [61,67]. The data from the present study suggests that BCFs of perfluorinated compounds increase linearly, then may drop only slightly for high molecular weight compounds having log $D_{ow} > 8$.

Péry et al.[200] recently presented a physiologically based toxicokinetic (PBTK) model for predicting uptake and distribution of organic chemicals in zebrafish. An important parameter in this model is the blood-water partition coefficient (λ_{bw}). For $\lambda_{bw,}$ the authors

utilized a regression equation, $\log(\lambda_{bw}) = 0.78.\log K_{ow} - 0.82$, which was derived from empirical blood : water partitioning data for a set of structurally diverse organic compounds [201-203]. Figure 5.6 illustrates a plot showing a comparison between observed log BCF_{ss} in zebrafish plasma (this study) and log λ_{bw} values for the various PFCs. The data show that the zebrafish PBTK model parameter, log λ_{bw}, works well to predict the observed plasma-water distribution (i.e., log BCF_{ss}) for many of the studied PFCs. However, the observed log BCF_{ss} of PFDPA was substantially lower than the predicted log λ_{bw} value for this mono-substituted perfluorinated phosphonic acid. Further, for several compounds (e.g., PFOS, 6,6/di-PFPA, 6/8 di-PFPA, 8/8 di-PFPA and 6/10 di-PFPA), the observed log BCF_{ss} in zebrafish plasma was 1 to 3 log units higher than the corresponding log λ_{bw} value from the zebrafish PBTK model.

The regression equation for λ_{bw} in the zebrafish PBTK model was derived based on blood-water partitioning data for several classes of organic compounds, including chloroethanes, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, phenols, as well as organochlorine and organophosphate pesticides, which ranged in log K_{ow} between approximately 1.5 to 8 [200]. The poor prediction of log BCF_{ss} for many PFCs in zebrafish plasma using λ_{bw} may be due to an underestimation of the partitioning and/or binding of these compounds in phospholipids, proteins and organic anion transporters [98,108,109,114,175,204]. Thus, more accurate information regarding PFC-specific liposome-water and protein-water partition coefficients (K_{lipw}, K_{pw}), along with phospholipid and protein contents may be required to enable better prediction of steady-state blood-water distribution of these compounds.

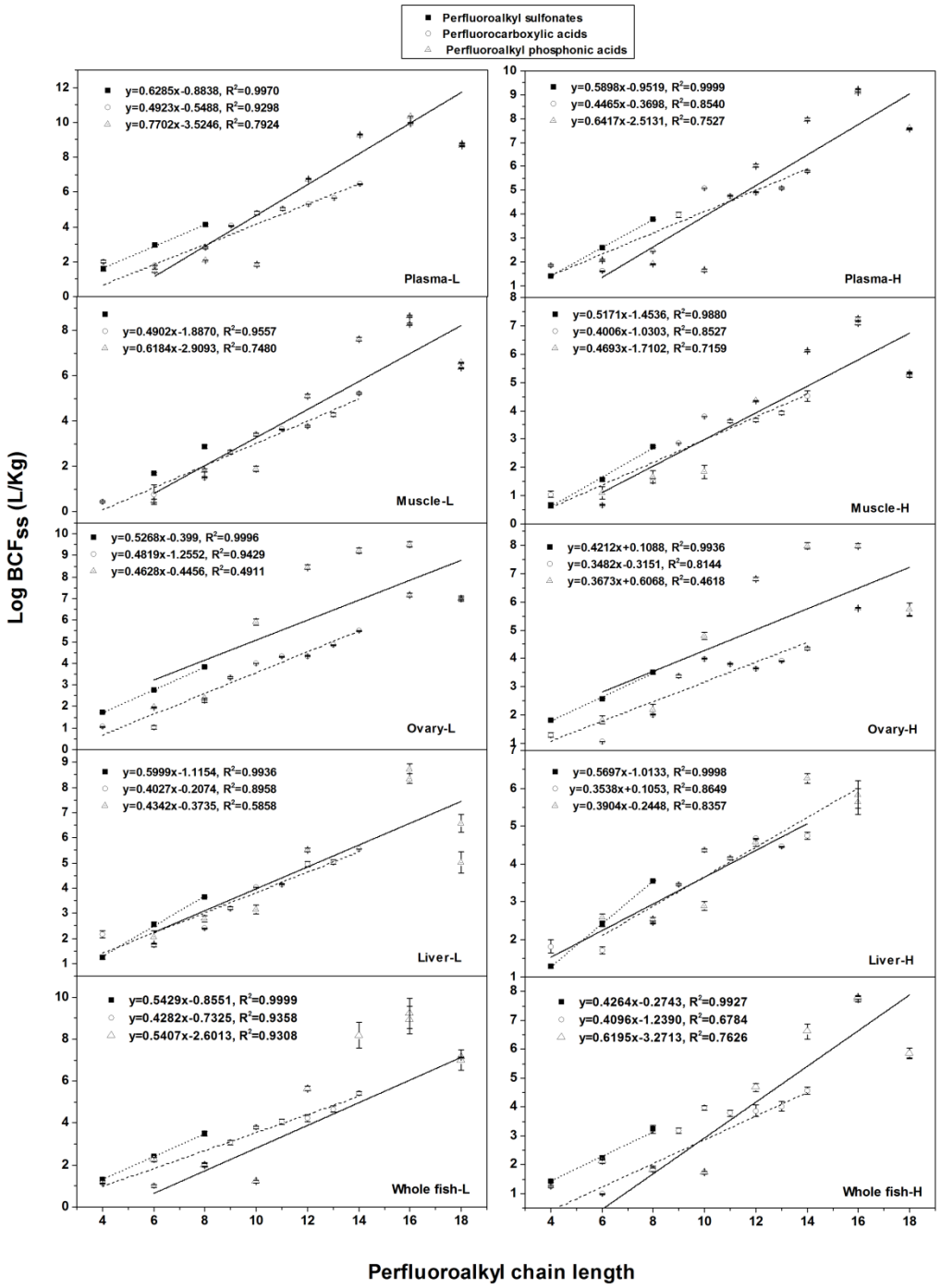


Figure 5.4 Relationships between LogBCF_{ss} and perfluoroalkyl chain length for plasma, liver, muscle, ovary and whole fish. Linear regression was applied and the resulting equations and coefficients of determination (R^2) are shown. Dotted line represents regressions for perfluoroalkyl sulfonates, dashed line represents regressions for perfluorocarboxylic acids and solid line represents regressions for perfluoroalkyl phosphonic acids.

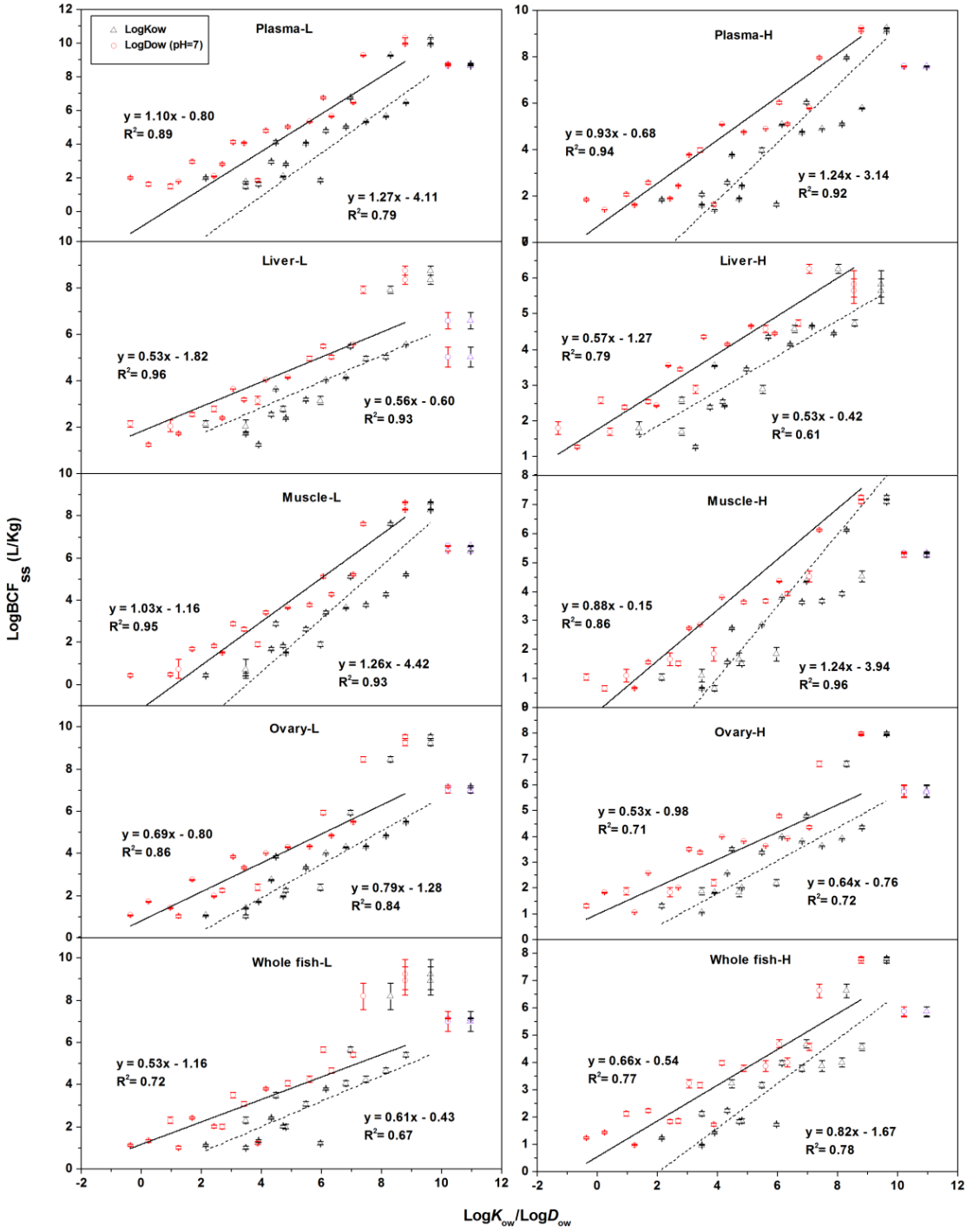


Figure 5.5 Relationship between log BCF_{ss} versus Log K_{ow} and Log D_{ow} for liver, plasma, muscle, ovary and whole fish for low dose group (L) and high dose group (H). Linear regressions do not include 8/10 di-PFPA and 6/12 di-PFPA data.

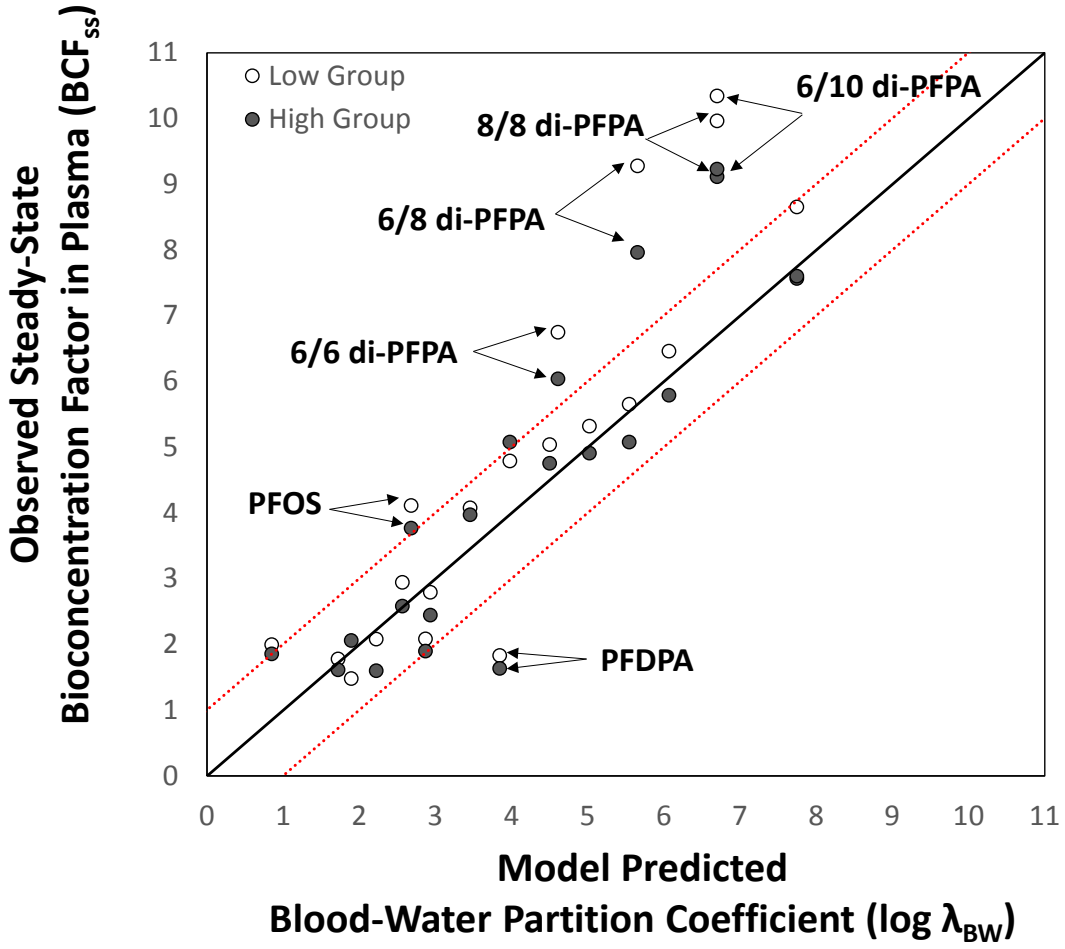


Figure 5.6 Relationship between observed steady-state bioconcentration factor (log BCF_{ss}) of individual perfluorinated compounds in zebrafish plasma (this study) and the chemical's predicted blood-water partition coefficient (λ_{bw}) from a physiologically based toxicokinetic model for zebrafish.

5.4 Conclusions

Overall, the results of this 48 day exposure of multi-class PFCs (PFCAs, PFSAs, mono- and di-PFPAs) in zebrafish demonstrate that bioaccumulation potential varies widely among test compounds and between different tissues. The di-substituted PFPAs exhibited an extremely high degree of bioaccumulation in zebrafish plasma and tissues. Both

mono- and di-PFPAs exhibited relatively long half-lives. Plasma clearance half-lives for mono- and di-PFPAs ranged between approximately 9 and 77 days, which is comparable to PFOS and long chain PFCAs. The di-anionic mono-PFPAs exhibited substantially lower BCFs, as compared to the more hydrophobic anionic di-PFPAs. To our knowledge this is the first report of uptake and elimination rates, half-lives and BCFs for mono- and di-PFPAs in fish. The results may help to improve understanding and forecasting of the bioaccumulation behaviour and exposure risks of these perfluorinated acids in aquatic systems.

CHAPTER 6 BIOACCUMULATION BEHAVIOR OF IONOGENIC PERFLUORINATED COMPOUNDS IN FISH: ASSESSING THE ROLE OF PROTEINS AND PHOSPHOLIPIDS

6.1 Introduction

In recent years, there has been increasing focus on perfluorinated compounds (PFCs) due to observations of their high degree of persistence, bioaccumulation potential and potential biological impacts in wildlife and humans [9,186-188,205-208]. Several field and laboratory based studies demonstrated the bioaccumulation potential of PFCs [17,18,21,79,84]. The majority of studies have focused on assessing the bioaccumulation potential and tissue distribution of perfluoroalkyl acids, including perfluorocarboxylic acids (PFCAs) and perfluorosulfonic acids (PFSAs), and the two major commercial PFCs, perfluorooctanoic acid (PFOA, $C_7F_{15}COOH$) and perfluorooctane sulfonic acid (PFOS, $C_8F_{17}SO_3^-$). In general, bioaccumulation potential of PFCAs and PFSAs with longer fluorinated carbon chains (>7 fluorinated carbons) are orders of magnitude higher than short-chain PFCs [79,174]. Observed bioconcentration factors (BCFs) of long-chain PFCAs (C_{12}-C_{14} PFCAs) range between 18,000-40,000 L/kg [79].

Information regarding the bioaccumulation of other PFC such as fluorotelomer acids (FTA), fluorotelomer sulfonates (FTS), perfluorosulfonamides (FSOA) and perfluoroalkyl phosphonic acids (PFPAs) are more limited. Further, a full mechanistic understanding and predictive models describing PFC bioaccumulation behavior are also lacking. Toxicokinetic studies indicate PFCs have a high affinity for plasma protein such as albumin thus tends to accumulate in the blood, liver, and kidney [55,79,209]. Some

studies have shown PFC tissue concentrations on a wet wt. basis are positively correlated with tissue protein content, indicating that theoretical or empirically derived protein-water partition coefficients (K_{pw}) may be a good predictor of internal concentrations, much like chemical K_{ow} is used for determination of lipophilic organic compounds [112,174]. However, other studies have observed no correlation between PFC concentration and tissue protein and find vastly different protein corrected concentrations in liver and muscle tissue [210].

The current state of knowledge regarding ionogenic organic contaminants (IOCs) such as PFCs indicates bioaccumulation behavior is likely related to partitioning of these relatively polar compounds in phospholipids and/or binding with proteins and organic anion transporters [98,105,108,109,114,174,175]. Consequently, two key parameters inherent to those proposed mechanisms are the compounds liposome-water partition coefficient (D_{lipw}) and protein-water distribution coefficient (D_{pw}).

The objective of the present study was to examine the role of protein and phospholipids in the bioaccumulation, tissue distribution and maternal transfer of ionogenic PFCs in zebrafish (*Danio rerio*). The study aims to provide new information regarding the influence of phospholipids and proteins on PFC bioaccumulation behavior. Specifically, the study involves (i) determination of apparent lipid-water, phospholipid-water and protein-water distribution coefficients of multiple PFCAs, PFSAs and PFPAs in zebrafish plasma and tissues (D_{lw}, D_{lipw}, D_{pw}) and (ii) a comparison of observed steady state bioconcentration factors (BCF_{ss}) of individual PFCs with predicted BCF using a recently proposed mechanistic bioaccumulation model for ionogenic organic contaminants. The relationship between physicochemical properties, plasma and tissue protein and lipid

composition and bioaccumulation behavior is explored and evaluated. The findings may benefit for future efforts to assess the bioaccumulation, tissue distribution and chronic exposure risks of PFCs in fish.

6.2 Theoretical Section

Details regarding the theoretical section are described previously in Chapter 4.

6.3 Experimental Section

6.3.1 Bioaccumulation experiments

Details regarding the experimental setup for flow-through bioaccumulation studies and chemical analysis are described previously in Chapter 5.

6.3.2 Determination of protein, phospholipid and neutral lipid content in plasma and tissues

Details regarding determination of total protein, phospholipids, triglycerides and cholesterol in zebrafish plasma and tissues are previously provided in Chapter 4.

6. 3.3 Data analysis

Details regarding data analysis are previously provided in Chapter 4.

6.4 Results and Discussion

6.4.1 Protein and lipid measurements

Measured protein, phospholipid, cholesterol and triglyceride concentrations (mg/g) are

presented previously in Chapter 4. Protein concentrations were highest in liver and ovary. Phospholipids were highest in plasma, relative to other tissues. Total neutral lipids (cholesterol + triglycerides) was highest in muscle tissue, followed by liver > ovary > plasma.

6.4.2 Observed bioaccumulation patterns of PFCs

The PCA results are shown graphically in Figure 6.1. For PCA with absolute concentrations in zebrafish plasma and tissue samples (Figure 6.1 A), PC1 explained the majority of the variability (71.9%). PC1 was represented by strong correlation of the majority of PFCs, which are shown to be highly related to plasma samples. Phospholipid content exhibited relatively high Pearson's *r* values (> 0.7) for correlation with those PFCs. PFBS and mono-PFPAs (PFHxPA, PFOPA, PFDPA) were exceptions and are shown to cluster separately from the other PFCs (Figure 6.1 A). Figure 6.1 B illustrates the PCA results when using BCF_{ss} as the response variable, against physicochemical properties. Together, PC 1 and PC 2, explains the majority of the variability (83.7 %). PC 1 is related to elevated 6/10 and 8/8 di-PFPA in tissue samples, while PC 2 involves strong correlation between PFCAs and PFSAs and chemical Log D_{ow}, Log D_{lipw} and Log D_{pw}, demonstrated by strong eigenvectors (> 0.4) and factor loadings (> 0.7) for these co-variables.

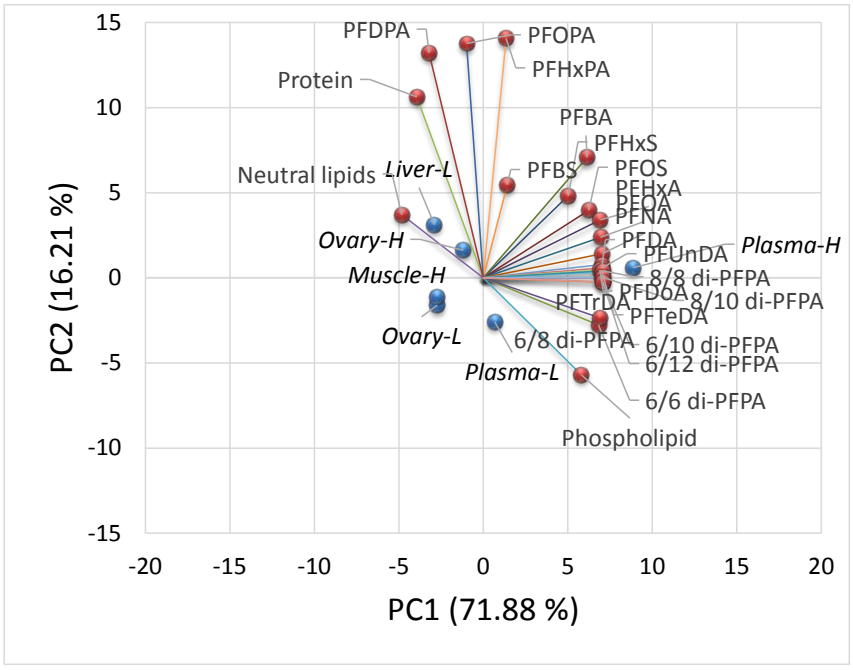
The PCA results are consistent with the observed pattern of the various PFCs in different tissues. Figure 6.2 illustrates plots of the relative distribution (%) of individual PFCs in plasma, liver, muscle and ovary for the low and high exposure group of zebrafish. As shown in the PCA results, the majority of the PFCs have a high distribution in plasma,

with the exception of PFBS and mono-PFPAs (PFHxPA, PFOPA, PFDPA), which tend to be higher in liver. In general, the distribution of the studied PFCs was ordered: plasma > liver > ovary > muscle, which is consistent with PFCs distribution in rainbow trout [79].

In the present study, phospholipids are relatively high in plasma (7.66 mg/g) while protein is high in liver tissue (409 mg/g). Ovary has moderate levels of protein (235 mg/g) and phospholipids (0.9 mg/g). The data for PFCAs and PFSAs in

Figure 6.2 indicates that with increasing hydrophobicity (log D_{ow} range between 0.25 and 7.08), there is an increased distribution in plasma coupled with a reduced distribution in ovary and liver. Mono-PFPAs (log D_{ow}'s between 0.96 and 3.89) exhibit very low plasma concentrations, relative to liver and ovary, while di-PFPAs are predominantly distributed in plasma and to a lesser extent ovary.

(A)



(B)

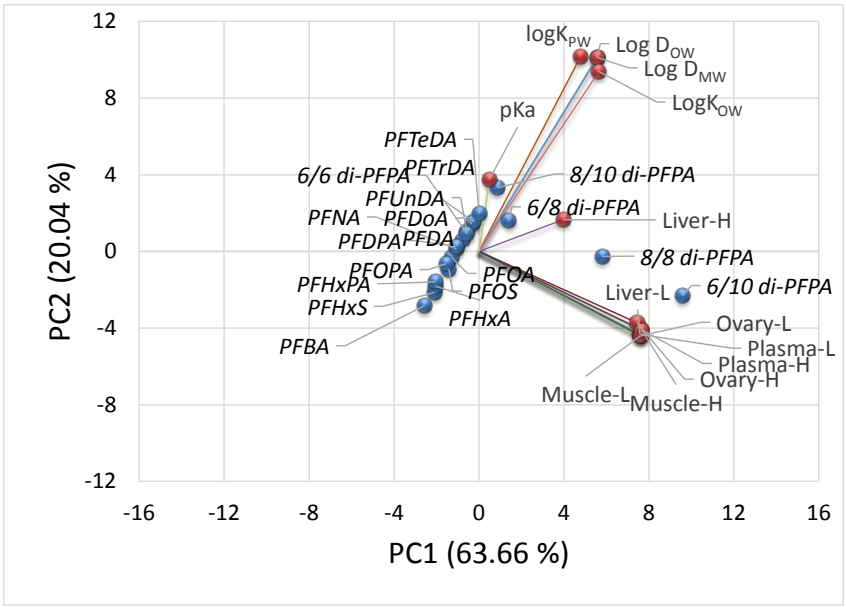


Figure 6.1 Biplots showing loadings (red) and scores (blue) of principal components (PC1 and PC2) for PCA conducted using (A) absolute concentrations or (B) BCF_{ss} of individual PFCs in different tissues as the observational response variable. Covariables included tissue, neutral lipid, phospholipid and protein content of the tissues, as well as different physicochemical properties (D_{ow}, D_{mw}, D_{pw}).

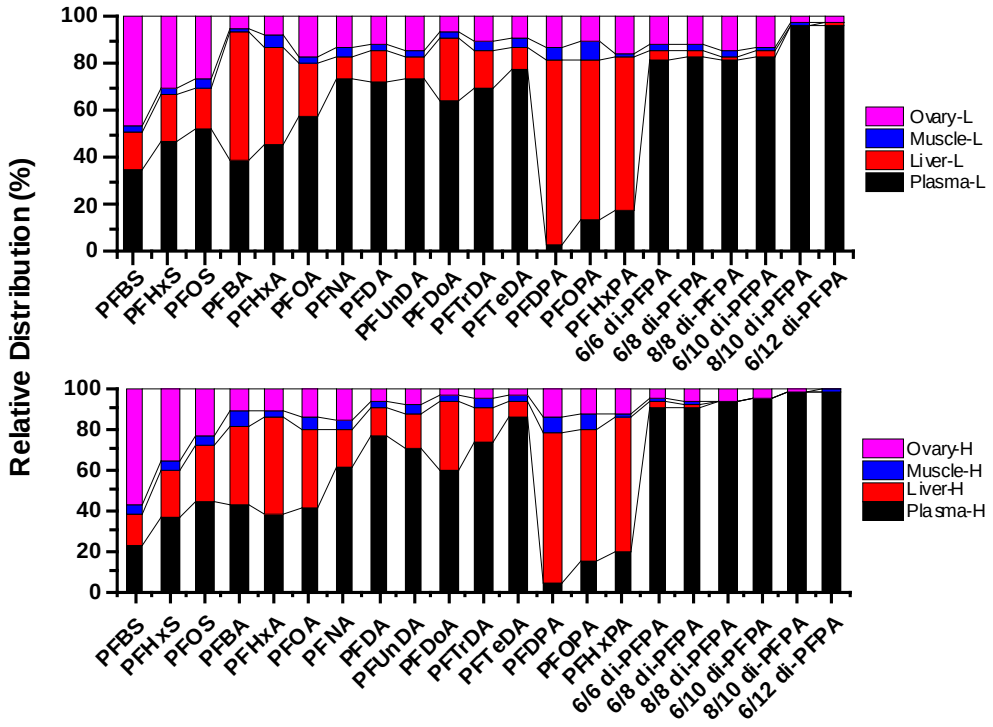


Figure 6.2 Relative distribution (%) of the studied PFCs in the different tissues of zebrafish for low (L) and high (H) exposure groups.

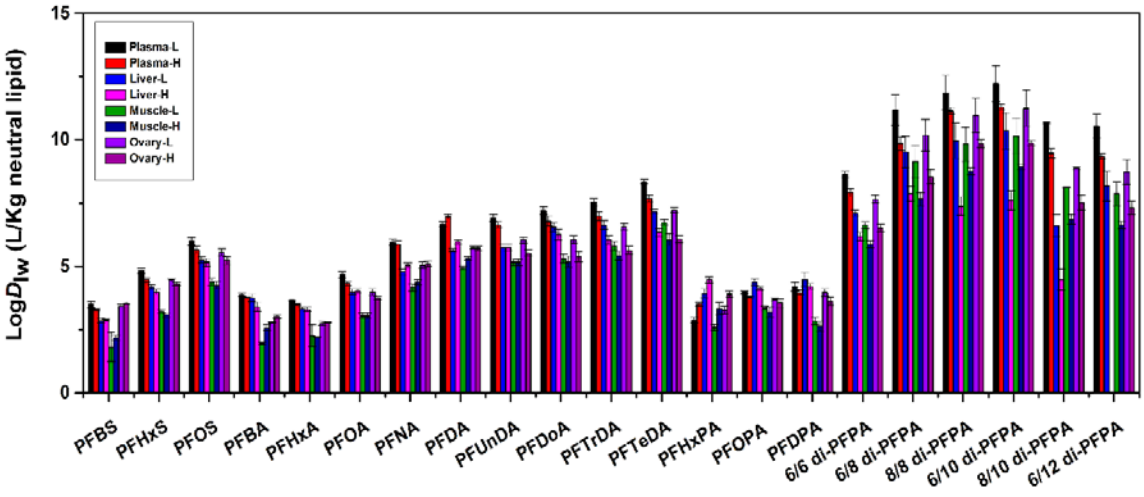
6.4.3 Maternal transfer and distribution in eggs

The observed data indicate some PFCs exhibit a high degree of maternal transfer and accumulation in eggs of zebrafish. In particular, PFBS, PFHxS and PFOS exhibit relatively high burdens in eggs. Potential reproductive dysfunction of these compounds may be of particular concern for wild fish, considering likely continuous chronic exposure conditions of these compounds. The results were consistent with Peng et al. [21]. Bioaccumulation of contaminants in eggs can result in reproductive and early developmental toxicity [21,182-184]. Further studies are required to better assess the potential reproductive impacts of these PFCs, which are shown to accumulate in eggs of female fish.

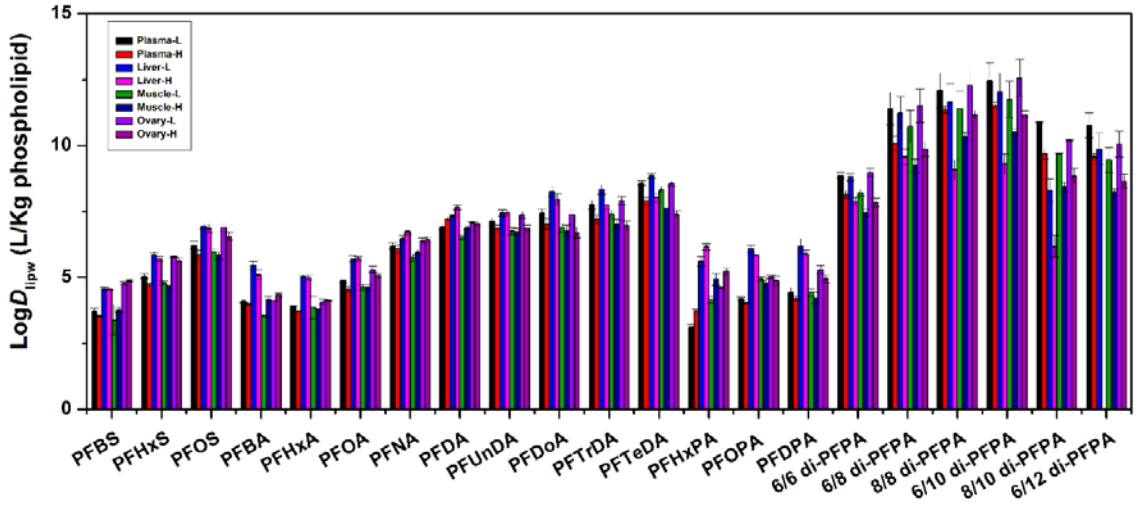
6.4.4 Apparent D_{lw}, D_{lipw}, D_{pw} of PFCs in zebrafish plasma and tissues

Figure 6.3 A-C illustrates the observed distribution coefficients for the various PFCs studied, including protein-water (D_{pw}), phospholipid-water (D_{lipw}) and neutral lipid-water (D_{lw}) in plasma, liver, muscle and ovary for low and high exposure groups. The results indicate that distribution coefficients for the various constituents vary widely among the various PFCs studied. The most hydrophobic of the PFCs studied, di-PFPAs with estimated log D_{ow}'s between 6.05 and 10.2, exhibited the highest partitioning to organic phase (i.e., lipids and proteins) within plasma and tissues. Mono-PFPAs, PFBS and PFHxS exhibited the lowest distribution coefficient values.

(A)



(B)



(C)

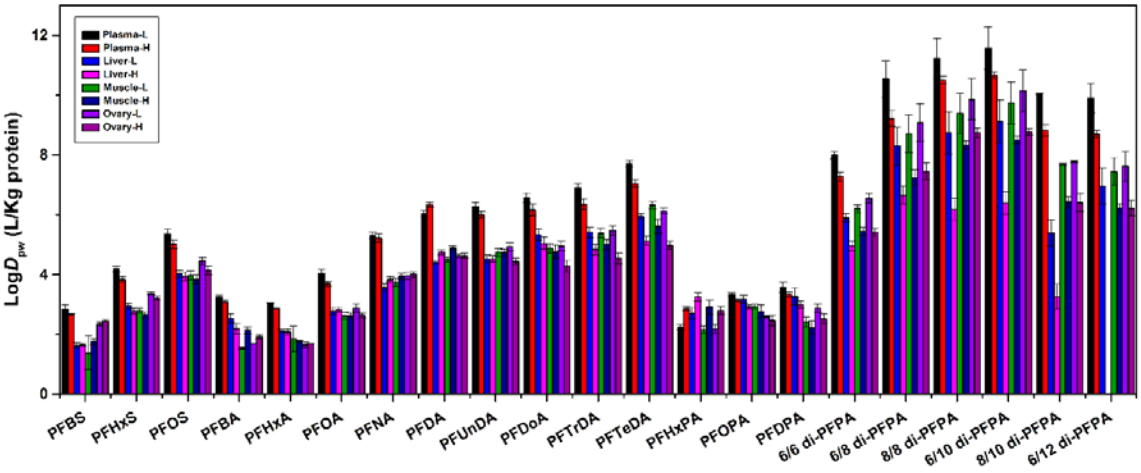


Figure 6.3 Observed steady state distribution coefficients, including (A) lipid-water distribution coefficient (D_{ow} ± SD), (B) membrane-water distribution coefficient (D_{lipw} ± SD) and (C) protein-water distribution coefficient (D_{pw} ± SD) for the various PFCs in different tissues.

The observed log D_{lw} and D_{lipw} values of the various PFCs, ranged between approximately 3-12, and 5-12, respectively. The observed D_{lw} and D_{lipw} values exceeded the range of estimated values based on chemical D_{ow}, indicating constituents other than

lipids contribute to the sportive capacity for PFCs in zebrafish tissues. In many cases, D_{lw} and D_{lipw} coefficient values of PFCs were significantly different among tissues.

Protein-water distribution coefficients (log D_{pw}) values of PFCs ranged between approximately 2 and 11 for the various PFCs. For a given compound, D_{pw} values also varied among different tissues, indicating other constituents are playing a role or specific proteins in the different tissues exhibit differential binding/sorptive capacity for PFCs.

Armitage et al. [175] argued that bulk protein fraction in tissues cannot explain the observed tissue distribution of ionogenic perfluoroalkyl acids in organisms, due to the fact that different tissues with similar bulk protein (e.g, liver and muscle) exhibit substantially different concentrations. This seems to be the case in the present analyses, as the observed protein-corrected concentrations and D_{pw} values PFCs in plasma were much higher than those in other tissues. Thus, D_{pw} alone may not be an adequate predictor for PFC bioaccumulation in the different fish tissues. There was no consistent correlation between tissue residue concentrations and the amount of protein or lipids present, which suggests that the sorptive capacity of the different tissues may due to a combined contribution of all constituents (neutral lipids + phospholipids + proteins).

6.4.5 Predicted tissue-specific bioaccumulation patterns of PFCs using a mechanistic bioaccumulation model for IOCs

Figure 6.4 illustrates the predicted distribution (% of total) of individual PFCs in neutral lipids, phospholipids, proteins and water of different tissues, following parameterization of the Armitage et al. [175] Model using measured values of neutral lipids, phospholipids and proteins (see Table 4.1) to represent f_{SL}, f_{PL} and f_{NLOM}, respectively for input into Eq.

5 in Capter 4. For the majority of PFCs, the model predicts PFCs to be distributed among phospholipids, neutral lipid, proteins and water in varying degrees for different tissues. High distribution in the aqueous phase mainly occurs for the more water soluble PFCs such as PFBA, PFBS, PFHxS and mono-PFHxPA. The remainder of the PFCs is mainly distributed in the organic phase (lipids and proteins). For zebrafish plasma, the model predicts a high distribution in phospholipids, with negligible fraction distributed in proteins. For liver, muscle and ovary, PFCs are distributed to proteins, phospholipids and neutral lipids to varying extents. The more hydrophobic di-PFPAs are shown to be extensively distributed (> 90%) in the neutral lipid fraction of plasma and tissues.

Figure 6.5 shows a comparison of observed steady state bioconcentration factors (BCF_{ss}, L/kg) versus the model predicted BCF_{ss}, which is assumed equivalent to the biota- or tissue-water distribution coefficient (D_{BW}, L/kg) proposed in the Armitage et al. model for IOCs. The model seems to substantially under-estimate the bioaccumulation potential of the studied PFCs by orders of magnitude, for all compounds in all tissues. The exception is for the very hydrophobic di-PFPA compounds, 8/10 di-PFPA and 6/12 di-PFPA (D_{ow} >10), which were observed to exhibit a substantial drop in BCF_{ss} compared to long-chain PFCAs. Similar observations of reduced BCFs for highly hydrophobic compounds have been attributed to reduced absorption efficiency, slow rates of membrane permeation and limited diffusive mass transfer [95,198].

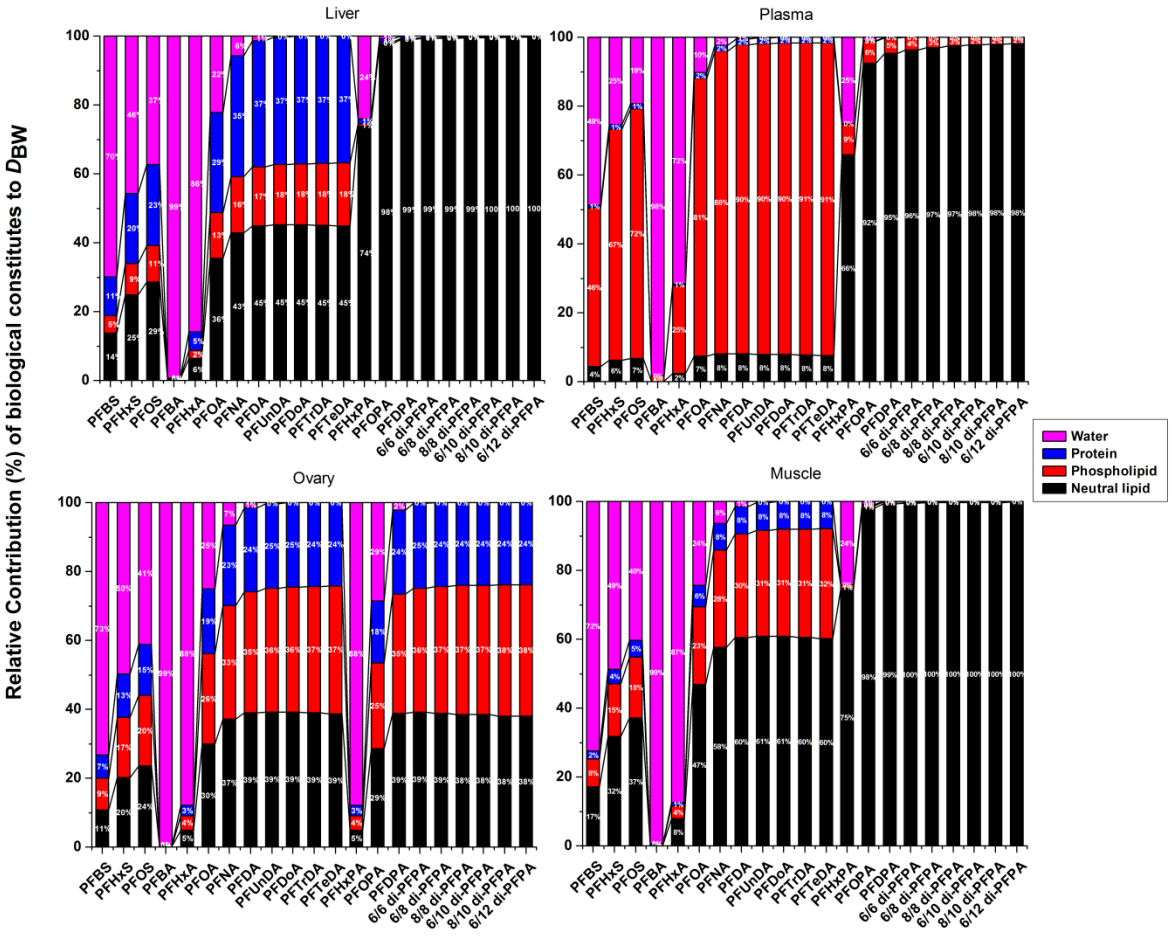


Figure 6.4 The predicted sorptive capacity contribution (%) of the various constituents (neutral lipids, proteins, phospholipids and water) comprising zebrafish plasma and tissues for the studied PFCs. The % contribution was determined using the equation for D_{BW} from the original Armitage et al. model, which incorporated a value of $0.05 \times D_{ow}$ to represent the protein-water distribution.

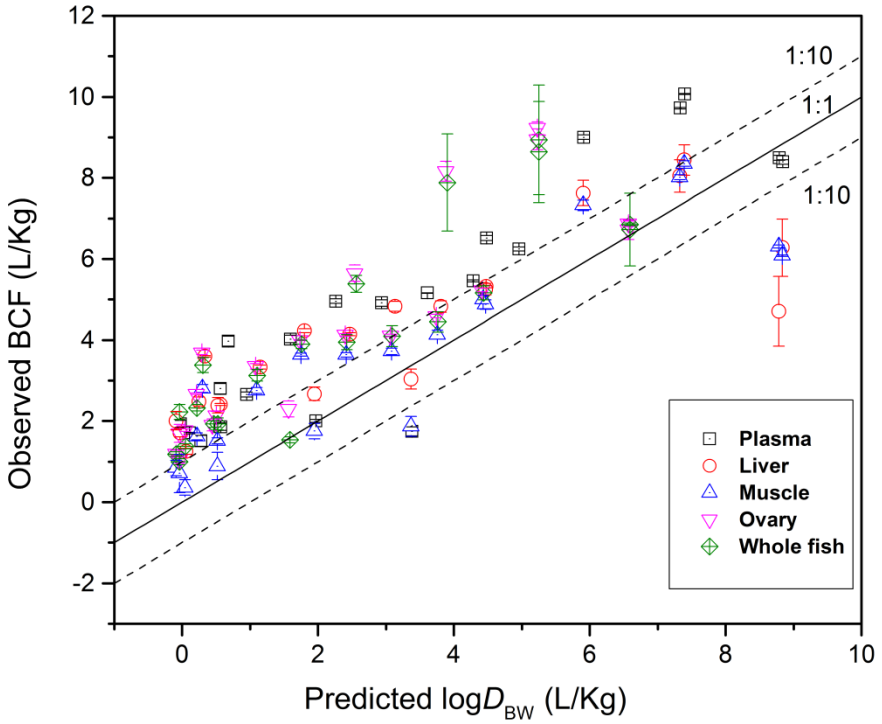


Figure 6.5 Observed log BCF (L/Kg) versus predicted log BCF (L/Kg) using the original Armitage et al model equation for ionogenic organic contaminants. Observed BCFs are shown as mean ± SD for PFCs. Model values were generated using the Armitage equation for the biota-water distribution coefficient (D_{BW}). The 1:1 line and factor of 10 lines are also indicated.

The model results indicate a considerable under-estimation of the sorptive capacity of zebrafish plasma and tissues for PFCs. As previous studies have observed very high binding affinities of PFCs with proteins, the under-estimation may be due to the term used to represent the sorptive capacity of proteins in the model. Specifically, the term $\rho_{NLOM}D_{OW}$ in Eq. 5 in Capter 4 may not be an accurate representation of protein-water distribution of PFCs. Experimental results presented by Bischel et al.[108] have demonstrated perfluoroalkyl carboxylates (PFOA, PFNA) suggest very high K_{pw} values for these PFCs, reporting log K_{pw}'s between 4.56 and 4.93, substantially greater than the octanol-water distribution coefficient for these compounds (log D_{ow}~2.69-3.42). In

comparison, based on a log D_{ow} = 3.42 for PFNA, the original term for protein sorptive capacity in the Armitage et al. model (i.e., $\rho_{NLOM}D_{OW}$), corresponds to a log K_{pw} for PFNA of 2.11, over 200 times lower than the observed K_{pw} reported by Bischel et al.[108]. Using this seemingly low estimate of K_{pw} for PFCs in this model appears to result in substantial under-estimation of PFC bioaccumulation in the zebrafish.

Debruyn and Gobas [107] conducted a comprehensive assessment of available data regarding sorptive capacity of animal protein and derived an empirically-derived equation for determination of log K_{pw}, based on chemical log K_{ow}, represented as:

$$\text{Log } K_{pw} = 0.57 \times \log K_{ow} + 0.69$$

For the purpose of this study, we modified the Armitage et al. model (Eq. 5 in Chapter 4) by substituting the original term for protein contribution ($f_{NLOM}\rho_{NLOM}D_{OW}$) with a value equal to $f_{protein} \times K_{pw}$, where K_{pw} was determined from the empirically derived equation reported by Debruyn and Gobas (Eq. 4 in Chapter 4). Figure 6.6 shows the revised model predicted distribution (%) for the various PFCs in constituents (neutral lipids, phospholipids and protein) in zebrafish plasma and tissues. In comparison to the original model predicted distribution (shown in Figure 6.4), it is clear the revised model now incorporates an enhanced influence of proteins towards the sportive capacity and distribution in different tissues, typically comprising > 99% of the total capacity.

Figure 6.7 illustrates the revised IOC bioaccumulation model predictions of BCFs for the various PFCs in zebrafish plasma and tissues versus the observed values. The revised model predictions show a general improvement with regards to deviation from observed

BCFs of the majority of PFCs. However, there are still a number of observed data exceeding the 10:1 boundary line of perfect model agreement. In particular, predicted BCFs for very hydrophobic di-PFPA compounds (8/10 di-PFPA and 6/12 di-PFPA) are still several orders of magnitude higher than observed BCF values. Conversely, observed BCF_{ss} values of other di-PFPAs (6/8 di-PFPA, 8/8 di-PFPA and 6/10 di-PFPA), in the range of 1×10^8 to 10^9 are shown to be orders of magnitude above the model predicted BCF (~1 x 10^6) (see Figure 6.7).

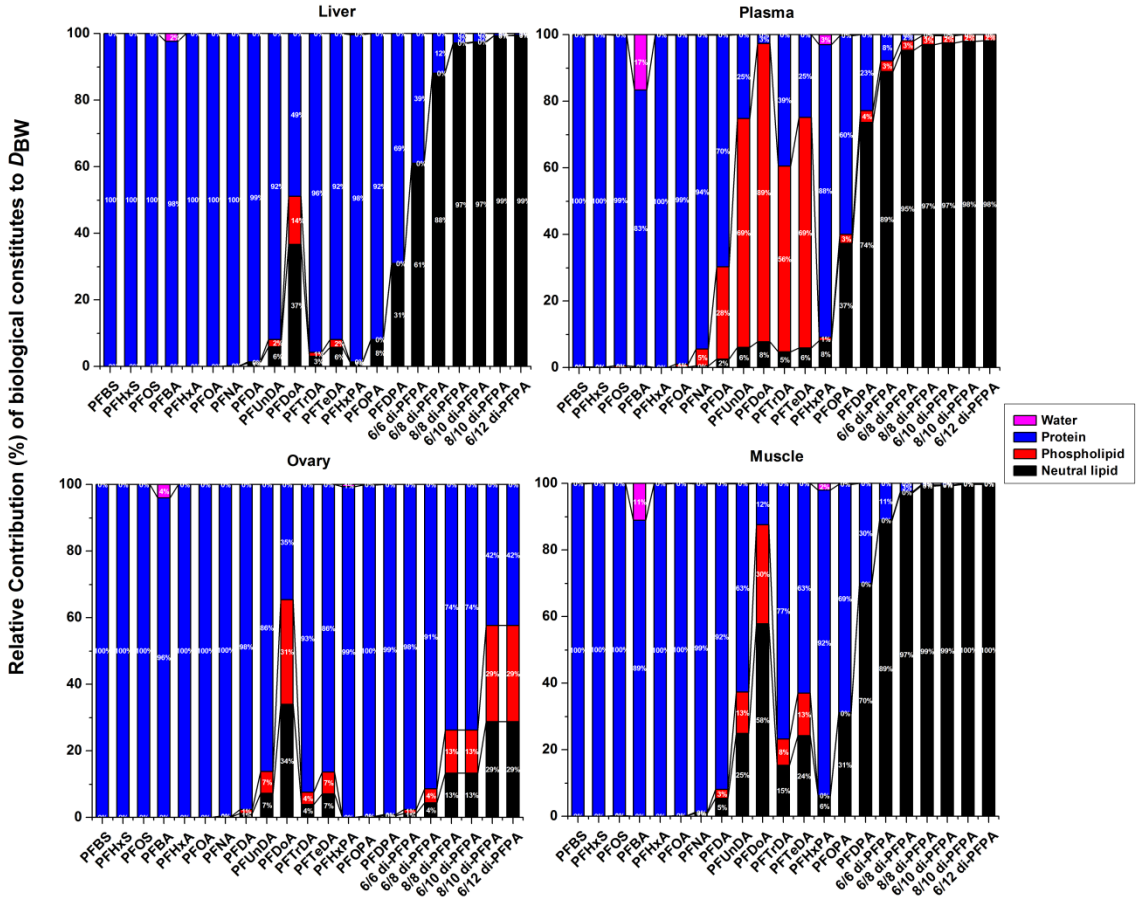


Figure 6.6 The predicted sorptive capacity contribution (%) of the various constituents (neutral lipids, proteins, phospholipids and water) comprising zebrafish plasma and tissues for the studied PFCs. The % contribution was determined using a modified version of the Armitage et al model, which incorporated the equation Log K_{pw} = 0.57 × log K_{ow} + 0.69 to represent protein-water distribution.

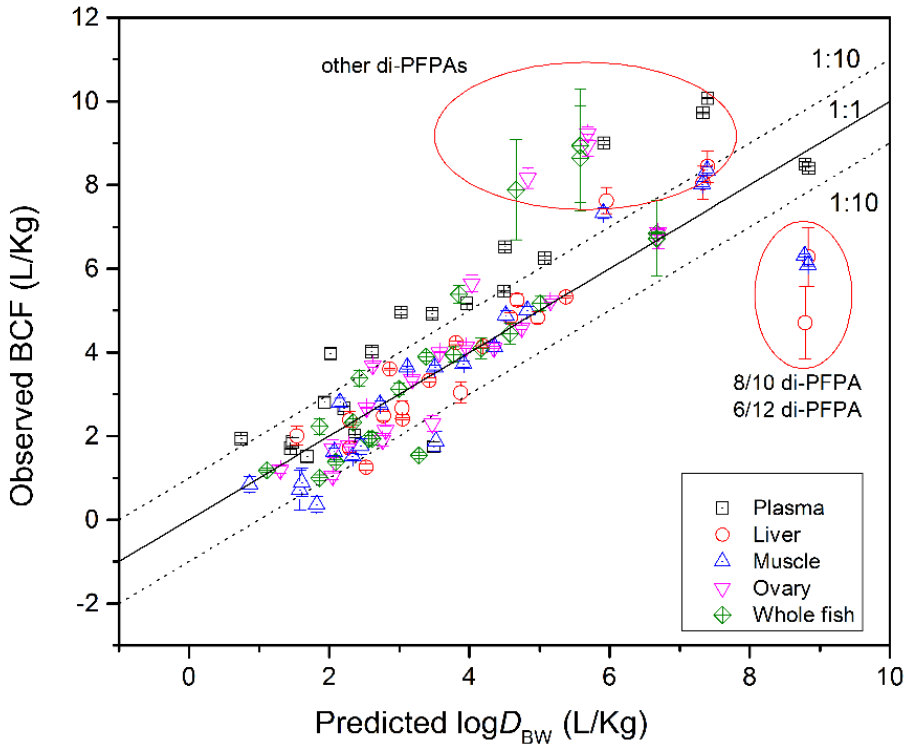


Figure 6.7 Observed log BCF (L/Kg) versus predicted log BCF (L/Kg) using the revised Armitage et al. model incorporating higher K_{pw} values for PFCs. Observed BCFs are shown as mean ± SD for PFCs. Model values were generated using the modified Armitage et al. equation for the biota-water distribution coefficient (D_{BW}). The 1:1 line and factor of 10 lines are also indicated.

6.5 Conclusions

Observed steady-state bioconcentration factors (BCF_{ss}) of individual perfluoroalkyl compounds (PFCs) in zebrafish plasma and tissues were correlated with octanol-water distribution coefficient (log D_{ow}). Several PFCs (PFBS, PFHxS and PFOS) exhibited a high degree of maternal transfer and preferential accumulation in zebrafish eggs, highlighting concerns regarding reproductive effects of these compounds in wild fish populations. Apparent values of D_{pw} / D_{lipw} / D_{lw} of individual PFCs varied among different tissues and were generally much higher than theoretical values reported in the

literature and physical-chemical property databases. The data suggest that no one constituent alone can adequately predict the bioaccumulation and tissue distribution of these compounds in zebrafish. Rather the results suggest distribution of PFCs is due to a combined contribution of several constituents (e.g, neutral lipids + phospholipids + proteins).

A recently presented bioaccumulation model for ionogenic organic contaminants (IOCs) was employed to predict tissue specific and whole-body BCFs in zebrafish, as well as explore the influence of proteins and phospholipids. The model incorporates a combined sorptive capacity of neutral lipids, phospholipids and proteins and calculates a biota-water distribution coefficient (D_{BW}, L/kg), which is equivalent to a steady state BCF_{ss}. Application of the original model equations, which utilizes a value of 0.05 × D_{ow} to represent protein-water distribution behavior (K_{pw}), substantially under-estimated the observed bioaccumulation potential of PFCs. Modification of the model, utilizing an empirically derived equation for K_{pw} enhanced the capacity of proteins in the model, resulting in higher BCF predictions, which were more comparable to the observed steady state BCFs. The findings suggest tissue specific bioaccumulation of PFCs can be explained by a relative contribution of neutral lipids, phospholipids and proteins, which combine to provide the overall sorptive capacity in aquatic organisms. Future studies of phospholipid-water and protein-water distribution (D_{lipw}, D_{pw}) of PFCs will help to advance our mechanistic understanding of the behavior of these important contaminants of concern.

CHAPTER 7 CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

Currently, there are two prevailing hypotheses regarding the bioaccumulation and tissue distribution of ionogenic organic contaminants (IOCs). One assumes that interactions with animal proteins including serum albumin, liver fatty acid binding proteins (L-FABP) etc. control the distribution and accumulation of IOCs [108-113]. The other asserts that partitioning to membrane phospholipids, which have a higher affinity for ionizable species than neutral storage lipids, also plays a major role in the bioaccumulation and tissue distribution of IOCs [98,106,114-116].

The key objectives of this thesis were to (i) evaluate the toxicokinetics and bioaccumulation behavior of several IOCs of emerging concern in zebrafish and (ii) assess the role of key biological constituents (proteins, phospholipids and neutral lipids) and influence of octanol-water, membrane-water and protein-water distribution coefficients (D_{ow}, D_{mw} and D_{pw}) on the bioaccumulation behavior of IOCs in zebrafish

To this end, the present thesis has provided novel measurements and observations to achieve a better understanding of the bioaccumulation behavior and tissues distribution of ionogenic PPCPs and PFCs in zebrafish (*Danio rerio*).

Contributions and findings of particular importance in this thesis include:

- In Chapter 2, details are provided regarding the development of a novel LC-ESI-MS/MS based analytical method that provides rapid identification and quantification of twelve commonly PPCPs in fish plasma micro-aliquots (~20 μL).

Quantification limits of the target PPCPs were in the low to sub pg/μL (ppb) range in plasma. The developed method may be useful for future biomonitoring programs, especially those employing small fishes with low blood compartment volumes, as well as toxicokinetic and/or toxicodynamic studies, which often require fast analysis of PPCP residues in fish plasma.

- Novel information regarding uptake and elimination kinetics and tissue-specific bioconcentration of twelve PPCPs in zebrafish is provided in Chapter 3. The study provides measures of key bioaccumulation metrics, including uptake rates (k_u), depuration rate constants (k_d), half-life and kinetically derived and apparent steady-state bioconcentration factors (BCF_k and BCF_{ss}). The results showed rapid uptake and depuration in zebrafish for many of the PPCPs. BCFs in fish tissues were low for most of PPCPs tested in this study. Half-lives ($t_{1/2}$) of the studied PPCPs ranged between 0.2 to 3.8 days. The whole-body steady state bioconcentration factors (BCF_{ss}) at steady state ranged between 0.2 and 465. The highest tissue-specific BCF_{ss} was observed for triclosan in liver (6,460 ± 1,560). The BCF_{ss} of triclosan was tissue dependent, with BCF values in liver (6,460 ± 1,560) exceeding those in muscle (135 ± 37) by approximately 50 times, which highlights the importance of conducting tissue-specific analysis in order to avoid under or over prediction of bioaccumulation potential and exposure. Relatively strong positive relationships were observed between BCF_{ss} and log D_{ow} ($r^2 > 0.6$), suggesting chemical D_{ow} may be a good predictor of PPCP bioconcentration in fish.

- A comprehensive investigation of the chemical and biological factors governing the bioaccumulation, tissue distribution and maternal transfer of PPCPs in zebrafish was provided in Chapter 4. The results showed that observed BCF_{ss} values of various PPCPs in zebrafish plasma and tissues exhibited a general increasing trend with increasing octanol-water distribution coefficient ($\log D_{ow}$), Also, several basic pharmaceuticals (i.e., diphenhydramine, fluoxetine, risperidone) that exhibit a high degree of maternal transfer and preferential accumulation in zebrafish eggs, indicating possible reproductive dysfunction related exposure risks of these compounds in wild fish. The study provides novel apparent estimates of protein-, phospholipid- and netral lipid-distribution coefficients (D_{pw} / D_{lipw} / D_{lw}) of individual PPCPs in fish plasma and tissues. The distribution coefficients varied among different tissues and were generally much higher than theoretical values reported in the literature and physical-chemical property databases. Application of a recently developed mechanistic model for bioaccumulation of ionogenic organic contaminants (IOCs) in fish, which incorporates the combined sorptive capacity of biological constituents, demonstrated reasonably accurate prediction of observed steady-state bioconcentration factors (BCF_{ss}) of PPCPs in zebrafish plasma and tissues.

- Chapter 5 provides novel measurements of uptake and elimination kinetics, half-lives and bioconcentration factors of mono-PFPAs and di-PFPAs, which to our knowledge currently do not exist. Strong positive linear relationships were

observed between log BCF_{ss} and perfluoroalkyl chain length and log D_{ow}. The findings also highlight that di-PFPAs exhibit a high degree of bioaccumulation, exceeding that of PFOS and long-chain (C_9-C_{14}) PFCAs. Both mono- and di-PFPAs exhibited very long half-lives. Plasma clearance half-lives for mono- and di-PFPAs ranged between approximately 15 and 80 days, comparable to PFOS and long chain PFCAs. The di-anionic mono-PFPAs exhibited substantially lower BCFs, as compared to the more hydrophobic anionic di-PFPA. Whole-body steady-state bioconcentration factors (BCF_{ss}) for di-PFPAs ranged between 5×10^4 and 2×10^9, while mono-PFPA BCF_{ss} values ranged between approximately 20 and 200.

- Chapter 6 examines the role of protein and phospholipids in the bioaccumulation, tissue distribution and maternal transfer of ionogenic PFCs in zebrafish. The results show that observed BCF_{ss} of individual PFCs in zebrafish plasma and tissues were correlated with octanol-water distribution coefficient (logD_{ow}). The study provides novel apparent estimates of protein-, phospholipid- and neutral lipid-distribution coefficients (D_{pw} / D_{lipw} / D_{lw}) of several PFCs in fish plasma and tissues. Application of the original model equations, which utilizes a value of 0.05 D_{ow} to represent protein-water distribution behavior (K_{pw}), substantially under-estimated the observed bioaccumulation potential of PFCs. Modification of the model, utilizing an empirically derived equation for K_{pw} enhanced the capacity of proteins in the model, resulting in higher BCF predictions, which were more comparable to the observed steady state BCFs. The findings suggest tissue specific bioaccumulation of PFCs can be explained by a relative contribution of neutral lipids, phospholipids and proteins,

which combine to provide the overall sorptive capacity in aquatic organisms.

In summary, this thesis demonstrates that bioaccumulation of IOCs in fish involves a complexity of chemical and biological factors. Biological constituents (proteins, phospholipids and neutral lipids) greatly influence bioaccumulation and tissue distribution patterns of IOCs. Chemical properties such as D_{ow}, D_{lipw} and D_{pw} were also found to play a key role in the bioaccumulation of IOCs.

7.2 Future work recommendations

Knowledge generated from this study provides novel insights into the bioaccumulation behavior of IOCs in aquatic organisms. Further research directed at understanding the role of phospholipids and proteins in IOC bioaccumulation, as well as determination of protein-water distribution coefficients (D_{pw}) and phospholipid-water distribution coefficients (D_{lipw}) for various IOCs of emerging concern would undoubtedly help to improve our mechanistic understanding of these and other contaminants of emerging concern.

Based on the work in this thesis, the following related research works are recommended for further study.

In the present study, the K_{pw} and D_{lipw} values in the model application are from the QSAR model. The comparison of the observed D_{pw} / D_{lipw} / D_{lw} with the reference modeled data suggests that the previous model equations for above distribution coefficients of IOCs are not accurate. PH-dependent measurements of D_{pw} / D_{lipw} / D_{lw} would be a very useful parameter to characterize. Concentration dependent of D_{pw} / D_{lipw} / D_{lw} for PFCs would be

also a very important parameter to characterize. This could be accomplished via either in vivo or in vitro study. In addition, in view of the protein / phospholipids / neutral lipids in different tissues are not equal regarding to sorption capacity, measurements of interactions of IOCs with different class of protein / phospholipids / neutral lipids in the different tissues should be investigated.

Finally, mono-PFPAs and di-PFPAs demonstrated unique behavior compared to PFCAs and PFSAs in the fish. The mono-PFPAs were observed substantially distribute in the liver whereas the di-PFPAs were observed substantially distribute in the plasma. Both of these observations suggest advanced study for them.

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PUBLICATIONS ARISING FROM PHD WORK

- F.F. Chen, Z.Y. Gong, Barry C. Kelly. Rapid analysis of pharmaceuticals and personal care products in fish plasma micro-aliquots using liquid chromatography tandem mass spectrometry. ***Accepted by J. Chromatogr. A***

- F.F. Chen, Z.Y. Gong, Barry C. Kelly. Assessing uptake and elimination kinetics of pharmaceuticals and personal care products in zebrafish (*Danio rerio*). ***Prepared manuscript.***

- F.F. Chen, Z.Y. Gong, Barry C. Kelly. Assessing the bioaccumulation potential of mono- and di-perfluoroalkyl phosphonic acids (PFPAs) in zebrafish (*Danio rerio*: comparison to perfluoroalkyl carboxylic (PFCAs) and perfluoroalkyl sulfonic acids (PFSAs) ***Prepared manuscript.***

- F.F. Chen, Z.Y. Gong, Barry C. Kelly. Assessing chemical and biological factors governing the bioaccumulation behaviour or pharmaceuticals and personal care products in fish. ***Prepared manuscript.***

- F.F. Chen, Z.Y. Gong, Barry C. Kelly. Bioaccumulation behavior of ionogenic perfluorinated chemicals in fish: assessing the role of proteins and phospholipids. ***Prepared manuscript.***

PRESENTATIONS ARISING FROM PHD WORK

- F.F. Chen, Z.Y. Gong, Barry C. Kelly. Bioaccumulation, Tissue distribution of Pharmaceuticals and Personal Care Products in Zebrafish (*Danio Rerio*). Poster. *SETAC Europe 24rd Annual Meeting, 2014*

- F.F. Chen, Z.Y. Gong, Barry C. Kelly. Bioconcentration of Pharmaceuticals and Personal Care Products in liver of Zebrafish (*Danio Rerio*). Poster. *SETAC North America 34rd Annual Meeting, 2013*

- F.F. Chen, Z.Y. Gong, Barry C. Kelly. Bioconcentration of Pharmaceuticals and Personal Care Products in blood plasma of Zebrafish (*Danio Rerio*). Poster. *SETAC Europe 23rd Annual Meeting, 2013*

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- F.F. Chen, Z.Y. Gong, Barry C. Kelly. Bioaccumulation, Tissue distribution and Maternal Transfer of Pharmaceuticals and Personal Care Products in Zebrafish (*Danio Rerio*). Poster. *SETAC North America 33rd Annual Meeting, 2012*

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