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**BIOACCUMULATION AND ECOTOXICITY STUDY OF
PERFLUORINATED CHEMICALS**

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BIOACCUMULATION AND ECOTOXICITY STUDY OF PERFLUORINATED CHEMICALS

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Abstract

Perfluorinated chemicals or perfluorochemicals (PFCs) are getting more and more attention. In recent investigations, PFCs are universally detected in environmental compartments, wildlife and human bodies worldwide. PFCs are thermally and chemically stable, resistant to biodegradation and therefore, extremely persistent in environment. Because of their global distribution and high persistency, PFCs were classified as the emerging chemicals of concerns. As newly emerged contaminants, knowledge on the ecotoxicological behaviors of PFCs remains scarce. Effective environmental management, however, requires the information about the environmental and ecological consequences of the contaminants. Therefore the objective of this study was to investigate the bioaccumulation behavior and to evaluate the environmental toxicity of four common PFCs, namely perfluorooctanesulfonate (PFOS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA) and perfluorodecanoic acid (PFDA) in a sentinel organism, green mussel *Perna viridis*.

The bioaccumulation behavior of PFCs was investigated, where the bioaccumulation factors and kinetics were studied to understand the compounds' accumulation, elimination and partitioning behavior between environmental media and biota. Strong bioaccumulation potential of the tested compounds was demonstrated, where long chain PFCs were found to be more bioaccumulative than short chain ones. More importantly, bioaccumulation of PFCs was also found to be exposure concentration dependent. A new kinetic bioaccumulation model based on the adsorption mechanism was proposed, which provides a more accurate description of the process.

The environmental toxicity of PFCs was assessed through biomarker-based toxicity study, where biomarkers at three biological levels (bio-molecular, cellular and physiological) were examined. The results demonstrated that PFCs could induce toxic responses across different biological levels and also through different toxic modes of actions. The compounds were found to be able to cause oxidative toxicity, genotoxicity, inhibition of xenobiotic metabolism, immunotoxicity and also deleterious consequences on the general well-being of the target organism. The systematic investigation provides a holistic idea and comprehensive understanding of the adverse effects of PFCs on an organism.

Toxic mechanisms were also explored by examining the dose-response relationships and toxicity kinetics. Toxicity models were established wherever applicable. The correlations of these biomarkers were investigated to elucidate relationships between toxic responses at various biological levels. The complex biomarker responses were also integrated by the Enhanced Integrated Biomarker Response (EIBR) system. The integrated biomarker assessment and structure-activity analysis revealed the diverse toxicity patterns of PFCs. Besides the commonly recognized chain length and functional group effects, several structural factors were also involved in the toxic action of PFCs. EIBR analysis demonstrated that it is the fluorinated chain length that governs the integrative toxicity of the compounds. Although the estimated half maximal effective concentration (EC_{50}) values based on integrated biomarker response results were higher than the environmental concentrations, most toxic effects were also found to increase with organism PFCs burden. Therefore, with prolonged exposure and bioaccumulation effect, these contaminants could induce severe long-term toxic effects even at low exposure levels.

To our knowledge, this is the first systematic investigation of environmental toxicity of PFCs. The evaluation of various toxic effects, integrative toxicity and toxic pattern provides new perspectives of environmental consequences of these pollutants. The observed structure-activity relationships also help to elucidate the gaps in understanding the mechanisms behind PFCs toxicity. The mathematical models and methods developed in the current study could also be applied in future ecotoxicity investigation of the compounds.

List of Publications

Liu CH, Gin KY, Chang VW, Goh BP, Reinhard M., Novel perspectives on the bioaccumulation of PFCs & the concentration dependency. Environ Sci Technol. 2011; 45(22):9758-64

Liu CH, Chang VW, Gin KY., Environmental toxicity of PFCs: An enhanced integrated biomarker (EIBR) assessment and structure-activity analysis. Environ Toxicol Chem. 2013; 32(10): 2226-33

Liu CH, Chang VW, Gin KY., Multi-biomarker responses in green mussels exposed to PFCs: effects at molecular, cellular, and physiological levels. Environ Sci Pollut R. 2014; 21(4):2785-94

Liu CH, Chang VW, Gin KY., Genotoxicity of perfluorinated chemicals (PFCs) to the green mussel (*Perna viridis*). Sci. Total Environ. 2014; 487: 117-122

Liu CH, Nguyen VT, Chang VW, Gin KY. Occurrence and ecotoxicity of PFCs in marine environment of Singapore. Environment & Health (Conference of ISEE, ISES and ISIAQ) 2013, Basel, Switzerland.

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List of symbols

$\hat{\epsilon} \log \text{BAF}$	Deviation in $\log \text{BAF}$
$\text{BAF}_{\text{kinetic}}$	Kinetic BAF from
BAF_{ss}	Steady state BAF
B_i	Biomarker response score
C_o	Organism concentration of PFCs
C_w	Water concentration of PFCs
EC_{50}	Half maximal effective concentration
EIBR	Enhanced integrated biomarker response value
F	Fisher criterion
k_e	Elimination constant
K_{ow}	Octanol water partitioning coefficient
k_u	Update constant
n	Binding sites
N_{fc}	Fluorinated carbon number/chain length
Q^2	Cross-validated correlation coefficient
R^2 or r^2	Regression coefficient
s	The root mean square error
	Binding affinity
	Proportion of bounded compounds
W_i	Weighting of biomarker
Y_i	Standardized biomarker response

List of abbreviations

APFO	Ammonium PFOA
BAF	Bioaccumulation factor
BCF	Bioconcentration factor
CAT	Catalase
EIBR	Enhanced integrated biomarker response
EPA	US Environmental Protection Agency
EROD	Ethoxyresorufin-O-deethylase
FR	Filtration rate
GPx	Glutathione peroxidase
GSH	Glutathione
GST	Glutathione-S-transferase
IBR	Integrated biomarker response
MN	Micronucleus
NRRT	Neutral red retention time
PBS	Phosphate buffered saline
PFCA _s	Perfluorinated carboxylates
PFC _s	Perfluorinated chemicals
PFDA	Perfluorodecanoic acid
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonate
PFSAs	Perfluorinated sulfonates

POPs	Persistent organic pollutants
QSAR	Quantitative structure activity relationship
RCF	Relative condition factor
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SPE	Solid phase extraction
TBARS	Thiobarbituric acid reactive substances

Chapter One Introduction

1.1 Background

In recent years, there has been growing concerns on the group of emerging contaminants known as perfluorinated chemicals or perfluorochemicals (PFCs). PFCs are synthetic compounds that have been applied in a broad spectrum of commercial products and industrial processes in the past few decades. Due to their unique water and oil repellent properties, PFCs are widely used as surfactants, refrigerants and as components of pharmaceuticals, lubricants, paints, fire fighting foams, cosmetics and food packaging (Houde et al. 2006, Lau et al. 2007, Suja et al. 2009). PFCs are released into the environment either through the usage of the above PFCs containing products, or by degradation of existing precursors.

Perfluorinated compounds are a group of organofluorine chemicals of which hydrogen atoms on the carbon chain are replaced by fluorine. They contain a functional group which, in most cases, is either a sulfonate or a carboxyl group. Among the various PFCs of concern, perfluorinated carboxylates (PFCAs) and sulfonates (PFSA) are the two commonly detected groups in the environment (Becker et al. 2008). The chemical structure of PFCs gives them unique properties including chemical inertness, thermal stability, low surface energy, hydrophobicity as well as lipophobicity (Hagenaars et al. 2011). On the other hand, these compounds show good binding affinity with proteins and have therefore, also been recognized as proteinophilic compounds (Liu et al. 2011, Rayne & Forest 2009). Due to the high energy of carbon-fluorine covalent bonds, PFCs are thermally and chemically stable, and are also resistant to biodegradation. Hence, PFCs are extremely persistent in the environment (Conder et al. 2008, Rayne & Forest 2009).

PFCs are universally detected in all environmental compartments: water, air and soil worldwide, even in remote regions such as the Arctic (Houde et al. 2006, Lindstrom et al. 2011, Nguyen et al. 2011, Prevedouros et al. 2006, Wu & Chang 2012). They have also been found extensively in wildlife and human bodies (Fernandez-Sanjuan et al. 2010,

Houde et al. 2008, Kelly et al. 2009, Kwadijk et al. 2010). Besides their ubiquitous distribution, PFCs impose risks on human and ecological health. In animal studies, PFCs were found to be linked to liver cancer, developmental and reproductive toxicity (Houde et al. 2008, Kelly et al. 2009, Kwadijk et al. 2010, Lau et al. 2007, Morikawa et al. 2006, Quinete et al. 2009). Selected perfluorinated compounds also demonstrated endocrine disrupting capacity (Martin et al. 2004). In humans, it was suggested that PFCs might be associated with woman infertility (Berger et al. 2009). U.S. EPA is also concerned that PFCs may cause adverse effects in human offspring due to the wide spread detection of PFCs in human breast milk (Kannan et al. 2001, Kwadijk et al. 2010).

Ecotoxicity is converging with the other term, "environmental toxicity". Ecotoxicology is the branch of toxicology concerned with the study of toxic effects that are caused by natural or synthetic pollutants to the constituents of ecosystems (Newman 2009). As its name implies, ecotoxicology emphasizes the impacts to the ecological entities and more importantly to the environment, which is reflected by the living organisms in the environment. The common term "toxicology" often refers to studies that focus on the toxic effects on humans. The test organisms are generally different as well: while conventional toxicity tests use human cell lines or rats as human surrogates, ecotoxicity studies normally use organisms from the natural environment. Ecotoxicity testing of environmental samples can be carried out at any biological organization levels: from bio-molecule, cell, through whole organisms, to populations. The majority of pollutants act initially at the molecular level following accumulation into the exposed organisms, with any effects then becoming apparent as physiological changes and effects on key individual parameters such as growth, reproduction or survival. Bioaccumulation is an integral part of an ecotoxicological study and generates insights into the "internal doses" of contaminants which are more ecotoxicologically relevant than environmental concentrations (Fernandez-Sanjuan et al. 2010). It is important to understand and to predict the accumulation behavior of contaminants in biota because effects are normally a consequence of concentration in target tissues or organs (Thompson et al. 2005). As an evaluation of environmental impacts, results of ecotoxicological studies provide useful information for environmental risk assessment. Therefore, for emerging contaminants such as PFCs, ecotoxicological data is urgently needed to facilitate effective environmental management of these pollutants.

1.2 Motivation

There are several reasons why PFC contaminations are receiving so much attention: 1) their ubiquitous existence in environment, wildlife and human; 2) their highly persistent characteristic and 3) their potential health risks. Despite numerous reports on environmental occurrence and toxicological concerns of PFCs, as a newly emerged group of contaminants, knowledge on the bioaccumulation behavior and ecotoxicological effects of PFCs remains scarce. Available toxicity data are mainly focused on the toxic effects on mammalian species especially rodents, where PFCs have been mostly studied as "human health hazards" rather than "environmental hazards". The lack of information about their environmental and ecological consequences makes it difficult for authorities to make discharge regulations and treatment guidelines for these contaminants.

1.3 Research questions

Although efforts have been made, ecotoxicity data of PFCs are still insufficient for a comprehensive understanding of environmental and ecological consequences of these compounds. There are a number of limitations regarding previous studies. The following questions were addressed in this thesis:

- A systematic ecotoxicity investigation of PFCs is still lacking. Available toxicity information is based on studies that examined different toxic effect of different compounds on different organisms under different conditions. The lack of consistency makes it difficult to interpret, correlate and extrapolate the toxicity data.
- Although some toxic effects of PFCs have been reported, mechanistic information regarding the compounds' toxic activities remains largely unknown. Such information includes toxic pathways, exposure concentration and time effects and structure-activity relationships.
- Most of the investigations examined PFOS and PFOA only, since these two normally comprise a major proportion of total PFCs. However, many other PFCs are also detected in the environment. A better understanding of the toxic mechanism of these compounds requires extending the investigation to other PFCs in the group.
- The previous toxicity examinations mainly focus on effects at molecular level.

Higher level toxic effects, such as alterations at cellular and physiological levels remain largely unknown. Higher level toxic effects should also be included in the investigation to generate toxicity data that are ecologically relevant for environmental risk assessment.

- Toxicity studies of PFCs still largely focus on human health effects and therefore, only limited animal species (e.g. rodent) were used as the target organism. PFC induced effects in natural living organisms are still not clear. The ocean is the final sink for many pollutants including PFCs, but the ecotoxicity of PFCs has seldom been examined in the marine environment.

The lack of ecotoxicity data and the ambiguity of toxic mechanisms suggest that there is an urgent need to examine the ecotoxicological effects of these contaminants.

1.4 Objective

With the above questions considered, the objective of this study was to investigate the bioaccumulation behavior and to evaluate the environmental toxicity of four common PFCs, namely perfluorooctanesulfonate (PFOS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA) and perfluorodecanoic acid (PFDA) in marine green mussel, *Perna viridis*. Long chain PFCs are more potent relative to short chain ones (Kleszczynski et al. 2007). PFCs with 7 or more fluorinated carbons are of special concern because they are bioaccumulative and can be biomagnified through the food web (Liu et al. 2011, Conder et al. 2008). The target compounds, including both perfluorinated carboxylates (PFCAs) and perfluorinated sulfonates (PFSAs), were selected to enable the comparison and understanding of structural influences on the chemicals' bioaccumulation and toxicological behavior. Specific objectives include:

- To examine the bioaccumulation behavior of the target PFCs. Bioaccumulation is an integral part of ecotoxicity study and generates insights into the "inner dose" of contaminant which is more closely related with the toxic effects.
- To investigate the PFC induced toxic effects in various toxic modes of action. Biomarkers at three biological levels (molecular, cellular and physiological levels) are examined in the test organism. The systematic investigation aims to provide a holistic idea and comprehensive understanding of how PFCs can affect an organism.

- To evaluate overall toxicity potential of target PFCs by integrating the toxicity tests results of different toxic modes of action from multiple biological levels.
- To reveal the underlying toxic mechanisms of PFCs by examining dose-response relationships, kinetic of toxic effects, toxic pathways and structure-activity relationships. The knowledge is crucial for the understanding of the toxic behavior of the whole group, in which compounds have similar structure and physico-chemical properties. The mechanistic information will greatly facilitate the environmental modeling and risk assessment.
- To establish models that can simulate the toxic behaviors of PFCs. The models can be useful tools for prediction and risk assessment of environmental impacts of other compounds in the group.

1.5 Test organism

The ocean has been considered as the final sink of persistent organic pollutants, and marine invertebrates are one of the major repositories of PFCs, as they are sessile and filter-feeding animals that tend to accumulate more organic pollutants (Villela et al. 2006). Bivalves, especially mussels, are also common indicators of water quality and environmental health in biomonitoring studies (Luengen et al. 2004). Therefore, a local species, green mussel *Perna viridis*, was selected as the sentinel organism and representative of marine wildlife.

1.6 Scope

This thesis contains six chapters. Chapters 1, 2 and 6 present the Introduction, Literature review and Conclusions, respectively. The results and discussions are presented in Chapters 3-5. There are two major parts in the study: bioaccumulation and ecotoxicity. Results of the bioaccumulation study are discussed in Chapter 3, and the major findings have been published in Environment Science and Technology (Liu et al. 2011). Results of ecotoxicity are discussed in Chapters 4 and 5. Some of the findings in these chapters have already been published in Environmental Toxicology and Chemistry (Liu et al. 2013), while others have also been submitted to Environmental Science and Pollution Research

for potential publication.

Chapter 3 investigated the bioaccumulation behavior of PFCs. The bioaccumulation factors and kinetics were studied to understand the compounds' accumulation, elimination and partitioning behavior between environmental media (water) and biota. Mathematical models were established to describe the mechanism of PFCs bioaccumulation.

In Chapter 4, the results of a series of biomarker-based toxicity tests were presented and discussed. Toxic effects of PFCs on the target organism were assessed with toxicity tests at different biological levels, namely molecular level, cellular level and physiological level. These tests also examined a number of toxic modes of action including oxidative toxicity, suppression of detoxification, genotoxicity, immunotoxicity and effects on general well-being.

In Chapter 5 toxicity test results from Chapter 4 were compiled by a proposed enhanced Integrated Biomarker Response system to examine the integrative toxicity of the compounds. Correlations between different toxic effects were examined to reveal the toxic pathways of PFCs. Structure-activity relationship were also analyzed based on the integrated toxicity results.

The data presented in this thesis contributes to the understanding of the environmental and ecological impacts of PFCs. The information will be useful for the prediction of environmental impacts of other compounds in the group. More importantly, the results would assist regulatory authorities in the environmental risk assessment and management of these contaminants.

Chapter Two Literature Review

2.1 Bioaccumulation of PFCs

2.1.1 Biomonitoring the occurrence of PFCs

PFCs has been manufactured and used in various industries and consumer products for over 60 years. The compounds have been released into the environment through their production, usage, as well as disposal. Besides the above mentioned direct source, indirect sources such as PFC precursors also contribute to the environmental PFCs once converted to their more stabilized final products (Prevedouros et al. 2006). Although PFCs production has been phased out since the 1990s, their extensive application and their persistence have already led to widespread occurrence in the environment, wildlife and even human bodies (Armitage et al. 2009, Fernandez-Sanjuan et al. 2010, Lau et al. 2007, Moon et al. 2010, Plumlee et al. 2008, Prevedouros et al. 2006). The global distribution in human and biota is the major reason that PFCs were classified as the emerging chemicals of concerns (Giesy et al. 2010). Environmental monitoring results have shown that PFCs are ubiquitous in all tested species. High concentrations of PFCAs and PFSAAs in invertebrates were reported from South China and Japan (Hekster et al. 2003). A series of long chain PFCAs were detected in the food web in Lake Ontario (Kennedy et al. 2004), where biomagnification was observed. PFCs were also detected in birds especially from coastal and industrialized areas (Houde et al. 2006). Biomonitoring studies of fish and mammals in Asia, Europe and America reported notable concentrations of PFCs ranging from 8.7ng/g to 180 000ng/g wet weight (Becker et al. 2008, US EPA 2006, 2009, Fromme et al. 2010, Mulkiewicz et al. 2007, Paul et al. 2009, Shaw et al. 2009). There were also detections of PFCs in wildlife from remote environments such as the Arctic, where atmospheric oxidation and transportation of precursors were proposed to be the dissemination mode (So et al. 2006). Studies of human exposure have detected PFCs, mostly in blood samples throughout the world (Berger et al. 2009, Kwadijk et al. 2010, Martin et al. 2004). A study in Japan showed that PFOS and PFOA concentration in human have increased by factors of 3 and

14 respectively in the past 25 years (Martin et al. 2004).

To date, biomonitoring of PFCs occurrence has covered a wide range of regions including Asia, Europe, North and South America, Russia and even Antarctica and Arctic, and different habitats (Houde et al. 2011). These assessments clearly indicate the widespread distribution of PFCs and exposure of wildlife species. Regardless of the contamination profiles in the surrounding environment, there is a predominance of PFOS and long chain PFCAs found in organisms (Braune et al. 2010, Butt et al. 2010, Houde et al. 2011, Ishibashi et al. 2008, Shaw et al. 2009). Although PFOS and PFOA are the predominant PFCs detected in the environment, other long chain PFCs such as PFNA and PFDA were also frequently detected and may account for higher proportions in organisms (Ishibashi et al. 2008, Nguyen et al. 2011). These observations also highlight the importance of bioaccumulation of these chemicals.

2.1.2 Bioaccumulation field studies

The aquatic ecosystem is suggested to be one of the major sinks of environmental PFCs, because of their high water solubility compared to traditional POPs (Mulkiwicz et al. 2007). Understanding the bioaccumulation of PFCs in aquatic organisms therefore becomes paramount (Conder et al. 2008). Many biomonitoring studies and field work on the bioaccumulation of PFCs have been conducted.

Quinete et al. (2009) studied the occurrence of PFCs in South America. PFCs were measured in Rio de Janeiro State (Brazil) from environmental samples including drinking water, river water, and sea water. The test organisms used were fish, dolphin, and mussel. The concentration and patterns of PFCs in these environmental and biological samples were determined, and PFCs were detected in all samples including drinking and surface waters as well the biota. It was found that the bioconcentration factor (BCF) in biota depends on the species. The results indicated a potential for bioaccumulation and biomagnification since the chosen test organisms in this experiment were from different levels in the food chain.

In another study of PFC contamination in marine mammals (Shaw et al. 2009), the exposure of harbor seals to PFCs (C₇-C₁₂ PFCA & PFSA) in the northwest Atlantic was characterized. The concentration, pattern, and trend of PFCs in the liver, which was previously identified as one of the target organs, were investigated. PFOS concentration

was found to be the highest in the liver, followed by PFUnDA. The concentration of PFCs was observed to be independent of seal gender. However, concentration in pups was significantly higher than in adults, which suggests the potential maternal transfer of PFCs.

A comparative study of PFCs and lipophilic organohalogenes was conducted by Kelly et al. (2009) to better understand the bioaccumulation behavior of PFCs. Concentrations of several PFCs in Arctic marine sediments and various organisms were measured and compared to those of persistent lipophilic organohalogenes in order to evaluate the trophic magnification and wildlife exposure levels of PFCs. Concentrations of PFCs were observed to increase significantly with increasing trophic level. It was found that PFOS and PFCAs are also highly bioaccumulative in the Arctic marine food web.

While most field works focus on the occurrence, monitoring and bioaccumulation of PFCs, there are also studies that focus on their environmental transformations. Simultaneous measurement of PFCs in water, sediment, and biota was conducted by Kwadijk et al. (2010) to determine the distribution coefficient for fate, bioaccumulation and biomagnification modeling. The distribution and time series of PFCs in aquatic systems in the Netherlands was investigated. The time trend study revealed a concentration increase followed by a decline, similar to the trend in many other industrialized countries. The distribution study implied that PFCs are mainly present in water. For the test organism, eel, the sorption and bioaccumulation factors were also found to be correlated with the PFC chain length.

Field study results demonstrated the bioaccumulation potential and trends of PFCs. They also provide valuable data on the compounds' environmental partitioning. These data are good references for controlled laboratory studies for the detailed mechanism of bioaccumulation of PFCs.

2.1.3 Laboratory bioaccumulation studies

Data from monitoring studies have demonstrated the potential of bioaccumulation and magnification of PFCs (Houde et al. 2006). In laboratory studies, it has been found that the bioaccumulation behavior of PFCs is comparable to short and medium chain fatty acids (Martin et al. 2003a), where the bioaccumulation potential and perfluoroalkyl chain length are positively correlated (Jeon et al. 2010, Moon et al. 2010). In aquatic organisms, dietary assimilation and direct uptake from water are the two major routes of accumulation (So et

al. 2004, Wang et al. 2010). The relative significance of the two could depend on factors such as salinity, body size, and trophic level (So et al. 2004).

In early controlled laboratory studies, Martin et al. (2003a, b) conducted a detailed examination of the bioaccumulation of a series of PFCs, including five perfluorinated carboxylates and two sulfonates. Juvenile rainbow trout were used as the test organism. Dietary accumulation and accumulation through water phase were examined, where PFCs were exposed through the food and the water respectively. To study the kinetics of uptake and elimination, trout were subject to an accumulation phase with PFCs spiked either in the food or in the water, and a depuration phase with clean food and water. Uptake and elimination rate constants were found through curve fitting and used to calculate the kinetic BCF and kinetic bioaccumulation factor (BAF). The results showed that dietary exposure was the major route for the uptake of PFCs. The tissue distribution of the accumulated PFCs was examined and found to follow the order of blood > kidney > liver > gall bladder in rainbow trout. Among the tested compounds, a positive relationship between bioaccumulation potential and chemical molecular chain length was also identified.

Although this study pioneered the controlled laboratory bioaccumulation study of PFCs, the most important impact, however, was not the findings but rather the method of using a kinetic approach to evaluate BCF and BAF. Compared with the fundamental steady-state approach, the kinetic approach is fast and can avoid the excessive cost of chemicals. In the steady-state approach, exposure has to last until steady state is reached, where BAF is calculated using the steady-state concentration. The kinetic approach, therefore, became popular in later bioaccumulation studies of PFCs.

In Higgins et al. (2007) study, the bioaccumulation of four common PFCs from sediments in the aquatic worm, *Lumbriculus variegates*, was assessed. The kinetic approach was adopted to estimate the steady-state biota sediment accumulation factors (BSAFs): the worms were first exposed to PFC-spiked sediments and then to clean sediments. The results suggest that PFCs in sediment are readily bioavailable and bioaccumulation from sediments does not continually increase with fluorinated chain length as with bioaccumulation from water. The elimination constants were found to be quite low, which could be explained by lack of metabolic transformation and conjugation assisted excretions.

Using the same kinetic approach, the salinity effects on the two main routes of

accumulation of PFCs (i.e. direct uptake from surrounding water and dietary accumulation from contaminated food) were examined in Jeon et al. study (2010). Pacific oysters were acclimated and exposed to four different salinities for 56 days (28-day exposure and 28-day depuration). The BCF and BAF were calculated from the mechanistic mass balance model with relevant parameters obtained from curve fitting. PFC accumulation was found to increase with increasing salinity, with long chain PFCs partitioning more into oyster tissues than short chain ones. BAF increased proportionally with salinity, while BCF exhibited no trend. Compared with freshwater fish, oysters exhibited lower accumulation and faster elimination rates, and hence, lower bioaccumulation factors.

Previous studies also suggests that bioaccumulation of PFCs could be influenced by several factors. Besides salinity, other factors such as exposure concentration could also affect the bioaccumulation process. Although a number of studies have been carried out, the reported bioaccumulation and/or bioconcentration ratios of PFCs showed a lack of consistency. There could be several reasons for this: 1) Different organisms were targeted in these studies and PFCs bioaccumulation could be species specific. PFCs are proteinophilic compounds that tend to accumulate in protein rich compartments. Different organism may have different protein content and distribution thus may result in variations in PFCs absorption; 2) Different exposure levels. For other persistent organic pollutants, the partitioning ratio between different phases is regardless of exposure concentration. However, for compounds like PFCs which have unique physico-chemical properties and thus unpredictable behavior, exposure level may also be one of the variables that can affect the bioaccumulation of the compounds and 3) Different laboratory conditions. These factors should be taken into consideration in future PFC bioaccumulation studies. Moreover, previous studies mainly focus on finding out the bioaccumulation constant. Little is known regarding the kinetics and underlying mechanism of this process. This information could be useful in biomonitoring, model development and risk assessment.

2.2 Toxicological studies of PFCs

2.2.1 Previous findings

In previous animal studies, it has been found that PFCs can trigger the increased production of reactive oxygen species (ROS), which could lead to significant cell damage

such as DNA damage and oxidation of amino acid in proteins (Liu et al. 2007b). Some PFCs were identified to be peroxisome proliferators and can exert biological effects such as beta-oxidation of fatty acids, increased frequency of cytochrome P-450 mediated reaction and inhibited secretion of cholesterol from the liver (Kawashima et al. 1994, Kudo et al. 2000, Kennedy et al. 2004). Exposure to PFCs might also induce alterations in plasma concentrations of both steroidal androgens and estrogens (Oakes et al. 2005). Some PFCs have also been shown to be able to induce the activation of nuclear receptors (Shipley et al. 2004), interference in mitochondrial bioenergetics (Berthiaume & Wallace 2002) and have neuroendocrine effects (Austin et al. 2003). *In vitro* studies also have demonstrated the endocrine disrupting capacity of selected PFCs (Maras et al. 2006). At physiological level, studies have suggested that PFCs have potential toxicity in development and reproduction processes (Mulkiewicz et al. 2007). Experiments demonstrate that PFCs could be responsible for decreased sperm production as well as sperm deformity (Fan et al. 2005). Subchronic exposure to PFCs was shown to linked with delays in lung maturation (Grasty et al. 2005), increased liver weight accompanied by hepatotoxicity, and body weight loss (Kudo & Kawashima 2003, Lau et al. 2004). These toxicity data were mainly from experiments on mammalian species, especially in liver and serums.

2.2.2 Molecular level effects

Normally toxic effects from pollutants are initiated from bio-molecular levels (Newman 2009). After entering the inner environment of the organism, pollutants can interact with biomolecules such as lipid, protein and DNA. Biochemical reactions may take place as a result of this interaction. These low level alterations, compared with physiological effects, are generally sensitive to the contamination level. Therefore molecular level biomarkers are popular in environmental monitoring and toxicity assessment. Bio-endpoints measuring protein, enzyme activity and DNA integrity have also been applied in PFC toxicity testing.

Oxidative toxicity and antioxidant enzyme activity are the most common target toxic modes of action in environmental toxicity studies of organic compounds. One of the reasons is that conventional organic pollutants generally induce oxidative stress through the production of free radicals during their metabolism inside the organism. Therefore the presence of organic pollutants is often associated with the induction of antioxidant

responses. Although PFCs have been identified as metabolic inert compounds that hardly go through any biotransformation, many studies have demonstrated that PFCs can induce significant oxidative stress to the exposed organism. Liu et al. in their study (2007a) examined a batch of antioxidant enzymes activities in primary cultured hepatocytes of tilapia after exposure to PFOS and PFOA. After a 24-hour exposure, a significant increase of ROS was recorded, accompanied with activation of catalases (CAT), superoxide dismutase (SOD) and glutathione reductase (GR). Deactivation of glutathione peroxidase (GPx) and glutathione-S-transferase (GST) and decrease in glutathione (GSH) were concurrently observed. The overall results of these enzymes show that both PFOS and PFOA are able to produce oxidative stress in the target cells (Liu et al. 2007a). The compounds have been shown to be able to increase the fatty acyl-CoA oxidase activity which could result in the excessive ROS production (Liu et al. 2007a, Oakes et al. 2005). CAT and SOD levels were also found to be increased in rats after exposure to PFDoA, where the level of Thiobarbituric acid reactive substances (TBARS) was significantly increased as well (Zhang et al. 2008). The results demonstrated that long chain PFCs may also cause excessive ROS production and result in oxidative stress. However, there were also some contradictory results. Liu et al. (2009) in their study reported a suppression of SOD activity and total antioxidant capacity in postnatal mice. This observation was explained by the distinction in the degree of oxidative damage at different developmental stages.

In addition to antioxidant enzymes, PFCs related DNA damage has also been examined. Liu et al. (2007a) demonstrated that PFOS and PFOA can induce apoptosis in fish hepatocytes via the caspase pathway. The occurrence of visible DNA laddering further confirmed the apoptotic development (Liu et al. 2007c). A clear DNA fragmentation was observed after a 48-hour exposure for both PFOS and PFOA. The PFC induced oxidative stress was suggested to be responsible for the DNA damage, where ROS may interfere with mitochondrial function and lead to the activation of caspase which initiates apoptosis (Liu et al. 2007b). In Kim et al.'s study (2010) DNA single strand breaks were found to be significantly induced by PFOS but not by PFOA, suggesting a higher genotoxicity potential of PFOS.

Besides direct examination of changes in the macromolecule of genetic material, gene expression is also often employed to screen for genotoxicity potential and to understand the toxic mechanism. Gene expression profiles have been used extensively to

characterize the toxic effects of PFCs in many studies (Bjork & Wallace 2009, Guruge et al. 2009, Rosen et al. 2008, Shipley et al. 2004). The change in mRNA expression of several antioxidant activity related enzymes, PPARs, acyl-CoA oxidase, SOD, CAT and GPx, were examined in Arukwe and Mortensen's study (Arukwe & Mortensen 2011). The results showed that PFC exposure can up-regulate the mRNA and thus, the expression of antioxidant enzymes in response to the increased ROS production (Arukwe & Mortensen 2011). Bjork et al. (2009) also found in their study that PFCs cause concentration and chain length dependent increase in the expression of gene targets related to cell injury and PPAR activation in primary rat hepatocytes. Transactivation of PPAR is a critical pathognomonic event in PFC-induced toxicity (Bjork et al. 2009). There are a number of biological activities ascribed to PPAR activation, including peroxisome proliferation, hepatomegaly and decreased serum cholesterol (Andersen et al. 2008, Kennedy et al. 2004). Rosen et al. (2009) in their study stated that most transcriptional changes induced by PFOS were actually related to activation of PPAR. Variables related to physical properties, such as altered fluid dynamics of pulmonary surfactant, may be associated with PPAR-independent modes of action.

In a bacterial gene profiling assay (Nobels et al. 2010), the induction of stress response genes by PFCs was observed. The study evaluated 14 PFCs, which were shown to be able to induce genes that were related to oxidative damage, membrane damage, cellular and osmotic damage and DNA damage. Oxidative stress was shown to be one of the major pathways affected after PFC exposure by showing the most significant gene induction. Hagenshaars et al. (2008) tried to study the mode of action of PFOS at molecular level. After a subchronic exposure (14 days) to PFOS, a microarray was constructed with a carp liver-specific cDNA library to examine the effects of PFOS on gene transcription in liver cells. The data revealed that PFOS can influence the expression of several liver genes that are involved in energy metabolism, reproduction and stress response (Hagenshaars et al. 2008). The microarray technology may be applied to other organs such as haemolymph, as a tool to investigate possible molecular targets with exposure to PFCs. Wei et al. (2009) also examined the genotoxicity of several PFCs using microarray techniques. Gene profiles of primary cultured hepatocytes from rare minnows were analyzed for individual PFC as well as PFCs mixtures. It was shown that both mixture and individual compounds consistently regulated a particular gene set. Genes implicated in the study include xenobiotic metabolism, oxidative stress, immune response, fatty acid metabolism and transport, cell

death, etc. These conserved genes were suggested to play a central role in the toxicity mediated by PFCs (Wei et al. 2009).

These gene profiling studies provide fundamental evidence of observed toxic effects, such as oxidative damage, reduced body weight, cellular toxicity, etc. Gene expressions are very sensitive, or perhaps even the most sensitive assay, to any environmental stimulation. Therefore, examination of gene expression is popular in ecotoxicity studies for providing observable results. However, alterations at gene expression levels may not necessarily link with adverse effects at higher levels. For example, an increase in the expression of CAT does not confirm the occurrence of oxidative stress. Therefore, a combination of gene profiling and cellular/physiological investigation may provide more solid information on toxicity.

Other than enzymes and DNA, PFCs have also been reported to affect hormone levels in organisms. PFCs were shown to be able to interfere with the thyroid hormone and related genes in both rodents and fish (Chang et al. 2008, Du et al. 2009, Lau et al. 2003, Seacat et al. 2002, Wei et al. 2008). Thyroid hormones play an important role in regulating growth and development (Power et al. 2001). Therefore, thyroid disruption at early development stages could affect the growth of organism. Significant reductions of serum thyroxine and triiodothyronine have been demonstrated in rodents exposed to PFOS (Chang et al. 2008, Seacat et al. 2002). PFOA has also been shown to reduce the thyroid hormone level in rats by disturbing the expression of the hormone (Martin et al. 2007). A similar effect from PFOA was also reported in rare minnow, a freshwater fish species by Wei et al. (2007) according to microarray results. It was suggested that PFCs may affect the biosynthesis and metabolism of the hormone (Du et al. 2009).

Potential endocrine-disrupting activity of PFCs has been investigated through molecular level biomarkers that examine VTG and VTG gene expressions (Du et al. 2009, Kim et al. 2010). Liver VTG of zebra fish was shown to be up-regulated by PFOS which confirms the possible estrogenic activities of these compounds (Du et al. 2009). The estrogenic activities of other PFCs were also assessed in the hepatocytes of tipilia, where similar results were observed (Wei et al. 2007). Blood VTG level in male carps exposed to PFOA was reported to be significantly increased in a concentration-dependent manner (Kim et al. 2010). However, in contrary to previously mentioned studies, no significant alterations after PFOS exposure was reported in Kim et al.'s study (2010). Hence the author concluded that the estrogenic effect of PFOA is stronger than PFOS. The

contradictions among these studies are likely to result from the species specific behaviour of the compounds. Therefore, more toxicity data is needed before a conclusion can be reached. Moreover, although PFCs were shown to be able to disturb VTG level or gene expression, changes in sex ratio or other physiological level effects have not been observed. To better assess the induced estrogenic activity by PFCs, a combination of different levels of biomarkers would provide more comprehensive information.

Transporter protein activity, as part of the multixenobiotic resistance (MXR) mechanism, has been used frequently in ecotoxicity evaluation. The toxicological effects of 11 PFCs on the p-glycoprotein (p-gp) cellular efflux transporter have been investigated (Stevenson et al. 2006). Compounds including PFOA, PFNA and PFDA were shown to significantly inhibit the activity of the transporter protein, while PFOS did not exhibit any effects even though it is more bioaccumulative than PFOA. It was suggested that the suppression of transporter protein may be independent of the concentration of the compounds, but from an indirect way, such as by blocking the ATPase activity of the transporter.

In some previous studies of environmental pollutants, a batch of molecular biomarkers that targeted enzymes, protein and DNA were assessed concurrently to enable an integrated biomarker assessment. The integrated biomarker response (IBR) is becoming more and more popular in assessing the toxicity of contaminants, given that more than one biomarker is generally observed by exposure to toxic compounds since not all compounds have the same toxic mechanism on organisms. This approach has been applied to the toxicity assessment of PFCs as well. Kim et al. (2010) applied the IBR method to PFOS and PFOA to evaluate the toxicity of the two compounds in common carp, *Cyprinus carpio* (Kim et al. 2010). Five biomarkers was used, namely 7-ethoxyresorufin-O-deethylase (EROD), DNA single-strand breaks (Comet assay), acetylcholinesterase (AChE), vitellogenin (VTG) and catalase (CAT). The results showed that PFOA can increase the VTG and CAT level significantly compared with PFOS, which implies that PFOA can induce stronger estrogenic effect as well as produce more reactive oxygen species (subsequent antioxidative response) than PFOS. On the other hand, the presence of PFOS can induce significant DNA single strand breaks while no substantial effect was observed for PFOA exposure. It was suggested that PFOS could influence the average DNA base-pair length and thus, interfere with the homeostasis of DNA metabolism (Hoff et al. 2003). However, some of the findings from this study are somehow different from earlier toxicity

studies of PFOS and PFOA. For example, it was shown that the activity of AChE are not influenced by either compound, which is contradict with a previous conclusion that PFOS was related to the regulation of norepinephrine concentrations in the central nervous system of rats (Austin et al. 2003). Moreover, earlier *in vitro* tests have shown that both PFOA and PFOS can induce CAT activity (Hu & Hu 2009, Liu et al. 2007b), although it was also reported in some studies that no effects were observed for both compounds. These disagreements could be caused by different test species or different exposure concentrations and exposure period. Nevertheless, IBR is still a very useful tool for a comprehensive assessment compared with a single test.

2.2.3 Cellular level effects

Cellular level effects refer to damages to cells and cellular organelles. Although evaluation of cell viability has been employed in many PFC ecotoxicity studies, these cell viability assays were only applied as auxiliary tests and not as a mean to look at the cytotoxicity of PFCs (Liu et al. 2007c, Mulkiewicz et al. 2007, Wei et al. 2009). In Liu et al.'s study (2007), loss of cell viability after PFOS and PFOA treatment was observed in fish liver, which was suggested to be mediated through ROS induction and thus, oxidative stress. The significance of cellular level effects is equivocal. They are not as sensitive as molecular effects, and are not as ecologically relevant as physiological ones. In the investigation of PFC toxicity, there are only a few studies that examine the cytotoxicity of these compounds in human cells.

In the series of Kleszczynski et al.'s studies (2007, 2009 & 2011) on human HCT 116 cells, selected PFCs were found to decrease the viability of HCT 116 cells. PFCs were demonstrated to be able to induce perturbations in the calcium distribution, which is an important link to mitochondria dysfunction which plays a crucial role in apoptotic cell death (Kleszczynski & Skladanowski 2011). In addition, PFCs were observed to alter membrane potential which could cause membrane structural damage and may also lead to programmed cell death (Kleszczynski & Skladanowski 2009). Freire et al. in their study (2008) examined the cytotoxicity by PFOA in human Vero cells. Besides decreasing Vero cell viability, PFOA was found to interfere with mitochondrial integrity which was identified through observed morphological changes under the microscope. At elevated PFOA exposure concentration, significant cell cycle arrest was also identified (Freire et al. 2008).

Cytotoxicity in rat liver, dolphin kidney and fish blood cells have also been identified where adverse effects at cellular level could be caused by inhibition of intercellular communication (important for maintaining tissue homeostasis) and increased membrane permeability towards hydrophobic ligands (Hu et al. 2002, Hu et al. 2003).

To date, data regarding the cytotoxicity of PFCs in living organisms is still lacking. In fact, cellular level biomarkers have been applied in the investigation of other contaminants and water pollutions (Hannam et al. 2009, Sheir & Handy 2010), and were shown to be useful indicators of pollutant level. Dorts et al. (2011) investigated enzyme activity using proteomic analysis. It was reported that a number of proteins displayed significant changes under PFOS exposure. Those proteins were related to important cellular processes such as general stress response, energy metabolism, actin cytoskeleton and ubiquitin-proteasome system. The results of this study provide important hints for possible cellular level toxic effects from PFCs. Therefore, in future studies, one may want to include the cellular level toxic effect to complete the missing toxicological data of these compounds.

2.2.4 Physiological level effects

Potential physiological toxic effects of PFCs have been investigated mainly in rodent species and some in fish species (Cui et al. 2009, Hagenaaars et al. 2011, Lau et al. 2004, Zhang et al. 2008). In brief, exposure to different PFCs could lead to significant weight loss, hepatotoxicity and also affect the survival and growth of offspring.

In the investigation of PFC induced physiological effects, PFOS and PFOA are still the most intensively studied compounds. Cui et al. (2009) in their histological examination of rat organs showed that, after a subchronic exposure to PFOS and PFOA, abnormal behaviour of the dosed rats was observed, including reduced activity and reduced food intake which can be related with the significant weight loss of the organism. Pathological alteration and death were also observed for high PFOS-dosed organisms. In other studies, developmental effects that have been reported include reduction of fetal weight, cleft palate, anasarca, delayed ossification of bones and cardiac abnormalities (Lau et al. 2004). Most of these abnormalities were found also in the group with the highest PFOS dose. In addition, exposure to PFOS can result in reduced energy stores for maintenance and survival and thus, directly affect the fitness of the organism (Hagenaaars et al. 2008). Developmental effects on the offspring have also been well documented. Significant lag of

weight gain in the offspring of ammonium PFOA (APFO) treated rats has been reported by Butenhoff et al. (2004). An increase in mortality was recorded in both male and female pups. The post weaning mortality as well as delays in pubertal onset could be partially explained by the reduced body weight (Butenhoff et al. 2004).

Liver, lung and kidney were identified to be the target organs of PFCs. Hepatic toxicity had been reported in several studies (Seacat et al. 2003), where the liver has been identified as the primary target organ for PFCs due to their proteinophilic nature. It is hypothesized that the peroxisome proliferation induced by these PFCs could also cause the formation of hepatocellular hypertrophy, which then leads to the commonly observed hepatomegaly (Cui et al. 2009, Kennedy et al. 2004). Increases in relative liver weight were reported in rodents, monkeys and fish that were exposed to PFOS and/or PFOA (Austin et al. 2003, Hoff et al. 2003, Martin et al. 2003a, Seacat et al. 2003, Seacat et al. 2002). The lung is another important target organ of PFCs (Cui et al. 2009, Grasty et al. 2003). PFC induced effects in lungs may be attributed to their surfactant properties. The damage to lungs is probably because exposure to PFCs could alter the surfactant biosynthesis pathways whereby PFCs may influence some essential enzyme during the course of phospholipids synthesis (Fisher & Dodia 2001).

The kidney is another important organ involved in metabolism. Adverse effects in kidneys such as renal hypertrophy and morphological changes, after PFOS and PFOA treatment have been reported (Cui et al. 2009, Kennedy et al. 2004). The disturbance in kidney function provides some explanation of metabolism disturbance observed in PFC-dosed rats (Cui et al. 2010). Splenic atrophy has also been reported in rodents exposed to PFCs (Yang et al. 2000).

Other than PFOS and PFOA, the hepatotoxicity of perfluorododecanoic acid (PFDoA), the PFC with 11 fluorinated carbon, has been examined in rats that were orally dosed with the target compounds for 14 days (Zhang et al. 2008). PFDoA was also found to increase the relative liver weight. However, a reduction of the absolute liver weight (up to 26% of initial weight) was identified, which could be a result of the significant body weight loss under the exposure. Changes in the liver ultrastructure were also observed after treatment with PFDoA. The findings of this study demonstrated the consistency in PFCs' hepatotoxic effects by providing toxicity data of compounds other than the most commonly studied PFOS and PFOA.

Currently, physiological toxicity data of other PFCs is still lacking. Pharmacokinetic

studies have shown that for those PFCs with longer carbon chain, for example PFNA and PFDA, the elimination rates in rodent species appear to be several folds lower than that of PFOA. Thus, the toxicity potential of these compounds is likely to be higher than PFOA (Lau et al. 2004).

As mentioned earlier, physiological level effects were mostly examined in rodent species where they were used primarily as surrogates for humans. These toxicity data was then extrapolated and interpreted for human health risk assessment. Although the findings among different studies show good agreement, these data may be less useful for environmental toxicity assessment. For example, these data of PFC induced toxicity on liver, lung and kidney will have limited use on organisms without these organs, such as aquatic invertebrates, which however constitute a large proportion of the total organisms on earth. To serve the purpose of understanding environmental effects, a few studies have also been carried out using aquatic organisms as the target (Ankley et al. 2005, Hagenaaars et al. 2008, Hagenaaars et al. 2011, Oakes et al. 2004).

In Hagenaaars et al.'s study (2008), the toxic effects of PFOS in the common carp were examined. PFOS was demonstrated to significantly affect the general well-being of the organism; the glycogen content was significantly lower and the relative condition factor and hepatosomatic index also dropped with increasing exposure concentration, which could be related to the observed decrease in the available energy reserves. A stressful environment could negatively impact the general well-being of organism, including growth and reproduction (Hagenaaars et al. 2008). The increase in energy expenditure in the detoxification of PFOS seems to negatively affect the normal survival of the organism. Du et al. (2009) examined the chronic effects of water-borne PFOS on zebrafish fry in a partial life-cycle test. A series of adverse effects were reported which included larvae malformation, growth suppression and histopathological alteration in the liver. Long term exposure to PFOS was suggested to result in offspring deformation and mortality, therefore ecological impacts of PFOS would likely impair offspring survival (Du et al. 2009). Hagenaaars et al. (2011) in their study compared the effects of four PFCs in the embryo of zebrafish. Malformations were observed from the onset of hatching in embryos exposed to PFOS and PFOA. For PFBS and PFBA, this became apparent only after 96 h post-fertilization. Among the tested compounds, only PFOA was found to delay the hatching at concentrations higher than 50 mg/L. Similarly to fish fry, suppression of growth was observed in the PFOS and PFOA treated group, with no effects from the

shorter chain PFCs. It was suggested in the study that some of the higher level toxicity could be directly related to lower level effects. For example, apoptosis of cells could be one of the reasons for the malformation.

Overall, toxicity data from mammalian studies (with rodents) and fish studies both indicate PFCs (especially PFOS) can inhibit growth. Compared with molecular effects, physiological level effects are less frequently applied in ecotoxicity studies. Part of the reason is that biomarkers at this level are less sensitive to environmental stress. Significant responses at physiological level may be better observed at elevated and/or prolonged exposure. However, physiological effects are directly linked with organism health and thus, is more ecological relevant with respect to an ecotoxicity assessment.

2.2.5 Ecotoxicity study in plants

Besides biota, flora is also an essential component of the ecosystem. For the purpose of environmental impact assessment, the toxic effects of PFCs have also been evaluated in plants and algae (Boudreau et al. 2003, Ding et al. 2012, Li 2009, Phillips et al. 2007). Inhibition of growth from PFOS exposure was observed on the floating macrophyte, *Lemna gibba*, where the concentration of 50% inhibition was 0.058mM wet weight (Boudreau et al. 2003). Root elongation and germination are the common biomarkers applied in toxicity studies in plants. In Li et al.'s study (2009), PFOS and APFO's adverse effects on root elongation and seed germination were identified in lettuce, cucumber and pakchoi. Ding et al. in their studies (2012) tested the relative toxicity of seven different PFCs and a positive relationship of toxicity and fluorinated chain length was demonstrated. Similar results were also reported in Phillips et al. study (2007). These results of structure-activity relationships showed good consistency with the animal studies. However, there are some variations in the resulting EC₅₀ values (based on root elongation) among the different experiments. The deviations may be caused by different genotypes and experimental conditions (Ding et al. 2012).

In previous studies, algae have been applied as the representative of aquatic plants in PFC toxicity testing (Latala et al. 2009, Liu et al. 2008). Growth rate, cell density and chlorophyll concentration are the most commonly used parameters in the evaluations, whereby 50% inhibition/effect concentrations were calculated. A study of six PFCs reported that the IC₅₀ of long chain PFCs was in the range of 0.134-0.261 mM. The observed toxicity again increased with the chain length of the compounds (Liu et al. 2008).

A similar conclusion was later made in another study by Latala's group (2009) where the EC_{50} ranged at much higher levels from 0.28-12.84mM.

Compared with animal studies, most of the investigations on flora examined only the acute toxicity. Unlike animal studies, the effective concentration was usually targeted rather than the toxic mode of action. Therefore, only a few well-established biomarkers were used in these tests. Nevertheless, some common toxic mechanisms can be found in PFC induced effects in both animals and plants. For example, it was suggested that PFCs could be absorbed to the algal membrane-water interface and thus, disrupt the integral membrane of the algae, leading to the observed toxic response (Latala et al. 2009). Cell membrane disruptions were reported in animal studies as well (Kleszczynski & Skladanowski 2009). This toxic effect can be a result of the surfactant nature of PFCs. Toxicity tests in algae further confirm the potential adverse impacts of PFCs in the aquatic ecosystem. However, studies that focus on ecotoxicological consequences in the aquatic environment are still very limited. This is especially true for the coastal environment.

2.2.6 Structure-activity relationship and QSAR

Structure-activity relationship is the relationship between the chemical structure and the biological activity of compounds. In ecotoxicity studies, it provides useful mechanistic information regarding the toxic behavior of target compounds. Structure-activity relationship is especially useful for a group of compounds with a basic structure and varying attached groups, such as PFCs and PAHs. For newly emerging contaminants such as PFCs, the structure-activity relationship can be used to predict the toxic behavior of compounds in the same group when individual toxicity testing is not practical. For example, structure-activity relationships have been examined when four or more PFCs were investigated. Mulkiwicz et al. (2007) in their evaluation of five PFCAs (C5-C9), concluded that "the longer the fluorinated chain, the more toxic the acid" in terms of acute toxicity. This is possibly due to the short chain PFCs being eliminated from the organism more quickly than the longer chain compounds (Mulkiwicz et al. 2007). In Kleszczynski et al.'s study (2007), a chain length dependency of EC_{50} of cytotoxicity has been observed, where the estimated EC_{50} value was found to decrease with the elongation of fluorinated carbon chain between C5-C13 PFCAs. However further elongation (C15, C17) did not deepen the effect (Kleszczynski et al. 2007). Other cellular level adverse effects including depolarization of the plasma membrane and acidification of cytosol were also found to be

positively correlated with fluorocarbon chain length in a later study by the same group (Kleszczynski & Skladanowski 2009). Examination of both PFCAs and PFSA by Bjork et al. (2009) showed that both groups caused a chain length dependent increase in the expression of gene targets related to cell injury and PPAR activation in rat hepatocytes. This observation was explained as a simple toxicokinetic phenomenon driven by internal dose of the respective PFCs (Bjork & Wallace 2009). In bacteria, both chain length and functional group effects were examined in Nobel et al. study (2010). Although effects seen at gene expression level were higher for the sulfonate than for the carboxylate, the effect of chain length is still more important than the functional group (Nobels et al. 2010). Similar conclusion was also reported in Hagenaars et al. study (2010) where PFCs with a sulfonate group were shown to have a larger toxicity potential. The chain length effects on PFCs toxicity has also been reported in microalgae (Latala et al. 2009). Among tested compounds, C5-C8 PFCA, distinct relationship between hydrophobicity and toxicity is demonstrated; for every extra perfluorocarbon, the acute toxicity increased twofold. Log EC₅₀ was found to be well linearly correlated with both carbon number and K_{ow} (Latala et al. 2009). The preferential adsorption of long chain PFCs to the algal membrane-water interface by hydrophobic interaction and accumulation were used to explain the observed structure-activity relationship.

Other than the above mentioned qualitative analysis of structure-activity relationships, several studies also examined the quantitative structure-activity relationship. Wang et al. (2011a) constructed two linear quantitative structure-activity relationship models using octanol-water partition coefficient and C₁₈-EmporeTM disks/water partition coefficient and demonstrated that hydrophobicity was an important parameter in describing the toxic activity of the compounds. A bioluminescence inhibition assay was applied to assess the acute toxicity of C3-C18 PFCAs. A tendency of increasing toxicity from C3 to C14 PFCA and a tendency of decreasing toxicity from C14 to C16 PFCA were also identified (Wang et al. 2011a). A cross species QSAR model has also been developed based on the toxicity results on root elongation in lettuce and photosynthesis on green algae (Ding et al. 2012). In this study, the number of fluorinated carbons was used as the molecular descriptor and a linear regression model was established. These QSAR models could be useful in environmental pollutant control and ecological risk assessment. However, to date, only a few QSAR models are available, and only PFCAs have been considered in these models.

2.3 Summary and implications

Although efforts have been made, there are a number of limitations of previous studies. Firstly, most of the investigations examined PFOS and PFOA only, since these two are the most commonly detected PFCs that comprise a major proportion of total PFCs. Due to the effort to phase out these compounds, the production of PFOS and PFOA has been significantly reduced. In recent environmental monitoring data, the pattern of PFCs distribution has also changed where the proportion of short chain PFCs and other long chain ones, such as PFNA and PFDA, has been increased. However, ecotoxicity data on these compounds remain scarce. The lack of data of different PFCs also makes it difficult for mechanistic analysis, such as the development of a QSAR model. Secondly, toxicity studies of PFCs still largely focus on human health effects and therefore, only limited animal species (e.g. rodent) were used as the test organism. Although there are also a few studies that aim to look at the ecological impacts in natural species such as fish. The available information is still very limited for a comprehensive understanding of the environmental and ecological consequences of PFCs. Thirdly, most of the ecotoxicity examinations only focus on molecular toxic effects. As chemically inert compounds, the toxic effects of PFCs are not as easily detected as other pollutants and therefore, molecular level alteration is frequently examined due to their good sensitivity. However, molecular level alteration may not necessarily lead to higher level adverse effects and damage, not to mention the health of the organism. Thus, molecular biomarkers are less ecologically relevant. More high level toxic effects should be included in the investigation to generate toxicity data that are relevant for environmental risk assessment. Last but not least, although different toxic modes of action have been examined in different organisms, these data are difficult to correlate and compile. Some contradictions and disagreements exist in the toxicity results due to different laboratory conditions or species specificity. A systematic investigation on a single species may thus be able to provide a holistic idea and comprehensive understanding of the effects of PFCs on an organism. It will generate consistent and relevant data, which enables the interpretation and correlation of results of different toxic effects and facilitate the understanding of toxic mechanism of the compounds.

Chapter Three Bioaccumulation of Perfluorinated Compounds

3.1 Introduction

Bioaccumulation is an integral part of an ecotoxicity study. It is important to understand and predict the accumulation behavior of contaminants in biota for several reasons. Effects are generally a consequence of concentration in target tissues or organs (Thompson et al. 2005), and bioaccumulation of contaminants is directly linked to toxicity to the affected organism or predators. A bioaccumulation study generates insights into an "internal dose" of contaminants, which is more ecotoxicologically relevant than the environmental concentration of pollutants (Fernandez-Sanjuan et al. 2010). Also, human exposure to environmental contaminants often occurs through the consumption of tainted food, for example polluted fish. In addition, bioaccumulation is part of the chemical's environmental fate. Understanding the bioaccumulation is crucial in the mitigation of possible risks related to the pollutants, as environmental safety levels generally take into account the toxicity of the substance, its persistence and also the ability to bioaccumulate. Bioaccumulation study will therefore, help to provide the environmental benchmarks against which environmental monitoring data can be assessed, and to set goals for the pollution controls.

As an emerging group of contaminants, information regarding the bioaccumulation of PFCs is insufficient to assess their partitioning behavior and environmental fate. It is also necessary to understand the bioaccumulation process of the compounds before further examination of their toxicity and ecological impacts. In this chapter, the bioaccumulation behaviors of PFCs in green mussels were investigated to understand the bioaccumulation mechanism of tested compounds and identify the bioaccumulation factors.

3.2 Detection and accumulation of PFCs in mussels in Singapore

Singapore is a highly urbanized, industrialized city, which potentially have high PFC contamination. The manufacturing processes of electronics, electrical goods, plastic goods and textile are also expected to be important sources of PFCs (So et al. 2004). Previous investigations have detected high levels of PFCs in Singapore's surface water (Nguyen et al. 2011). Detectable concentrations were also reported in the coastal environment (Hu et al. 2011). The presence of these contaminants is threatening to the health of the aquatic ecosystem. However, little is known about the environmental distribution of PFCs, especially in biota. Even less is known regarding the toxicological effects of PFCs on organisms exposed to them.

The purpose of the current study was to preliminarily examine the bioaccumulation behavior of two commonly detected PFCs, namely PFOS and PFOA, in tropical environments through both field study and controlled laboratory study. A local species of green mussel, *Perna viridis*, was used as the target organism.

3.2.1 Materials and Methods

3.2.1.1 Chemicals and standards

Potassium perfluorooctanesulfonate (PFOS, 98%), perfluorooctanoic acid (PFOA, 96%), were purchased from Sigma-Aldrich (St. Louis, MO). The internal standards, perfluoro-n-[1,2,3,4-¹³C₄]octanoic acid (MPFOA, 99%) and sodium perfluoro-1-[1,2,3,4-¹³C₄]octanesulfonate (MPFOS, 99%) were purchased from Wellington Laboratories (Guelph, ON). Optimal grade methanol and HPLC grade acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA). Supelclean ENVI-Carb 120/400 was from Supelco through Sigma-Aldrich. The stock solution was prepared with PFOS and PFOA at identical concentrations in optimal grade methanol. The stock solution was stored at 4 °C.

3.2.1.2 Controlled laboratory experiment

Green mussels, *Perna viridis* (protein content: 12%; lipid content: 1.4%) from a local fish farm were firstly acclimated for three days in laboratory conditions. After that, mussels with shell length of 60-70 mm were transferred to tanks filled with artificial seawater spiked with 0.4 ng/ml PFCs (0.2 ng/ml PFOS + 0.2 ng/ml PFOA). Duplicated tanks were used for both exposure set and the control set. The exposure period was 96-hr and the tanks

were cleaned and refilled every 24-hr. Mussels were sampled every 24-hr for testing.

3.2.1.3 Field sample collection and preparation

Environmental samples (mussels and seawater) were collected from six locations around Singapore coastline in late June (Figure 3-1). They were stored in polypropylene (PP) bags in an ice box during transportation to the laboratory.

Figure 3-1 Sampling locations around Singapore coastal waters.

For both laboratory and environmental samples, the gills of mussels were excised and freeze-dried at -80°C for 72 hours. The extraction method has been described elsewhere (Stevenson et al. 2006). In brief, homogenized dry gill tissues were transferred to a 50ml PP centrifuge tube and 3ml of acetonitrile was added. The mixture was vortexed and sonicated in a heated water bath for 10 mins. The samples were then centrifuged at 1500 rpm for 10 mins and decanted to 15 ml PP tubes. The above steps were repeated three times, and all centrifuged extracts were combined. 1.8 ml of the extracts was transferred to a 2 ml centrifuge tube, and acidified by adding 50 μl glacial acetic acid (1% v/v). The extracts were then purified by adding 25 mg of dispersed sorbent (ENVI-Carb, 25-50mg). The tubes were vortexed and centrifuged for 20 min at 18000 rpm. Purified extracts were diluted 1:1 in ultra pure water (100 μl purified extracts + 100 μl UPW into sample vial). Prior to analysis, 20 μl of internal standards were also added.

3.2.1.4 Analytical method

Concentrations of PFCs in mussel tissues were determined using high-performance liquid chromatography coupled with tandem mass spectrometry (LC MS/MS) (LC: Shimadzu LC-10 AD; MS/MS: API3000 AB Sciex, Toronto, Canada). The MS/MS was operated in negative electrospray ionization multiple reaction monitoring (MRM) mode. A volume injection of 20 μ L was injected into a Targa Sprite C18 column (3.5 μ m pore size, 40 mm $\times$ 2.1 mm ID, Higgins Analytical, CA, USA) using the following chromatography program: methanol was increased from 35% to 100% from 0 to 7.5 min, held at 100% to 10 min, then returned to 35% until 15 min at a flow rate of 0.25 mL min⁻¹ with 2 mM ammonium acetate as the second mobile phase (Higgins et al. 2005).

3.2.2 Results and Discussion

3.2.2.1 PFC levels in environmental green mussels

In the environmental samples, the overall tissue concentrations of PFOA and PFOS ranged from 0.05 to 0.28 ng/g wet weight (ww) and 0.52-1.74 ng/g ww, respectively (Table 3-1). The resulted concentration could be slightly lower if whole body concentration was used instead of gill concentration. Compared with a study of mussels from South China and Japan (So et al. 2006), the level of PFOS in green mussels is about 2-3 times higher in Singapore coastal waters. The PFOS level is also higher than those detected in clams in the eastern Arctic marine food web (0.28 ng/g ww) (Tomy et al. 2004). However, based on a review of biomonitoring results of global PFCs, the level of PFOS in Singapore is still considered low, since it is below the 2 ng/g ww benchmark identified in previous studies (Houde et al. 2008). Elevated concentrations of PFOS (9-877 ng/g ww) were detected in invertebrates from the United States and European countries (Houde et al. 2006). Compared with PFOS, PFOA was rarely detected in marine invertebrates (Houde et al. 2008) or it was detected at very low levels like in the current study. However PFOA was predominant in fish sampled in Europe and Asia (Houde et al. 2008).

Table 3-1 PFOA and PFOS concentrations in mussels around Singapore coastline.

No.	Location	PFOA ng/g wet weight	PFOS ng/g wet weight	Total PFC (PFOA+PFOS)
S1	Tuas	0.24	1.74	1.98
S2	Laborador Park	0.17	1.70	1.87
S3	East Coast	0.05	0.52	0.57
S4	Changi	0.28	1.46	1.74
S5	Punggol	0.16	1.53	1.69
S6	Lim Chu Kang	0.08	1.01	1.09

In Singapore coastal waters, the highest overall tissue PFC concentration (PFOS+PFOA) was detected at Tuas (1.98 ng/g ww), which is an area with dense industry. Industrial influent is identified as one of the major sources of perfluorinated chemicals (Plumlee et al. 2008). Elevated levels of PFCs have been detected in coastal water in industrial areas in Hong Kong, South China, Korea and Singapore (Hu et al. 2011, So et al. 2004). Mussels from Punggol, Changi and Laborador Park also contain high concentrations of PFCs. These sites are either close to ship maintaining yards or busy shipping lanes, where lubricant, paints and surfactants used in the ship industry could contribute to the PFCs pollution. The East Coast of Singapore is a recreational area with less industry, and hence, is less contaminated by perfluorochemicals. The lowest PFC concentration (0.57 ng/g ww) was detected at this location (Table 3-1).

There could also be a seasonal variance of PFC levels in mussels, although not measure in the current study. Singapore is characterized by the Northeast and Southwest Monsoons and the inter-monsoon period. Higher concentrations of PFCs might be expected during the wet season (typically Dec to Feb) mainly because there will be more surface runoff inject to the sea, which carries significantly heavier load of PFCs than the seawater (Hu et al. 2011), and meanwhile possible atmospheric deposit by rainwater.

3.2.2.2 Comparison between PFOA and PFOS

In mussel tissues, the concentrations of PFOS are about 5-10 times higher than the concentrations of PFOA in the test results. However, in coastal water, the concentration of

PFOA was generally predominant over PFOS. The percentages of compounds in seawater and mussel tissue are shown in Figure 3-2. For example at S4, PFOA accounts for 71% of total PFC (PFOA+PFOS) concentration in seawater, while in mussel tissues, this compound makes up only 16% of the total PFC concentration. At S5, although PFOA and PFOS were detected at similar levels in the water, the concentration of PFOS in mussel tissues was about ten times higher than that of PFOA. Similar results were observed in the remaining locations as well. Previous monitoring studies have also reported that PFOA was generally detected at lower level than PFOS in wildlife (Houde et al. 2008).

Figure 3-2 Comparison of percentage composition of each PFC in seawater and mussel tissue.

One possible explanation is that concentrations in seawater fluctuate with time, while tissue concentration is relatively steady and may not respond to environmental levels so quickly. However, the most probable reason is that PFOS is more bioaccumulative in mussel tissues when compared with PFOA. This conclusion is further supported by the controlled laboratory experimental results.

3.2.2.3 Bioaccumulation potential of PFCs

The controlled laboratory exposure experiment showed that both PFOS and PFOA were accumulated in mussel gills during the exposure period. The time trends of bioaccumulation are shown in Figure 3-3.

Figure 3-3 Bioaccumulation trends in mussel tissues during 96-hr exposure.

During the 96-hr exposure, the concentrations of both PFOS and PFOA in mussel gills increased in a linear manner. The rate of bioaccumulation of PFOS, which is represented by the slope of the line, is approximately 17 times higher than PFOA. At the end of the exposure period, the concentration of PFOS was approximately 20 times higher than that of PFOA. These results imply that PFOS has a greater bioaccumulation potential and serves to explain the monitoring results where the concentration of PFOS in mussel tissues was higher than PFOA, even though PFOA is prevailing in the ambient environment. In a study of oysters in temperate waters, accumulation of PFOS in tissues was found to be about 10 times that of PFOA after 28-d exposure (Jeon et al. 2010). A similar result was also found in a study of freshwater invertebrates, after exposure to PFOS for 60 days where the organism concentration reached about 5 times as much as PFOA (Higgins et al. 2007).

There are some possible explanations for the higher bioaccumulation potential of PFOS. Firstly, the fluorinated chain for PFOS is longer than PFOA (Table 3-2). Since the fluorinated chain is the hydrophobic part of a perfluorinated compounds, longer chain length may result in enhanced hydrophobic interaction. In addition, the functional group of PFOS ($-\text{SO}_3^-$) provides stronger ionic interactions than the functional group of PFOA ($-\text{COO}^-$).

COO⁻). Both of these structural features favor the partitioning process for PFOS.

Table 3-2 Chemical properties of PFOA and PFOS.

Compounds		Chemical structure	Fluorinated chain length	Formula	Molecular weight
PFOA	perfluorooctanoic acid		C7	C ₈ HF ₁₅ O ₂	414.07 g/mol
PFOS	perfluorooctane sulfonate		C8	C ₈ HF ₁₇ O ₃ S	500.13 g/mol

3.2.2.4 Mussels as monitoring tool

Results of this study suggest that mussels could be a suitable water quality monitoring tool. Previous occurrence studies of PFCs only measured the compounds concentrations in the water phase (Hu et al. 2011, Nguyen et al. 2011). However, these results normally fluctuate with time and thus, can only reflect the water quality at specific sampling times. Moreover, levels of contaminations in the water phase may not help the assessment of the health state of aquatic ecosystem. On the contrary, mussels are sessile organisms. A measure of tissue concentration could reflect the water condition over a period of time and thus, the accumulated effects from their living environment. Therefore mussels could be a good indicator of PFC contamination and also the sentinel organism to study the ecotoxicological effects of the compounds.

In summary, although Singapore is highly industrialized and urbanized, its marine contamination of perfluorinated compounds still remains at a low level when compared to the US and European countries. Heavy industry and busy shipping areas are more likely to be contaminated by PFCs. In mussel tissues, PFOS was found to be the dominant compound although PFOA was normally detected at higher concentration than PFOS in the surrounding seawater. This observation suggests a higher bioaccumulation potential of PFOS than PFOA in green mussels. To our knowledge, this study represents the first measurement of accumulation of PFCs in invertebrates in the tropical marine environment. The results fill in the gaps in the monitoring data of PFCs in marine invertebrates. The preliminary study also highlights the need for extensive bioaccumulation investigation, as bioaccumulation results are critical for environmental risk assessment.

3.3 Bioaccumulation model of PFCs – the concentration dependency

It is important to understand the behavior of PFCs in the environment: their source, fate and toxic effects, in order to mitigate possible risks. Hence the study of bioaccumulation, which is part of environmental fate, is crucial. From a toxicological point of view, bioaccumulation *per se* is not necessarily of concern. What is of concern is that bioaccumulation can cause toxicity to the affected organism or predators of that organism. Aquatic organisms can accumulate PCBs a 100,000 times that of the water concentration. Clearly, chemicals which bioaccumulate have the potential to cause unusual impacts, travel unusual pathways and exert severe toxic effects (MacKay 2001). Bioaccumulation study generates insights into the internal dose of contaminants which is more ecotoxicologically relevant than the environmental concentration (Fernandez-Sanjuan et al. 2010). Continuous accumulation of certain compounds at low environmental level could lead to elevated internal concentration. For example, notable concentrations of PFCs were detected in marine organisms although their concentrations in seawater are relatively low. Moreover, as a result of their unique properties, an assessment of PFCs bioaccumulation requires different approaches compared with those well studied POPs. PFCs are both hydrophobic and oleophobic. They possess high affinity to protein albumin, and therefore, are sometimes referred to as proteinophilic (US EPA 2009, de Vos et al. 2008, Rayne & Forest 2009). Both monitoring and laboratory studies have demonstrated that PFCs accumulate mostly in protein-rich compartments such as livers and blood (Kelly et al. 2009, Kennedy et al. 2004, Shaw et al. 2009). The accumulation mechanism and exposure routes of PFCs are thus different from other hydrophobic persistent organics, such as PCBs. Thus, the commonly used octanol-water partition coefficient K_{ow} is inappropriate and inaccurate to predict the bioaccumulation of PFCs.

Discrepancies in the reported bioaccumulation data (especially the bioaccumulation factor (BAF)) of PFCs exist among laboratory studies (Kelly et al. 2009, Lau et al. 2007). There could be several reasons for this. Firstly, bioaccumulation of PFCs could be species dependent (for example when considering the protein content) and easily affected by environmental variables. Furthermore, most studies adopted kinetic approaches which involve a number of assumptions, estimations and curve fittings (Higgins et al. 2007, Jeon et al. 2010, So et al. 2004, Wang et al. 2010). While the underlying mechanism of PFC bioaccumulation is still unclear, direct measurement of the bioaccumulation factor or the

fundamental steady-state approach may elicit greater and more accurate insight. Moreover, in some review papers, it has been pointed out that PFCs bioaccumulation is likely to be concentration dependent (Conder et al. 2008, Kelly et al. 2009). However exposure concentration has never been considered as a parameter in previous laboratory studies.

With these questions in mind, the purpose of the current study was to examine the bioaccumulation mechanism of four common PFCs, namely PFOS, PFOA, PFNA and PFDA, in green mussels *Perna viridis*, and, in particular, to characterize the concentration effects on the bioaccumulation process. The validity of the commonly used kinetic approach was also assessed in this study.

3.3.1 Materials and Methods

3.3.1.1 Chemicals and standards

PFOS (98%), PFOA (96%), perfluorononanoic acid (PFNA, 97%) and perfluorodecanoic acid (PFDA, 98%) were purchased from Sigma-Aldrich (St. Louis, MO). The internal standards, MPFOA (99%), MPFOS (99%), perfluoro-n-[1,2,3,4,5-¹³C₅]nonanoic acid (MPFNA, 99%) and perfluoro-n-[1,2-¹³C₂]decanoic acid (MPFDA, 99%) were purchased from Wellington Laboratories (Guelph, ON). Optimal grade methanol and HPLC grade acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA). The stock solution was prepared with PFOS, PFOA, PFNA and PFDA at identical concentrations in optimal grade methanol. The stock solution was stored at 4°C.

3.3.1.2 Bioaccumulation experiment set-up

To examine the effects of concentration on bioaccumulation, two exposure concentrations were used: a total PFC concentration of 4 gL⁻¹ and a total PFC concentration of 40 gL⁻¹, with 1 gL⁻¹ and 10 gL⁻¹ of each test compound respectively. A 70 liter PP tank was used as the test chamber. 60-65 mussels with shells 60-70mm in length were raised in artificial seawater. Two sets of duplicate tanks were used for each exposure concentration. In one set, mussels were exposed to PFCs for up to 56 days; in the other set, mussels were subject to a 24-day exposure followed by a 24-day depuration phase. The purpose of this experimental design was to find out and compare the bioaccumulation factor through both steady-state (former set) and kinetic (later set) approaches (Figure 3-4). Besides exposure tanks, another duplicate tanks were set as the control, where no PFCs were present. All tanks were cleaned and refilled every two days. Mussels were sampled

from each tank every two, four and eight days. Aqueous samples were also taken every four days.

Figure 3-4 The schematic of experiment set-up for bioaccumulation of PFCs in green mussels.

3.3.1.3 Mussel Rearing

Mussels were purchased from a local fish farm. They were acclimated to laboratory conditions for one week before the PFC exposure experiment. Mussels were raised in artificial seawater by mixing commercial sea salts and distilled water. The water temperature was maintained at 25°C, and salinity was 30ppt. A 12-h light cycle was used. Commercial dense algae (Reed Mariculture Inc. Campbell, CA) were used to feed the mussels. The dense algae were approximately 2×10^9 cells/ml (dry weight 9%). Mussels were fed twice a day. The feeding density was set as 1.25×10^6 cells/L after a trial feeding test, which was aimed to achieve maximum food conversion/utilization.

3.3.1.4 Sample preparation and extraction

The entire mussel tissues were removed from the shells with scalps. The tissues were cleaned thoroughly in Milli-Q water to rinse off exposure media. Tissues were then freeze dried at -80°C for 72hr. Dry mussel tissues were ground and homogenized with petal and mortar. Homogenized tissues were stored at -80°C until extraction.

A few extraction methods have been described in the literature (Stevenson et al. 2006, Wang et al. 2010). In order to validate the precision and accuracy of PFC determinations in mussel tissues, two popular extraction methods, acetonitrile extraction (Stevenson et al.

2006) and alkaline digestion with SPE (So et al. 2006) were tested and compared. A pre-extraction spike experiment showed that for the acetonitrile extraction method, recoveries for the four analytes were within 75-100%; and for the alkaline digestion and SPE extraction method, 95-110%. Therefore, the latter was applied in this experiment.

Homogenized dry mussel tissues were transferred to a 50ml PP centrifuge tube. 30ml of KOH (0.01mol/L in methanol) was added. The mixture was vortexed and shaken at 300 rpm at 25°C for 18 hours. Digested samples were then centrifuged at 4000 rpm for 15mins. 0.5 ml of supernatant was diluted to 50ml with Milli-Q water and vortexed. The diluted extracts were then extracted using Oasis HLB cartridges (0.2g, 6 cm³; Waters). Prior to loading, cartridges were preconditioned by eluting with 5ml methanol followed by 5 ml Milli-Q water. Diluted extracts were eluted at 1 drop/sec. Cartridges were vacuum-dried before elution using 15 ml methanol. Elutes were dried by gentle nitrogen gas (99.99%, Soxal, Singapore) and reconstituted to 2 ml with methanol. Prior to analysis, 200µl of final extracts were transferred to a sample vial. 20 l internal standards were then added.

3.3.1.5 Instrumental analysis

Concentrations of PFCs in mussel tissues were determined using high-performance liquid chromatography coupled with tandem mass spectrometry (LC MS/MS). The procedures have been described in Section 3.2.1.

3.3.1.6 Quantitation and QC/QA

Calibration curves were constructed from the analysis of 16 calibration standards (range 0.2-60 ng/ml). Active points on calibration curves were calculated to be within 10% of their true values. The correlation coefficient was >0.99 for all calibration curves. Calibration was performed at the beginning and end of every sample batch. The limit of quantitation (LOQ) of each compound was the lowest concentration in the calibration curve with a signal to noise ratio (S/N) larger than 3. Solvent blanks were analyzed with every 12 samples to monitor instrument background. Matrix spike was performed in selected samples. No significant matrix interference was found (spike recovery within 10%). Contact with glassware and Teflon surfaces was avoided to minimize losses due to sorption and contamination. All containers were cleaned with methanol followed by Milli-Q water before usage.

3.3.1.7 Data analysis

Bioaccumulation factor was calculated according to

$$BAF = \frac{C_o}{C_w} \quad (3.1)$$

where C_o is the PFC concentration in the organism at steady state (ng/g); C_w is the PFC concentration in water, or the exposure concentration (g/l); BAF is in L/kg. Steady state was assumed when three or more consecutive measurements were not statistically different, or when the normalized slope of the fitted line of three or more consecutive measurements was less than 0.005 (1/day). One-way ANOVA was applied to determine the statistical significance.

3.3.2 Results and Discussion

3.3.2.1 Bioaccumulation results

Among the tested compounds, the long chain perfluorinated carboxylate and the perfluorinated sulfonate were found to have the highest bioaccumulation potential. PFDA possess the largest BAF followed by PFOS (Figure 3-5). PFOA, on the other hand, is the least accumulative compound with a steady state concentration about 20 times lower than PFOS. This is consistent with the observation that PFOS is generally detected at higher level in wildlife than PFOA, although the environmental concentrations of the two are comparable (Houde et al. 2008, Hu et al. 2011). Compounds with higher BAF take longer to reach steady state, which is also consistent with previous studies (Martin et al. 2003a, b, Conder et al. 2008), and their depuration is generally slower too. Field BAF can also be calculated for PFOS and PFOA using data in previous field experiment (section 3.2). The results are 191-305 L/kg and 8-13 L/kg for PFOS and PFOA respectively. These values are comparable but slightly lower than the laboratory BAFs. It could be due to the interactions from other pollutants in the real environment, especially interactions from each other, for example, competing for the binding sites.

PFC depuration follows a first-order (exponential) model with rates increasing in the order of PFDA<PFOS<PFNA<PFOA (Figure 3-6). The fast elimination may be facilitated by the presence of PFCs in the circulating blood, coupled with extensive blood-water exchange at mussel gills during respiration (Kelly et al. 2009). It also implies that there should be a continuous exposure of PFCs at a certain level to maintain an observed tissue

concentration.

Figure 3-5 Bioaccumulation of PFCs in green mussels at exposure concentrations of 1ppb and 10ppb during 56-day exposure. Data points are steady-state experimental results; curves are Matlab curve fitting.

Figure 3-6 Depuration kinetics of PFCs after exposure to concentrations of (a) 1 ppb and (b) 10 ppb.

3.3.2.2 Concentration dependency of PFCs bioaccumulation

Figure 3-5 shows that when exposure concentration (C_w) changes from low (1 gL^{-1}) to high (10 gL^{-1}), steady state organism concentrations (C_o) do not increase proportionally. The BAF values (Equation 3.1) of each compound are different at the two exposure concentrations ($p < 0.01$, t test) and decrease with increasing exposure by a factor of 1.3 for PFOA and 1.8 for PFDA (Figure 3-5, Table 3-3B). For each compound, the time to reach steady state is longer under the lower exposure concentration where the BAF is also higher. These results show that the bioaccumulation of PFCs is concentration dependent.

Although the concentration dependency of PFCs bioaccumulation has been mentioned in previous studies (Morikawa et al. 2006, Giesy et al. 2010), insight into the underlying factors have been lacking. A possible explanation of the observed results is that bioaccumulation of PFCs is an adsorption-like process in which PFC molecules adsorb to the surface of quasi-solid materials, and the rationale is that PFC molecules are surface active chemicals (Conder et al. 2008). The conventional bioaccumulation model only views tissues as a bulk phase and the biological uptake as simple partitioning process. The mechanism of chemical adsorption is shown as follows (Sylvin 2005):

M = chemicals S = free binding sites MS = bonded chemicals

k_u = uptake rate constant k_e = elimination rate constant

For PFCs, the binding sites are most likely at the surface of hemocytes and liver cells. As mentioned previously, PFCs tend to accumulate in these protein-rich compartments. In adsorption, the binding sites limit the amount of adsorbate, and the fractional surface coverage of adsorbent, θ , depends on the concentration of adsorbate:

$$\begin{aligned} k_u [M][S] &= k_e [MS] \\ [MS] &= \frac{k_u}{k_e} [M][S] = \alpha [M][S] \\ \therefore \theta &= \frac{[MS]}{[MS] + [S]} = \frac{\alpha [M][S]}{\alpha [M][S] + [S]} \\ \theta &= \frac{\alpha [M]}{\alpha [M] + 1} = \frac{\alpha M}{1 + \alpha M} \quad \alpha = \frac{k_u}{k_e} \end{aligned} \quad (3.3)$$

Table 3-3 Bioaccumulation factors and curve fitting parameters.

	perfluorinated chain length	A		B		C	D – Proposed model		E – Old model			
		k_e (1/d)		$BAF_{ss} = C_o/C_w$ (L/kg)		α (L/kg)	$BAF_{kinetic} = \frac{nk_u}{k_u C_w + k_e}$ (L/kg)		k'_u (L/kg/d)		$BAF'_{kinetic} = k'_u/k_e$ (L/kg)	
		1ppb	10ppb	1ppb	10ppb		1ppb	10ppb	1ppb	10ppb	1ppb	10ppb
PFOS	8	0.05±0.01 (0.95)	0.05±0.01 (0.96)	378±29	235 ±13	0.07±0.02	386±37* (0.96)	236±17 [#] (0.97)	25.4±22.9 (0.77)	19.3±3.9 (0.99)	472±447* (0.77)	384±126 [#] (0.99)
PFOA	7	0.10±0.04 (0.95)	0.10±0.04 (0.96)	15±1	12±0	0.03±0.01	15±1 (0.97)	12±1 (0.94)	1.9±0.4 (0.99)	1.9±0.4 (0.99)	19±8 (0.99)	18±7 (0.99)
PFNA	8	0.09±0.04 (0.92)	0.09±0.04 (0.93)	144±14	109±14	0.05±0.02	149±12 (0.97)	105±7 (0.96)	10.4±0.0 (1.0)	13.1±0.0 (1.0)	112±50 (1.0)	146±64 (1.0)
PFDA	9	0.05±0.01 (0.93)	0.04±0.01 (0.95)	838±66	464±25	0.10±0.03	859±93 (0.97)	473±40 (0.96)	62.1±16.8 (0.98)	40.6±3.5 (0.99)	1375±569 (0.98)	965±297 (0.99)

*A: k_e estimated from elimination phase where $C_o = A \cdot \exp(-k_e t)$; B: BAF_{ss} was the average of steady-state results; D: nk_u and $(k_u C_w + k_e)$ are directly obtained from curve fitting results; E: k'_u estimated from initial uptake phase (Jeon et al. 2010); n=12; All curve fittings were carried out by Matlab. Data are provided with standard error ($\pm SE$) and R^2 in parentheses. *[#] highlight that data in the columns with the same superscript are significantly different at $p < 0.01$.*

Therefore the amount of adsorption depends on the chemical concentration. From this adsorption model, the major findings in this experiment can be well explained: 1) exposure concentration dependency of BAF, 2) correlation between BAF sensitivity to exposure concentration and fluorinated chain length, and 3) discrepancy between the kinetic and the steady-state approach.

(1) Concentration dependency of BAF

As illustrated in Figure 3-5, BAF decreases as exposure concentration C_w increases. In the adsorption model given by Equation 3.3, M is equivalent to C_w . If n is the total number of binding sites, C_o is equivalent to $n\theta$; and BAF is obtained by substituting Equation 3.4 into the BAF definition (Equation 3.1):

$$\begin{aligned}
 C_o &= n\theta, C_w = M \\
 \theta &= \frac{\alpha M}{1 + \alpha M} \\
 BAF &= \frac{C_o}{C_w} = \frac{n\theta}{C_w} = \frac{n\alpha M}{(1 + \alpha M)C_w} = \frac{n\alpha C_w}{(1 + \alpha C_w)C_w} \\
 BAF &= \frac{n\alpha}{1 + \alpha C_w} \tag{3.4}
 \end{aligned}$$

Equation 3.4 shows that BAF is an inverse function of the exposure concentration C_w . An increase in C_w will lead to a decrease in BAF. Therefore, when concentration C_w increased from 1 gL^{-1} to 10 gL^{-1} , the BAF decreased from $n / (1 + \cdot 1\text{ppb})$ to $n / (1 + \cdot 10\text{ppb})$, which explains the observed results that BAF became lower at the higher exposure concentration.

(2) Chain length effect

Binding energy vs. chain length. In Equation 3.3, the constant α is determined by $\alpha = \frac{C_o}{C_w \theta}$ and directly proportional to binding energy (Sylvin 2005). The α value of each compound can be calculated by Equation 3.3 when C_w and C_o are known:

$$\begin{aligned}
 C_{o1} &= n\theta_1 \\
 C_{o2} &= n\theta_2
 \end{aligned}$$

From Equation 3.3

$$\theta = \frac{\alpha M}{1 + \alpha M}$$

$$\frac{\theta_1}{\theta_2} = \frac{(1 + \alpha C_{w2}) C_{w1}}{C_{w2} (1 + \alpha C_{w1})} = \frac{C_{o1}}{C_{o2}}$$

The results of follow the order of PFDA>PFOS>PFNA>PFOA (Table 3-3C). This is consistent with the conclusion that the longer chain perfluorinated acids have enhanced binding affinity, as observed in many field and laboratory studies (Armitage et al. 2009, Jeon et al. 2010, Kwadijk et al. 2010, Martin et al. 2003a, b). A linear relationship between and fluorinated chain length is shown in Figure 3-7. Bioaccumulation of PFCs was suggested to be related to the chemical's hydrophobicity (Martin et al. 2003a, b). As the hydrophobic portion in the PFC molecule increases with the fluorinated chain length, the stronger the hydrophobic interaction and thus, the higher the binding energy. Woodcraft et al. (2010) in their work also demonstrated that increased hydrophobicity creates more binding sites for the compounds. Besides, it is also believed that bioaccumulation is governed to some degree by the contribution of ionic interaction of functional groups (Woodcraft et al. 2010), which explains higher binding energy by sulfonate than carboxylate for the same fluorinated chain (Figure 3-7: PFOS and PFNA).

Figure 3-7 Linear relationship between binding affinity and fluorinated chain length.

Concentration sensitivity vs. chain length. The concentration induced change in logBAF ($\hat{e}$ log BAF) was found to be linearly related to chain length, as demonstrated in Figure 3-8 and Figure 3-9. Among the carboxylates, BAF of long chain PFC is affected

more significantly by the concentration change. The magnitude of $\hat{e} \log \text{BAF}$ follows the order of increasing chain length. The $\hat{e} \log \text{BAF}$ of sulfonate is larger than that of carboxylate with the same fluorinated chain length (Figure 3-8: PFNA and PFOS). These results can be well explained by involving the binding affinity. In Equation 3.4, dividing both the denominator and the numerator by C_w we have:

$$\text{BAF} = \frac{n}{\frac{1}{\alpha} + C_w} \quad (3.5)$$

As the number of total binding sites, n , is constant, the effect of C_w on BAF, or in other words the BAF's sensitivity to C_w , depends on α value. When $\alpha \rightarrow 0$, $1/\alpha \rightarrow \infty$, therefore C_w will have greater influence on the BAF. Hence the larger the α value, the more sensitive the BAF to changes in C_w (i.e. the larger concentration induced change in $\log \text{BAF}$). From a physico-chemical point of view, when concentration increases, compounds possessing higher binding affinity are more likely to be adsorbed than those with low binding affinity. Thus, the amount of accumulation with respect to concentration change is more significant. The concentration-induced change in $\log \text{BAF}$ was found to be linearly correlated with α , too (Figure 3-9). Since the binding affinity α is closely related with chain length as discussed earlier, it is feasible to relate chain length with $\hat{e} \log \text{BAF}$ through α , i.e. longer chain $\Rightarrow$ larger $\alpha \Rightarrow$ greater influence by C_w .

Figure 3-8 Relationship between $\log \text{BAF}$ and fluorinated chain length: concentration induced change in $\log \text{BAF}$ increases with fluorinated chain length.

Figure 3-9 Linear relationship between $\Delta \log \text{BAF}$ and binding affinity (lower curve, left axis); and with fluorinated chain length (upper curve, right axis).

(3) Comparison of kinetic approach and steady-state approach

Besides the fundamental steady-state approach (Equation 3.1) to determine BAF, the kinetic approach has been popular in laboratory bioaccumulation studies (Martin et al. 2003a, b, Jeon et al. 2010). The kinetic BAF is estimated as the quotient of uptake and elimination rate constants (Equation 3.7), with the assumption that both uptake and elimination of chemicals are first order reactions (Tolls et al. 1994):

$$\frac{dC_{o(t)}}{dt} = k_u C_w - k_e C_{o(t)} \quad (3.6)$$

Solving the above first order differential equation for $C_{o(t)}$ yields:

$$C_{o(t)} = \frac{k_u C_w}{k_e} [1 - \exp(-k_e t)]$$

At steady state: $\frac{dC_{o(t)}}{dt} = 0$

$$k_u C_w - k_e C_o = 0$$

and the kinetic BAF is

$$BAF'_{kinetic} = \frac{C_o}{C_w} = \frac{k_u}{k_e} \quad (3.7)$$

Controlled laboratory studies have shown that the kinetic approach can generate similar results as the steady-state method for many POPs (Tolls et al.1994). However, the validity of the kinetic approach had never been verified for PFCs before it was applied in several laboratory studies (Jeon et al. 2010, Kwadijk et al. 2010, Martin et al. 2003a, b). In this study, the kinetic method was for the first time compared with the steady-state method and the result shows that it is not suitable for the assessment of bioaccumulation of PFCs.

As discussed previously, the BAF is exposure concentration dependent. However, the expression of Equation 3.7 itself suggests that the kinetic BAF ($BAF_{kinetic}$) should be a constant value independent of exposure concentration. This fundamentally contradicts the experimental results. Moreover, following the above mentioned kinetic approach as described in a previous study (Jeon et al. 2010), the resulting uptake rate constant in Equation 3.7, k_u , varies for the two exposure concentrations (Table 3-3E), which suggests that k_u here is not a constant as defined. Hence, the previous assumption of first order uptake reaction is inappropriate in the case of PFCs bioaccumulation. Even though two kinetic BAF values can be calculated from the variant k_u , the results were shown to deviate from the steady-state BAF (BAF_{ss}) (Table 3-3B&E).

Based on the experimental results and the special surfactant property of perfluorinated compounds, we hereby propose a new kinetic equation to describe the bioaccumulation of PFCs, incorporating the adsorption model (Equation 3.2). Compared with the old kinetic model (Equation 3.6), accumulation is no longer first order reaction: the rate of accumulation of PFCs depends on both the exposure concentration and free binding sites:

$$\frac{dC_{o(t)}}{dt} = k_u C_w S_{(t)} - k_e C_{o(t)} \quad (3.8)$$

where $S_{(t)}$ is the free binding sites at time t (Equation 3.2). The expression for organism concentration at time t ($C_{o(t)}$) can be obtained by solving Equation 3.8 as:

$$C_{o(t)} = \frac{nk_u C_w}{k_u C_w + k_e} \left\{ 1 - \exp \left[- (k_u C_w + k_e) t \right] \right\} \quad (3.9)$$

And the kinetic BAF can be obtained as:

$$BAF_{kinetic} = \frac{nk_u}{k_u C_w + k_e} \quad (3.10)$$

Compared with Equation 3.7, the exposure concentration effect is incorporated in Equation 3.10. The proposed expression of the kinetic BAF is consistent with the one obtained from the steady-state approach (Equation 3.4).

$$\alpha = \frac{k_u}{k_e}$$

$$\frac{nk_u}{k_u C_w + k_e} \quad (3.10)$$

$$= \frac{n k_u / k_e}{k_u C_w / k_e + 1}$$

$$= \frac{n\alpha}{\alpha C_w + 1} \quad (3.4)$$

Curve fittings using Equation 3.9 show agreement with both the steady-state (Figure 3-5) and kinetic batch (Figure 3-10) experimental results with good reliability, and so does the resulted $BAF_{kinetic}$ with the steady-state BAF, i.e. BAF_{ss} ($R^2 \times 0.94$; $p < 0.01$, F test) (Table 3-3B&D).

Figure 3-10 Kinetic batch results with curve fitting at exposure concentration of 10 ppb: (a) PFOS (b) PFOA (c) PFNA and (d) PFDA

The maximum value that $BAF_{kinetic}$ can potentially reach is when the exposure concentration is infinitely low ($C_w \rightarrow 0$), where

$$\lim_{C_w \rightarrow 0} BAF_{kinetic} = \lim_{C_w \rightarrow 0} \frac{nk_u}{k_u C_w + k_e} = \frac{nk_u}{k_e} = n\alpha \quad (3.11)$$

From Equation 3.11, the maximum BAF for the tested compounds were calculated as 415, 16, 157 and 944 L/kg for PFOS, PFOA, PFNA and PFDA respectively.

Time to reach steady state. As mentioned previously, the time to reach the steady state, t_{ss} , varies between the two exposure concentrations. Therefore, t_{ss} also appears to be concentration dependent. Mathematically the time to reach steady state is infinity. However, the time for C_o to reach 95% of steady state concentration, $t_{ss}^{95\%}$, can be used as a performance metric and derived from Equation 3.9 as:

From

$$C_o(t) = \frac{nk_u C_w}{k_u C_w + k_e} \{1 - \exp[-(k_u C_w + k_e)t]\} \quad (3.9)$$

The steady state concentration is

$$C_o = \frac{nk_u C_w}{k_u C_w + k_e}$$

At 95% of the steady state concentration, we have:

$$C_o(t_{ss}^{95\%}) = 95\% \cdot C_o(t_{ss})$$

or

$$\frac{nk_u C_w}{k_u C_w + k_e} \left\{ 1 - \exp \left[- (k_u C_w + k_e) t_{ss}^{95\%} \right] \right\} = 95\% \cdot \frac{nk_u C_w}{k_u C_w + k_e}$$

Solving the above equation yields the time to reach 95% of the steady state concentration as:

$$t_{ss}^{95\%} = \frac{-1}{k_u C_w + k_e} \ln(1 - 0.95) \quad (3.12)$$

Equation 3.12 shows that when exposure concentration C_w increases, t_{ss} will decrease accordingly, which explains the observed results of longer t_{ss} at lower exposure. However, if the old kinetic method (Equation 3.6) is followed, the $t_{ss}^{95\%}$ will be:

$$t_{ss}^{95\%} = \frac{-1}{k_e} \ln(1 - 0.95) \quad (3.13)$$

where t_{ss} will depend only on k_e and thus, will be a constant value for each compound.

Taken together, the bioaccumulation of PFCs appears to follow an adsorption model. In consideration of the special partitioning behavior of PFCs, the conventionally used kinetic model appears to be inappropriate for this group of chemicals. The fundamental assumption that both uptake and elimination of PFCs are first order reactions merits further scrutiny.

To our knowledge, this is the first study that demonstrates the concentration dependency of the bioaccumulation of perfluorinated compounds, and describes the relationships among various factors using mathematical models. Examination of the concentration dependency revealed the inadequacy of the conventional kinetic model and a new model based on the adsorption mechanism was accordingly proposed. This model provides a more accurate description of the bioaccumulation process and thus, the fate of PFCs.

It is also noted that protein binding and adsorption have similar mechanisms, both of which can be described by Equation 3.2. Protein-water partitioning has been suggested to be useful in evaluating the bioaccumulation of PFCs. As proteins have been considered as major reservoirs for PFCs, it is expected that protein binding could dominate the bioaccumulation process. It is therefore, also possible that the observed results in our study are attributed to this dominant process.

Partitioning coefficients used to predict the environmental distribution of chemicals, such as K_{ow} , are independent of concentration. Although more species- and environmental factor-dependent, the BAF in many ways is similar to these partitioning coefficients. Hence, in previous studies, the BAF of perfluorochemicals were always treated as a constant, regardless of the exposure concentration. In other words, bioaccumulation was assessed without considering concentration as an influencing parameter. The unique properties of PFCs, however, suggest that this approach may not be appropriate. This argument is further supported by the results of this study which has shown the importance of specifying the environmental concentration when carrying out bioaccumulation studies of PFCs. Literature reviews have found a lack of consistency in the BAF data from both laboratory and field studies. This inconsistency may likely be caused by ignoring the concentration effects as discussed in this study.

3.4 Conclusion

The bioaccumulation behavior of PFCs was examined in controlled laboratory studies. Bioaccumulation potential of the tested compounds was demonstrated, where long chain PFCs were found to be more bioaccumulative than short chain ones. More importantly, this process was also found to be exposure concentration dependent. For all compounds, the bioaccumulation factor is larger at the lower dosage. This concentration dependency can be explained by a nonlinear adsorption mechanism. The sensitivity of BAF to exposure concentration was found to be positively related to fluorinated chain length and the binding affinity of the compounds. Bioaccumulation of long chain PFCs are more easily affected by concentration changes. A new kinetic bioaccumulation model based on the adsorption mechanism was proposed, which provides a more accurate description of the bioaccumulation process of PFCs.

Toxic effects are directly linked with the inner concentration of the compounds. The knowledge of the bioaccumulation mechanism of PFCs provides a better understanding of the partitioning behavior and the inner concentration of these contaminants. With the help of the bioaccumulation model, toxicity test results could be better explained and understood. It should also be noted that in the real environment, PFC levels are normally 3-4 magnitudes lower than the tested concentration, therefore BAF values are expected to be even higher for individual compounds based on current bioaccumulation model. However,

in real environment there will be also mixture effect from other PFCs and environmental pollutants, which could affect the prediction power of the proposed model.

Chapter Four Ecotoxicity of PFCs and different modes of action

4.1 Introduction

Although the production of common PFCs have been phased out by their main producers, the lack of incentive and desirable performance characteristics of PFCs makes it difficult to control the production worldwide, especially where economic development exceeds environmental concerns (Lindstrom et al. 2011). Hence, PFCs will remain in our environment for a long period of time, and it is therefore important to understand the environmental and ecological consequences and the associated risks of these contaminants. As mentioned earlier, to date, available toxicity data of PFCs is still very limited with respect to target compounds and test species, and the underlying toxic mechanisms of these emerging pollutants remain unclear. The lack of toxicity information makes it difficult for an environmental management agency to create regulations and guidelines regarding the emission and treatment of PFCs in many regions of the world, including Singapore.

The ocean is the final sink for many persistent organic pollutants (POPs) including PFCs (Theobald et al. 2011), and thus, the health of marine wildlife is always of great concern. Previous studies clearly indicate exposure and the bioaccumulation potential of PFCs in marine organisms (Liu et al. 2011, Nguyen et al. 2011, Vestergren & Cousins 2009). Therefore, examination of toxic effects of PFCs on marine wildlife is of great importance. However at present, knowledge of the toxicological effects of PFC exposure is largely based on studies on mammalian species. PFC mediated adverse effects on marine invertebrates have hardly been addressed, and there is still a lack of toxicity data for a comprehensive ecotoxicological assessment.

In this context, the purpose of this chapter was to evaluate the environmental toxicity of four commonly detected PFCs, namely PFOS, PFOA, PFNA and PFDA on green mussel *Perna viridis*, through biomarker-based toxicity study. Pollutants can affect a biological system at many levels. Starting with interaction with biomolecules, the effects can cascade

through molecular →cellular →physiological →individual →population levels (Newman 2009). In order to have a holistic idea of how PFCs could affect the organism, biomarkers from three different bio-organization levels (biomolecular, cellular and physiological) were employed. In addition, organic contaminants generally have several classes of toxic mode of action, including oxidative damage, DNA damage, general cell lesions and membrane damage (Nobels et al. 2010). In this chapter, six possible toxic modes of action of PFCs were investigated. In the order of ascending bio-organization level, the examinations included oxidative toxicity, xenobiotic metabolism, genotoxicity, immunotoxicity and general health state. The underlying mechanism of toxic actions was also investigated, where dose-response relationship and exposure time effect were examined. Toxic response model and structure-activity relationship model were established wherever applicable.

Biomarkers are measureable bio-endpoints at different biological organization levels: biomolecular, cellular, tissue and organ, etc. For example, Neutral red retention time measures the lysosome membrane stability, and is a cellular level biomarker. Biomarkers measure sublethal effects in organisms and provide early warning signals for remedial or preventive action to be taken (Sarkar et al. 2006). Due to the complexity of contaminants in the environment and the variety of responses that they induce in organisms, it is essential that multi-biomarkers are employed (Brooks et al. 2009). A series of biomarkers from different biological levels can provide a holistic understanding of toxic effects on target organisms. The biomarkers and their corresponding toxic mode of action and biological level are illustrated in Figure 4-1.

Figure 4-1 Summary of biomarkers applied in the study and their corresponding toxic mode of action and biological levels.

CAT = catalase SOD = superoxide dismutase GPx = glutathione peroxidase GSH = glutathione GST = glutathione-S-transferase EROD = ethoxyresorufin-O-deethylase MN = micronucleus NRRT = Neutral Red retention time SC = spontaneous cytotoxicity RCF = relative condition factor

4.2 Oxidative toxicity

An imbalance of the redox state of cells can trigger the production of reactive oxygen species (ROS) which will cause severe damage to cell structures including proteins, lipids and DNA. This oxidative stress was also shown to be involved in the development of diseases (Wells et al. 2009). Some PFCs have been identified as peroxisome proliferators and were accused to impose oxidative stress on exposed organisms (Yang 2010). Previous studies demonstrated that oxidative stress and excess ROS production could be one of the

major toxic mechanisms for many POPs, including PFCs (Nobels et al. 2010). PFCs are ubiquitous contaminants in the ocean environment. However, antioxidant response and oxidative toxicity of PFCs in marine organisms have seldom been examined.

The purpose of the current study was to investigate the oxidative stress and oxidative toxicity induced by PFCs in green mussels through the examination of antioxidant enzyme activity and oxidative damage biomarkers. Six biomarkers were tested which included enzyme CAT, SOD and GPx, antioxidant GSH, DNA fragmentation and lipid peroxidation. The antioxidant enzymatic system plays an important role in protecting cells from oxidative damage by catalyzing the reduction of ROS through a series of chain reactions. Their activities under the exposure compared with the normal healthy state can be used as an indicator of oxidative stress. DNA fragmentation and lipid peroxidation are both linked with excessive ROS in the cell. Therefore, they are commonly used as biomarkers of oxidative toxicity related damage.

In order to better understand the toxic mode of action, the structure-activity relationship of the compounds was examined and quantitative structure-activity relationship (QSAR) models were developed to describe the oxidative toxicity of PFCs. K_{ow} has been generally used as the measure of physico-chemical property of POPs, based on which many QSAR models are established (Lee & Chen 2009, Zvinavashe et al. 2009). However, K_{ow} has been proved to be inappropriate to evaluate the partitioning behavior of PFCs, and thus the validity of K_{ow} based structure-activity relationship is in question. In the current study, the bioaccumulation factor was first introduced as a molecular descriptor to establish QSAR model. The QSAR can also be applied to predict the toxic behavior of other members in the PFC group when toxicity testing of each individual compound is not feasible.

4.2.1 Materials and Methods

4.2.1.1 Chemicals

Potassium perfluorooctanesulfonate (PFOS, 98%), perfluorooctanoic acid (PFOA, 96%), perfluorononanoic acid (PFNA, 97%), perfluorodecanoic acid (PFDA, 98%) were purchased from Sigma-Aldrich (St. Louis, MO). Chemicals used in the toxicity test were also purchased from Sigma-Aldrich unless otherwise specified.

4.2.1.2 Mussel acclimation and maintenance

In the current study, green mussels, *Perna viridis*, were selected as the target organism because in a previous study, they were shown to have great potential to bioaccumulate PFCs. In addition, mussels have been conventionally used as the sentinel organisms for environmental monitoring, as they are sessile and filter-feeding organisms that are in direct contact with contaminated compartments, and can provide a time integrated indication of contamination with measurable cellular and physiological responses (Izquierdo et al. 2003). Mussels were purchased from a local fish farm in Singapore. Only mussels with a shell length of 60-65 mm were selected for the experiment. Mussels were acclimated to laboratory conditions for one week before the exposure experiment. They were raised in artificial seawater made by mixing sea salts with distilled water. The water was maintained at 25°C with salinity at 25ppt. A 12-hr light-dark cycle was employed to simulate the diurnal variation of sunlight. Commercial marine micro algae (Reed Mariculture Inc. Campbell, CA) were used to feed the mussels every two days two hours before the water change.

4.2.1.3 The exposure experiment

Six exposure concentrations of individual PFC were applied: 0.1, 1, 10, 100, 1000 and 10000 g/L. The typical PFC level in ocean water is approximately a few hundred pg per liter (Cai et al. 2012). These compounds have been detected in oceanic water from 17.8 to 192ng/L in Asian waters (Hu et al. 2011, Wang et al. 2012). In the current study, the exposure concentration range was selected to include concentrations that were environmentally relevant, and also concentrations that were high enough to elicit distinguishable effects in order to elucidate possible modes of action (Arukwe & Mortensen 2011). Mussels were raised in polypropylene (PP) tanks. For each exposure concentration, duplicate tanks were used. Another set of duplicate tanks were engaged as the control, where no PFCs were present. The exposure period was 7 days (Kim et al. 2010; Yang et al. 2010). All tanks were cleaned and refilled every two days. Mussels were sampled at the end of exposure. The measured exposure concentrations and concentration in mussels were reported in the Appendix.

4.2.1.4 Sample preparation

Mussel haemolymph was extracted from the anterior adductor muscle with a

hypodermic syringe filled with physiological saline. The physiological saline was prepared by mixing HEPES 4.77 g, NaCl 25.48 g, MgSO₄ 13.06 g, KCl 0.75 g, CaCl₂ 1.47 g and distilled water to 1 L. The pH was adjusted to be 7.4-7.5 with NaOH. The haemolymph mixture was then transferred to a microcentrifuge tube before analysis. Mussel soft body was cut into pieces and homogenized in 100 mM phosphate buffer (pH 7.4, KCl 100 mM, EDTA 1 mM) using a tissue homogenizer. Protease inhibitor (Complete Protease Inhibitor, Roche) was also added. The homogenate was then centrifuged at 500xg for 20 min at 4°C. The supernatant was subsequently transferred into clean tubes and centrifuged again at 2000xg for 30 min at 4°C. Finally the supernatant was ultra-centrifuged at 100000xg for 90 min at 4°C. The final supernatant was transferred to clean tubes on ice before analysis. The protein content of the cytosolic extraction was quantified using the Bradford protein assay using Bovine Serum Albumin as the standard (Bio-Rad).

4.2.1.5 Biomarkers of antioxidant activity

CAT activity was measured in the whole soft tissues as described elsewhere (Binelli et al. 2009). The reaction was initiated by adding 20 μ l of diluted H₂O₂ to microplate wells with 20 μ l of cytosolic extract, 30 μ l of methanol and 100 μ l phosphate buffer. After 20 min incubation at 25°C, 30 μ l KOH and 30 μ l of chromogen were added subsequently. After incubation, KIO₄ was added and the plate was incubated for 5 min before reading the absorbance at 540nm. The results of CAT activity were expressed in terms of micromole formaldehyde per milligram protein per min.

SOD activity was determined as the inhibition of the rate of cytochrome c reduction, measured at 550 nm, by superoxide anion generated from the xanthine oxidase/hypoxanthine reaction. The reaction takes place in 0.05 M Na₂PO₄/NaHPO₄ buffer, pH 7.4, with 48 μ M xanthine/0.2 units of xanthine oxidase and with 96 μ M EDTA. One unit of SOD activity is defined as the amount of sample that inhibits by 50% the reduction of cytochrome c. The SOD activity was expressed as U/mg protein (Vlahogianni et al. 2007).

GPx activity was determined by measure the consumption of NADPH at 340 nm during the formation of reduced glutathione by glutathione reductase. The enzyme activity was expressed as nmol NADPH oxidized/min/mg proteins using a molar extinction coefficient of 6.22 mM⁻¹cm⁻¹

GSH content was quantified by the measurement of TNB, a product in the enzyme

recycling reaction. The sulfhydryl group of GSH reacts with DTNB and produces the yellow color TNB. The rate of TNB production is directly proportional to GSH concentration in the cytosolic fractions. Absorbance of TNB was measured at 405nm. GSH concentration was then calculated according to the standard curve.

4.2.1.6 Oxidative toxicity biomarker

Comet Assay The Comet Assay was performed using the Comet Assay Kit from Cellbiolabs, Inc. In brief, the haemolymph was extracted as previously described. The suspension was centrifuged at 700xg for 2 min and the supernatant was discarded. The cells were washed and resuspended in ice-cold PBS at 1×10^5 cells/ml. The cell sample was then mixed with pre-liquified agrose at 1:10 (v/v). 75 μ l of the mixture was transferred immediately onto microscope slides. The slides were prepared as triplicate for each cell sample. The slides were then transferred to 4°C in the dark for 15 min and maintained horizontally. After gelation, the slides were immersed in ice-cold lysis buffer and in ice-cold alkaline solution each for 30 min at 4°C in the dark. Electrophoresis was performed in alkaline solution for 30 min at 1 volt/cm, 300mA. After electrophoresis, the slides were first washed twice in ice-cold DI water and then immersed in 70% ethanol for 5min. 100 μ l of Vista Green DNA dye was applied to each slides and incubated at room temperature for 15 min before the slides were observed under epifluorescence microscopy.

Lipid peroxidation The level of lipid peroxidation was evaluated by the measurement of malondialdehyde (MDA) by recording the amount of thiobarbituric acid reactive substances (TBARS). 2 ml of the reaction mixture (thiobarbituric acid (0.375%), trichloroacetic acid (15%) and hydrochloric acid (0.25 N)) were mixed in 1:1:1 ratio and added to 1 ml of the heat denatured supernatant. TBARS levels was estimated at 535 nm using MDA as standard. The concentration of lipid peroxidation compounds was expressed as nmol of MDA per mg of tissue protein (Vlahogianni et al. 2007).

4.2.1.7 Integrated biomarker response

The integration of all measured biomarker responses into one general index was performed as previously described with modifications. Biomarker response data were first standardized to allow direct comparison at different exposure concentrations:

$$Y_i = \frac{X_i - m_i}{s_i} \quad (4.1)$$

where Y_i is the standardized biomarker response; X_i is the response value of each biomarker; m_i and s_i are the mean and the standard deviation of the biomarker, respectively. The minimum value ($\min_i$) and maximum value ($\max_i$) for each biomarker was also calculated from the standardized response value. The normalized score of each biomarker response (B_i) was computed as:

$$B_i = \frac{Y_i + |\min_i|}{|\max_i|} \quad (4.2)$$

Finally, the enhanced integrated biomarker response value (EIBR) was calculated as the average summation of the individual biomarker score:

$$EIBR = \sum_{i=1}^n B_i / n \quad (4.3)$$

4.2.1.8 Statistical analysis

Data were checked for normality and homogeneity of variance using Kolmogorov Smirnov and Levene's test. One way ANOVA followed by Tukey's post-hoc tests were performed to compare variables between the control and the exposure samples. The significance level was set at $p < 0.05$. Statistical analysis was performed using SPSS 19.

4.2.2 Results and Discussion

4.2.2.1 Antioxidant response and oxidative stress

As shown in Figure 4-2, the tested PFCs triggered a series of antioxidant enzyme activity. These activities include activation of CAT and SOD, inhibition of GPx and reduction of GSH content. Generally speaking, the antioxidant response increases with the exposure concentration of PFCs, and significant responses were normally observed at approximately 100 µg/L. The induction of antioxidant activity indicates an imbalance of the cellular oxidative homeostasis and excessive production of ROS in the cells. The presence of excessive ROS under PFC exposure may then result in oxidative stress related toxicity. PFCs are suspected peroxisome proliferators (Rosen et al. 2008). They may bind to and activate the peroxisome proliferator activated receptor protein and promote the catabolism of fatty acids which leads to excessive ROS production (Arukwe & Mortensen 2011, Yang 2010). Antioxidant responses for PFOA and PFOS have been reported in freshwater fish in *in vitro* studies (Liu et al. 2007c), where a higher range of exposure level (1-30 mg/L) was

adopted. The current study shows that besides PFOA and PFOS, exposure with other long chain PFCs, such as PFNA and PFDA, can also impose oxidative stress in organism. Similar increase of antioxidant activity was also observed in organism exposure to toxic metals and PCBs as part of their antioxidant enzymatic defense system against free radicals (Faria et al. 2009).

At the initial increase of PFC concentrations (from 0 to 100 μ g/L), activation of enzymes was observed for CAT and SOD, accompanied with a reduction in GSH content (Figure 4-2). ROS production under PFC exposure was shown to be dose-dependent and increase with the exposure concentration (Liu et al. 2007a). The increasing antioxidant enzyme activity is an adaptive response under this situation to remove the excess ROS, where ROS can be reduced through the antioxidant chain reactions: SOD first catalyzes the dismutation of superoxide to O_2 and H_2O_2 . CAT then catalyzes the reduction of H_2O_2 to H_2O . H_2O_2 can also be reduced by oxidizing GSH to GSSG which is catalyzed by GPx. Antioxidant enzymes play an important role in detoxifying PFC induced ROS. Activation of these enzymes under mild PFC exposure helps to prevent excess ROS from causing deleterious cellular effects. However, no significant induction of GPx was observed under mild PFC exposure (0-100 μ g/L) in the current study (Figure 4-2). It is also noticed that, unlike other enzymes, there is no significant lag phase of SOD activity. This enzyme was activated even at low exposure concentration of PFCs.

Figure 4-2 Response of antioxidant enzyme activity in green mussels under the exposure of PFCs. (a) CAT, (b) SOD, (c) GPx and (d) GSH. The points represent test results and the lines are dose-response curve fitted by Matlab.

When PFC exposure concentrations further increase (100-10000 $\mu\text{g/L}$), a decrease

was observed in both SOD and GPx activities. This possibly indicates that under elevated exposure of PFCs, the ability of the organism to respond in an adaptive manner has been compromised. There is also reported evidence that at high level of contamination, SOD activity could be suppressed by excessive ROS production (Parolini et al. 2010). In addition, the superoxide radicals that are not detoxified by SOD may then directly inhibit the GPx activity (Faria et al. 2009).

Significant increases in DNA strand breaks (Comet tail moment) and lipid peroxidation were also observed at high exposure levels (Figure 4-3). The two biomarker results show that the oxidative stress related damage was induced. It seems that under high PFC exposure, the ROS production exceeds the antioxidant capacity and the induced oxidative stress subsequently results in oxidative damage. The initial adaptive response becomes an adverse response where the antioxidant fails to protect cells by effectively removing the excessive ROS.

Figure 4-3 Response of oxidative damage biomarkers under the exposure of PFCs (a) Lipid peroxidation and (b) Comet assay. The points represent test results and the lines are dose-response curve fitted by Matlab.

Oxidative stress vs. oxidative damage: It is noticed that there are some common observations in the antioxidant enzymes activities. The magnitude of adverse response at same exposure generally followed the order of PFOA<PFNA<PFOS<PFDA (Figure 4-2). The results indicate the increased toxicity potential of these compounds follow the same order, which is consistent with their bioaccumulation potential identified in Chapter 3. It is possible that antioxidant enzyme activities are correlated with the tissue concentration of the contaminants. Antioxidant enzymes work directly with free radicals, their response to contaminants thus might be less specific to the compounds but more related with the accumulated concentration. Also, the tested PFCs triggered antioxidant enzyme activities in a similar manner as shown by their dose-response curves that have a same pattern (Figure 4-2). This could be due to the fact that these PFCs share similar mechanisms in inducing the ROS production, as discussed earlier.

The same relationships do not apply to the oxidative damage biomarker results. Figure 4-3 shows that PFOS exposure is able to cause the most severe oxidative stress related DNA injuries compared with other compounds. On the other hand, for lipid degradation, oxidative lipid damage posed by the perfluorinated carboxylates is more

significant than that from the sulfonate, PFOS. The results imply that PFCs may have different mechanism in causing oxidative damage. PFOS exposure is more likely to lead to DNA damage while PFCAs tend to cause lipid degradation and potential membrane damage. This observation supports a previous finding that PFCAs demonstrate higher toxicity than PFOS in terms of compromising membrane integrity (Kleszczynski & Skladanowski 2009). The reason for the disparities between antioxidant enzymes and oxidative stress biomarkers could be that, although ROS is responsible for the oxidative damages, the reactions could also be affected by parameters such as ambient pH, composition of cytosol and most probably direct interference from the compounds.

Dose-response curve: The assessment of dose-response relationship is critically important to determine the magnitude of effect and risks associated with pollutants. The shape of dose-response curves helps to better understand the toxic behavior of PFCs. Dose-response curves of chemicals with target biomolecules are usually sigmoid shape (Conolly & Lutz 2004). In previous studies of PFCs, sigmoid curve was used to simulate the toxicity results (Latala et al. 2009). However, our experimental results show that the best fitting curve of the biomarker results is not always sigmoid. Sometimes the curve can be even non-monotonic (Figure 4-2b). Dose-response relationships of CAT, GPx and GSH content show the common sigmoid shape behavior, which can be described by the sigmoid model. However, the curve for SOD is an inverted U shape, which is better modeled by a quadratic function. Non-monotonic curves can be attributed to diverse biological reactions that are initiated in different dose ranges (Gao et al. 2009), in this case the ROS promotion and inhibition, binding effect etc. The non-monotonic behavior is worth noting because a measured low response in the biomarker result may not only indicate an insignificant toxic effect, but also severe stress to the organism.

4.2.2.2 Structure-activity relationship

In order to have an overall assessment of the oxidative toxicity, the integrated biomarker response analysis was applied and the Enhanced Integrated Biomarker Response (EIBR) values were calculated based on all biomarker results (Equation 4.3). It was found that the EIBR values of each PFC are positively related to the fluorinated chain length or fluorinated carbon number, N_{fc} (Figure 4-4). The result demonstrates that long chain PFCs potentially impose more oxidative stress to the organism. Oxidative toxicity was also found to increase with the bioaccumulation potential of PFCs, where compounds with larger BAF

impose higher oxidative toxicity.

Figure 4-4 Integrated oxidative toxicity induced by PFCs: compounds comparison according to (a) bioaccumulation factor and (b) perfluorinated carbon number

To further examine the structure-activity relationships of the PFC induced oxidative toxicity, QSAR models were constructed. Different from conventional QSAR models, the

EC₅₀ evaluated from the dose-response curve of integrated oxidative toxicity (EIBR values) was used as the descriptive summary statistic in this study. BAF and N_{fc} are not common descriptors for QSAR model. However, considering their observed relationship with the PFC induced oxidative toxicity, they were also included in the initial descriptor pool.

The descriptive summary statistic: In conventional QSAR models, the descriptive summary statistic, EC₅₀ or LC₅₀, is mostly based on a single test result, which may potentially introduce bias. Taking PFCs as examples, PFOS was found to exhibit higher genotoxicity than PFCAs with similar chain length, while PFCAs were proved to possess higher cytotoxicity potential than PFOS with respect to cell membrane damage (Kleszczynski & Skladanowski 2009). Moreover, PFCs could display different toxicity potential even for the same toxic mechanism, as described earlier in the current study where the compounds demonstrated different toxicity potential as shown by their variant performance in oxidative stress biomarkers. Therefore, the EC₅₀ from a single test may not be a good representation of general toxicity evaluation. On the contrary, EC₅₀ of an integrated assessment of multiple toxicity tests provides a more conclusive evaluation of the toxicity potential. Therefore, the dose-response curves of integrated oxidative toxicity were constructed using EIBR values (Figure 4-5), from which the EC₅₀ was determined.

Figure 4-5 Dose-response relationships for integrated oxidative response of individual PFC.

The descriptor: Molecular descriptors were obtained through E-Dragon server (<http://www.vcclab.org/lab/edragon/>) using CORINA with the SMILES of the compounds as the input. 1664 molecular parameters were generated by the program. BAF and fluorinated carbon number (N_{fc}) were also added. Constant values and pairwise correlated descriptors were excluded in the first step (Papa et al. 2009) where 160 descriptors were left. By applying the Sequential Feature Forward Selection method the descriptors were then eliminated down to three. However, considering the number of observations, only one descriptor should be the used for final QSAR model (Cronin & Schultz 2003). Therefore correlation analysis was applied to find out the descriptor that alone is capable of providing good descriptions of the toxicity of the compounds.

The model results: Two best fitting QSAR models were obtained (Equation 4.4 & 4.5), with BAF and N_{fc} as the descriptors respectively.

In previous studies, the K_{ow} based QSAR model was developed for PFCAs (Wang et al. 2011a). Hydrophobicity is an important physico-chemical property of organic pollutants which is important in the uptake and elimination of compounds (Li et al. 2012, Zvinavashe et al. 2009). Therefore K_{ow} has been a commonly used descriptor to establish the QSAR (Ding et al. 2011, Wang et al. 2011a). However for PFCs, K_{ow} has been recognized as inappropriate for assessing the partitioning behavior of these compounds in many literatures (Kelly et al. 2009, Liu et al. 2011). Unlike common POPs, PFCs tend to accumulate in protein-rich compartments and compartments that are high in phospholipids content (Giesy et al. 2010, Quinete et al. 2009, Armitage et al. 2013). Considering the special ðproteinophilicö nature, a direct use of the bioaccumulation factor seems more appropriate to provide an accurate description of the uptake of PFCs from environment to biota. Besides, as discussed earlier, the oxidative toxicity of target PFCs was observed to be positively related with BAF. BAF was shown to be a good descriptor for the QSAR model:

$$\log EC_{50} = -0.442 \log BAF + 3.419 \quad (4.4)$$

$$n = 4, r^2 = 0.999, s = 0.012, F = 2233, Q^2 = 0.997$$

where r^2 is the correlation coefficient, s is the root mean square error, F is the Fisher criterion and Q^2 is cross-validated correlation coefficient.

BAF is an important parameter in describing both uptake and binding behavior of PFCs, where high bioaccumulation potential also implies high protein binding affinity of

the compounds (Liu et al. 2011). Therefore, high bioaccumulation potential favors toxicity in two aspects: 1) high inner concentration of PFCs, where toxicity is often the result of concentration as illustrated in Figure 4-2; and 2) strong interaction with the active proteins. A number of toxic effects imposed by PFCs are resulting from the compounds binding with active proteins (Arukwe & Mortensen 2011). As mentioned earlier, ROS production is associated with fatty acid oxidation that is triggered by PFCs binding with peroxisome proliferator activated receptor proteins. PFCs with higher protein binding affinity potentially have the ability to induce more ROS production. Moreover, protein binding also depends on the density of compounds (Vuignier et al. 2010). A high inner concentration of PFCs also favors the binding process and thus ROS production. Therefore, between the compounds with the same fluorinated chain length, PFOS displayed higher oxidative toxicity potential than PFNA, probably because of its higher bioaccumulation in organisms. Some of the previous studies concluded that PFSAAs are more toxic than the corresponding PFCAs (Nobels et al. 2010). The bio-endpoints measured in these studies could also be linked with binding affinity or inner concentration of the tested PFCs.

Other than BAF based QSAR model, the oxidative toxicity may also be described by the fluorinated chain length, N_{fc} :

$$\log EC_{50} = -7.201 \log N_{fc} + 8.915 \quad (4.5)$$

$$n = 4, r^2 = 0.899, s = 0.132, F = 17.8, Q^2 = 0.467$$

In previous studies, the bioaccumulation potential of PFCs has been shown to increase with chain length (Liu et al. 2011). Therefore, a larger N_{fc} generally results in a higher bioaccumulation rate. The QSAR model based on chain length offers a more practical and convenient option to predict and assess the toxicity potential of other PFCs in group, as the BAF of individual PFC is not always available, and BAF data are still largely obtained from experimental results. However, there are also some limitations associated with the chain length based QSAR model. Firstly, the functional group is not taken into consideration, and thus the actual EC_{50} for PFSAAs may be lower than the estimated value by the chain length based QSAR (This also explains the lower Q^2 value). Secondly, the bioaccumulation rate may not always increase with chain length (Conder et al. 2008). When N_{fc} is larger than 14, bioaccumulation of PFCs may decrease. In this situation the N_{fc} based QSAR model may not be appropriate.

In summary, PFC exposure can induce oxidative stress to organisms and lead to a

series of antioxidant responses and oxidative damage in green mussels. The oxidative toxicity was found to be exposure concentration dependent. At low exposure levels, activation of antioxidant enzymes is an adaptive response to low levels of ROS induced by PFCs. At high PFC concentration, inhibition of certain enzymes was observed where the organism's ability to respond in an adaptive manner was compromised. The oxidative stress under high contamination level of PFCs also leads to lipid and DNA damages.

The PFC induced oxidative toxicity was found to be positively related to the bioaccumulation potential of the compounds. High bioaccumulation potential promotes oxidative toxicity not only because of the resulting high inner concentration in organism, but also because for ϕ proteinophilic PFCs, high bioaccumulation also means high binding affinity with proteins. Both of these conditions favor the excessive ROS production. Besides bioaccumulation and perfluorinated chain length, the functional group can also affect the oxidative toxicity. For compounds with same perfluorinated chain, the sulfonate displays higher toxicity than its correspondence carboxylate. This could be due to the higher polarity of the $-\text{SO}_3^-$ group which may bring in stronger bindings and interactions. QSAR models were established to describe the toxicity potential of the compounds, where the oxidative toxicity of PFCs was found to be well related with BAF and fluorinated chain length. While the former provides a more accurate model description, the latter is more convenient in the prediction and risk assessment with certain limitations. Recent studies have suggested that binding to phospholipids can be an important pathway for the sorption of PFCs. Therefore the membrane-water partition coefficient, which was used to characterize sorption to phospholipids, could also be a useful descriptor of PFC bioaccumulation once the values of this coefficient are established.

4.3 Inhibition of xenobiotic metabolism

Xenobiotic metabolism is the biologically mediated modification of exogenous compounds through a specialized enzyme system. It is a form of biotransformation that leads to enhanced elimination and sequestration of compounds foreign to an organism's normal biochemistry, such as drugs, poisons and environmental pollutants (Newman 2009). During xenobiotic metabolism, lipophilic compounds are generally converted to more hydrophilic products that are more amenable to excretion from the organism. Therefore, xenobiotic metabolism is an important detoxification pathway of contaminants absorbed by

the organism from the ambient environment. The process is mediated and catalyzed by a series of xenobiotic metabolizing enzymes such as cytochrome P450 (CYP)-dependent mixed function oxidase and glutathione S-transferase (GST).

In the literature, the activities of these enzymes have been used as important biomarkers in the assessment of the functionality of xenobiotic metabolism and thus the health status of organisms (Kammann et al. 2005, Ramos & Garcia 2007). For example GST conjugating activity was found to be positively related with PAH levels in *Perna viridis* (Faria et al. 2009). Induction of CYP in organism was also observed in many studies under exposure to PCBs, PAHs and organochlorine pesticides (Ramos & Garcia 2007, Sarkar et al. 2006). Ethoxyresorufin-O-deethylase (EROD) has been successfully used as a biomarker for marine biomonitoring of fish in a number of studies (Kammann et al. 2005, Skouras et al. 2003). EROD is a highly sensitive indicator of contaminant uptake, providing evidence of receptor-mediated induction of cytochrome P450-dependant monooxygenases (the CYP1A1 subfamily specifically) by xenobiotic chemicals (Whyte et al. 2000). Studies demonstrated that induction of metabolizing enzymes do not merely indicate exposure to contaminant but is also correlated to harmful biological effects (Yuen & Au 2006).

Considering its significance in the biological detoxification process, investigation of xenobiotic metabolism through examination of metabolizing enzyme activities could bring some insights of the potential adverse effects of PFCs. With this in mind, the activity of two enzymes, EROD and GST were examined after exposure to the PFOS, PFOA, PFNA and PFDA, to identify the PFC induced adverse effects in the organism's detoxification process.

4.3.1 Materials and Methods

The chemicals, exposure experiment, mussel maintenance, sample preparation and statistical analysis were described in Section 4.2.1. The exposure concentrations were 0.1, 1, 10, 100 and 1000 µg/L of individual PFC. The exposure period was 7 days.

Biomarker analysis

Glutathione S-transferase (GST) activity. GST was measured using 1-chloro-2,4-dinitrobenzene (CDNB, 1 mM) as a substrate. The change in absorbance was recorded at 340nm. The enzyme activity was expressed as µmol CDNB conjugate formed/min/mg

protein using a molar extinction coefficient of $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$

7-ethoxy resorufin O-deethylase (EROD) activity. EROD activity was measured as described elsewhere (Jönsson et al. 2002), where mussel gill arches were excised and placed in ice cold HEPES-Cortland buffer (pH 7.7). The tip pieces were isolated by a cut above the septum of the gill filaments which resulted in tips pieces about 2 mm long. For each mussel, 2 mm pieces were selected and groups of ten pieces were placed in microcentrifuge tubes. 0.5 ml of reaction buffer was added and pre-incubated for 10 min. The reaction buffer consisted of 7-ethoxyresorufin ($1 \text{ } \mu\text{M}$) and dicumarol ($10 \text{ } \mu\text{M}$) in HEPES-Cortland buffer. The reaction buffer was then removed and replaced repeatedly with 0.7 ml fresh reaction buffer with an incubation period of 10 min and 30 min at 25°C . Triplicates of 0.2 ml aliquots were transferred from each tube to a fluorescent 96 well microplate. Resorufin standards (0.5-250 nM) were also included on each plate in triplicates of 0.2 ml aliquots. The standards were diluted from stock solution (10 mM in methanol) using reaction buffer. The fluorescence was determined using a microplate reader at 544 (ex) and 590 (em) nm. The EROD activity was expressed as picomole of resorufin per milligram protein per min.

4.3.2 Results and Discussion

Inhibition of enzyme activity has been observed for both biomarkers (Figure 4-6). Xenobiotic metabolism can be separated into two phases of reaction. Phase I is modification, where enzymes act to introduce reactive and polar groups into their substrates to increase the reactivity, normally hydrophilicity. One of the most common modifications is hydroxylation by the CYP group. Phase II is conjugation, where activated xenobiotic metabolites from Phase I are conjugated with charged species to foster the elimination of the compounds. One of the most common Phase II reactions is the GST catalyzed conjugation. The observed inhibition implies that PFCs are able to interrupt the normal activity of both Phase I and II xenobiotic metabolizing enzymes.

Measurement of CYP in terms of EROD activity has been commonly used as a biomarker of exposure to persistent organic pollutants. The mixed-function oxygenase (MFO) system along with its main component, the CYP group of enzymes, plays a fundamental role in the biotransformation of exogenous compounds. CYP catalyzes the oxidation of organic substances. It is the major enzyme in xenobiotics metabolism and accounts for 75% of the total number of different metabolic reactions. The induction of

CYP is often an adaptive response to the presence of xenobiotics. Induction of EROD in marine invertebrates has been frequently observed under the exposure of PCBs, PAHs and organochlorine pesticides (Binelli et al. 2006, Ramos & Garcia 2007, Sarkar et al. 2006). However in the current study, there is a lack of significant induction of EROD activity for the tested compounds at most of the exposure concentrations (Figure 4-6). As EROD activity describes the rate of CYP mediated xenobiotics metabolism, the current results imply a lack of biotransformation of the tested PFCs in the organism, which probably results from the high oxidation state of the functional group and the strong covalent carbon-fluorine bond. The observed results, however, support a previous conclusion that PFCs are metabolically inert and are resistant to biodegradation (Freire et al. 2008).

Decreases in the mean value of EROD activity were observed at 1000 $\mu\text{g/L}$ for PFOS, PFNA and PFDA. Previous studies showed that PFCs can modulate the gene expression of CYP enzymes (Hagenaars et al. 2008, Hu et al. 2005). It is possible that at higher exposure concentrations, PFCs can induce inhibition of EROD activity through gene modulation.

Figure 4-6 Response of xenobiotic metabolizing enzyme activity under PFC exposure. Significant responses ($p<0.05$) are marked with *.

Phase II enzymes play important roles in the detoxification and clearance of many xenobiotics. They protect cells by conjugating and neutralizing electrophilic compounds and metabolites from Phase I metabolism, rendering them as more water-soluble products, and therefore actively transported (Faria et al. 2009). GST is considered a major Phase II enzyme which is also an important detoxifying enzyme. GST activity is considered a sensitive biomarker of exposure to a broad range of contaminants in mussels (Faria et al. 2009). Compounds could be glutathione-conjugated by GST in a direct way or subsequently through the weakly active Phase I biotransformation enzyme. Similarly with Phase I enzyme, GST can be activated in the presence of xenobiotics. GST conjugating activity has been shown to increase with PAHs levels in *Perna viridis* (Faria et al. 2009). However in the current study, GST was inhibited under PFC exposure and the activity of the enzyme was found negatively correlated with PFC concentration in green mussels (Figure 4-6). PFC induced inhibition of GST was also reported in Liu et al. study (2007), where both PFOS and PFOA could significantly reduce the enzyme activity. Down-regulation of GST can also result in a disturbance in normal redox state which will then cause oxidative stress through the production of peroxides and free radicals. GSH is a common antioxidant. ROS can be reduced by oxidizing GSH. Moreover GSH is also one of the co-factors in GST-catalyzed conjugation. A decrease in GSH content is concomitant

to the decrease in GST. Suppressed GSH content under elevated PFC exposure has already been demonstrated in the previous section.

Studies have shown that PFCs have the ability to interfere transcription factors for metabolizing enzymes and may affect the transcription of several isozymes and their detoxification activities (Kennedy et al. 2004, Mortensen et al. 2011, Yeung et al. 2007). The decrease of EROD or GST activity could be due to the inhibition from the tested compounds, where PFCs may modulate the gene expression or down-regulate the transcription of the metabolizing enzymes (Hagenaars et al. 2008, Hu et al. 2005).

The observed deactivation of the enzymes indicates suppression in the cellular detoxification process under PFC exposure. The results not only suggest a lack of biotransformation of PFCs in the organism, but also imply that PFC exposure could have indirect adverse effects on organism health. The PFC induced inhibition of detoxifying enzymes places potential health risks to the organisms by weakening their self defense system, especially when other toxic pollutants are present.

4.4 Genotoxicity

In previous section, PFCs were found to be able to induce oxidative stress and subsequently cause damage to DNA molecules. It has been suggested that some PFCs could induce disturbance in DNA metabolism homeostasis (Arukwe & Mortensen 2011). Studies have reported that gene expressions of important biological functions such as energy consumption and reproduction can be influenced by PFOS exposure (Hagenaars et al. 2008). In other studies, PFOA was also found to suppress the expression of genes that were related to inflammation and immunity, and induce apoptosis (Cui et al. 2009, Freire et al. 2008). Occurrence of genetic damage after PFC exposure was also reported by flow cytometric analysis studies (Nobels et al. 2010). The results of these studies clearly indicate a genotoxicity potential of these compounds.

Genotoxicity refers to the damage by a physical or chemical agent of genetic materials such as chromosomes or DNA (Newman 2009). DNA damage can result in gene mutation, which could lead to serious biological dysfunctions, and even diseases, and the effects on the health of an organism normally take a long time to appear (Hagenaars et al. 2008, Villela et al. 2006). The ability of compounds to interfere with DNA integrity and

gene expression renders them potentially mutagenic or even carcinogenic. Therefore the examination of genotoxicity of PFCs can provide important information on their potential impacts on organism health.

The purpose of the current study was to investigate the genotoxicity potential of PFOS, PFOA, PFNA and PFDA. Genotoxicity of PFCs was assessed through three genotoxicity biomarker assays, including Comet Assay, DNA diffusion assay (Halo assay) and Micronucleus Tests. The time and concentration effects on the compounds' toxic behavior were examined. The organism burden of PFCs was also measured so that toxic behavior could be related to the inner concentration of the compounds.

4.4.1 Materials and Methods

The chemicals, experimental set-up, sample preparation and extraction have been described in Section 4.2.1.

4.4.1.1 Exposure and depuration experiment

The exposure levels were 0.1, 1, 10, 100 and 1000 g/L of individual PFC. The exposure period was 7 days, followed by another 7 days for the depuration period during which no PFCs were present. Mussels were sampled every 24-hour for biomarker tests and concentration analysis.

4.4.1.2 Biomarkers

Comet assay The Comet assay was performed using the Comet Assay Kit from CellbioLabs, Inc. In brief, the haemolymph suspension was centrifuged at 700xg for 2 min and the supernatant was discarded. The cells were washed and resuspended in ice-cold PBS at 1×10^5 cells/ml. The cell sample was then mixed with pre-liquified agarose at 1:10 (v/v). 75 μ l of the mixture was transferred immediately onto microscope slides. The slides were prepared in triplicate for each cell sample. The slides were then transferred to 4°C in the dark for 15 min and maintained horizontally. After gelation, the slides were immersed in ice-cold lysis buffer and in ice-cold alkaline solution each for 30 min at 4°C in the dark. Electrophoresis was performed in alkaline solution for 30 min at 1 volt/cm, 300mA. After electrophoresis, the slides were firstly washed in ice-cold DI water and then immersed in 70% ethanol for 5 min. 100 μ l of Vista Green DNA dye was applied to each slide before the slides were observed under epifluorescence microscopy.

Halo Assay Following the same procedures for Comet assay, after lysis in the lysis buffer, the slides were immersed in alkaline electrophoresis buffer for 5 min. After the unwinding, each slide was immersed twice in separate neutralizing solution for 30 min. The neutralizing solution was prepared by mixing Tris-HCl (20mM pH 7.4), spermine (1mg/ml) and ethanol (50% v/v) in DI water. Finally the slides were immersed in absolute ethanol for 5min and air dried. 50 μ l of diluted YOYO-1 dye (0.25 M YOYO, 2.5%DMSO, 0.5% sucrose dissolve in DI water) was applied for 15 min to stain the slides. The slides were then observed under epifluorescence microscopy.

Micronucleus (MN) test 50 μ l of the haemolymph suspension was then spread on a slide and left for 15min in a humidity chamber at room temperature to allow the hemocytes to settle down. The hemocytes were then fixed with glutaraldehyde (1% in PBS) for 5 min. The slides were then air dried. Each slide was stained with 50 μ l of bisbenzimidazole 33258 (1mg/ml) for 10 mins. The slides were then washed and mounted in glycerol: Mcllvane (1:1 v/v) buffer (pH 7) and kept in the dark at 4°C before being examined with fluorescence microscope with submerged lens at 100x magnification. Only intact cells with distinct nuclear and cellular membranes were scored. The micronucleus was identified according to: 1) spherical cytoplasmatic inclusions with a sharp contour; 2) diametric smaller than one third of the nucleus; and 3) the color and texture resembling the nucleus, and no contact with the nucleus.

4.4.1.3 Instrumental analysis

Mussel samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS, triple quadrupole 8030, Shimadzu, Japan) to quantify the organism concentration of the tested PFCs. Prior to injection, 100 μ l of internal standard solution 6 μ g/L containing [$^{13}\text{C}_2$] PFNA (for quantifying PFNA), [$^{13}\text{C}_2$] PFDA (for quantifying PFDA), [$^{13}\text{C}_2$] PFOS (for quantifying PFOS) and [$^{13}\text{C}_2$] PFOA (for quantifying PFOA) (Wellington Laboratories, ON) was added. 20 μ L of aliquot was then injected into a C18 column (3.5 μ m, 40mm $\times$ 2.1mm ID, Higgins Analytical, CA, USA) with the following chromatography program: binary gradient start with a flow rate of 0.25 mL/min and 30% methanol (Optima grade, Fisher, USA), equilibrated for 1 min then increased to 100% over 1 to 5 min, held at 100% to 10 min, then returned to 30% at 12 min and finished at 13 min. Aqueous 2 mM ammonium acetate (Sigma-Aldrich, Germany) was used as the other mobile phase.

Target compounds were optimized in negative ESI mode with the following instrumental parameters: ionization voltage 4.5kV, DL temperature 250 °C, nebulizing gas 3 L/min, heat block temperature 400 °C, drying gas flow 16 L/min, CID gas 230 kPa, interface current 26 µA. Table 4-1 shows the optimized parameters of individual PFC and their mass labeled compounds for instrumental analysis.

Table 4-1 Mass spectrometry parameters for target native and mass-labeled compounds.

	Parent transition	Daughter transition	Dwell (ms)	Q1 Pre Bias (V)	CE	Q3 Pre Bias (V)
PFOA	413.1	369.0	4	29	12	25
	413.1	169.0	4	29	18	30
PFOS	499.1	80.3	4	18	47	15
	499.1	99.2	4	17	38	18
PFNA	463.1	418.9	4	16	12	29
	463.1	219.0	4	16	18	23
PFDA	513.0	469.0	4	24	14	22
	513.0	218.8	4	24	20	14
[¹³C₂] PFOA	415.1	369.9	4	19	10	25
[¹³C₂] PFOS	503.0	80.0	4	24	41	30
[¹³C₂] PFNA	468.1	422.9	4	16	11	29
[¹³C₂] PFDA	515.0	24	4	24	11	22

4.4.1.4 Recovery test and blanks

Representative samples were spiked with each target PFC and analyzed using the method described above. For aqueous sample, 40ml of artificial seawater was spiked in with PFC standard to make 1 and 10 g/L (ppb) solution. For organism samples, 1 g of freeze dried and homogenized mussel tissue was spiked with 150 L of 100 ng/mL PFC standard. For all analytes reported, recoveries were between 90 and 110%. Blank samples consisted of Milli-Q water and spiked Milli-Q water.

4.4.1.5 Quantitation of PFCs

PFCs were quantified using 9 points calibration curves with internal standards, as

described above. Calibration curves were acquired for every batch of samples; linearity correlation coefficients (r) were > 0.99 . Blanks (Milli-Q water) were analyzed in triplicate using the same procedure and included filtration. The triplicate blank test was carried out in Milli-Q water and in solvent (methanol) blanks, where PFC concentrations were below the instrument detection limit. The limit of quantitation (LOQ) of each compound was the lowest concentration in the calibration curve with a signal to noise ratio (S/N) larger than 9, corresponding to an accuracy of 70-130%. The criteria have been previously described elsewhere (Higgins et al. 2005, Plumlee et al. 2008).

4.4.1.6 Data analysis

The statistical analysis of data and integrated biomarker analysis by EIBR system has been described previously in Section 4.2.1.

4.4.2 Results and Discussion

Genotoxicity can be assessed by the level of damage to genetic material. It has been suggested that DNA damage could be one of the major toxic modes of action of PFCs, and it has already been reported that some PFCs are able to affect gene expressions in the exposed organisms (Nobels et al. 2010, Rosen et al. 2008, Wei et al. 2009). In the current study, three genotoxic bio-endpoints were examined which included DNA single strand breaks and fragmentation (Comet assay, comet tail moment), chromosomal breaks (MN test, γ -micronucleus) and apoptosis (Halo assay). The results show that the tested PFCs were able to induce significant genotoxic responses in the target organism (Figure 4-7). The adverse responses generally increased with the exposure concentration and demonstrated a concentration dependency. Compared with the control, significant responses normally occurred only at elevated exposure concentrations (Figure 4-7).

Figure 4-7 Response of genotoxicity biomarkers in green mussels under PFC exposure. Values represent the mean $\pm$ standard error. Significant responses ($p < 0.05$) are marked with *. (a) Comet assay, (b) Halo assay and (c) MN test

Comet tail moment provides a measure of preliminary DNA fragmentation. DNA fragments and strand breaks were separated from intact DNA, yielding a classic comet tail under microscope examination. Current results show that all the tested PFCs were able to cause significant DNA fragmentation at 1000 g/L. Only PFOS and PFDA exhibited some induction at lower exposure levels. A larger comet tail moment normally indicates more DNA fragmentations and thus, more severe DNA injury. At each exposure level, PFOS exhibited higher induction of comet tail moment than other compounds and displayed distinct genotoxicity potential. Significant DNA fragmentation has also been shown to be induced by other organic contaminants such as common pharmaceuticals (Binelli et al. 2009a, Parolini et al. 2010).

Micronucleus (MN) test screens for potential genotoxic compounds, and detects permanent DNA damage. The MN test indirectly evaluates both chromosomal breaks and mitosis dysfunction (Villela et al. 2006). The results of the MN test were similar to the Comet assay results, with PFOS giving the maximum MN frequency, followed by PFDA, PFNA and PFOA. Significant MN induction has also been found in mussels exposed to carcinogenic PAHs (Siu et al. 2004). Micronuclei are cytoplasmic bodies having a portion of chromosome which was not carried to the opposite poles during the anaphase. The

observed formation of micronucleus may result in daughter cells lacking a part of the chromosome. It has been suggested that there could be a cause and effect relationship between DNA strand break and chromosome damage (Siu et al. 2004). This helps to explain the similar dose-response patterns of the results of Comet assay and MN tests.

Halo assay also detects fixed DNA damage in cells. It is a sensitive DNA diffusion assay for quantification of apoptosis. Nuclear DNA of apoptotic cells has abundant sites and under alkaline conditions, small pieces of DNA generated diffuse and giving the halo appearance. Normally a higher percentage of apoptosis indicates more severe DNA damage. The current result shows that PFOS, PFNA and PFDA are able to induce fixed DNA damage at exposure concentrations of 100 g/L and higher. The observed Apoptosis could be triggered by oxidative stress and related disruption in mitochondrial (Liu et al. 2007a). Among the tested compounds, PFOS again demonstrates marked genotoxicity potential by inducing more than 30% apoptosis than other PFCs. It is also noticed that the highest responses were not always observed at the highest exposure level. Under elevated exposure of contaminants, there is a slight decrease in apoptotic cells for PFOS. The reason is that apoptosis is induced only under mild genotoxic stimuli (Singh 2005). Therefore, when contaminant concentration is too high, necrosis may occur instead of apoptosis and the programmed cell death become natural cell death. Necrosis was detected in human cells exposed to benzo(a)pyrene and atrazine-based herbicide (Jiang et al. 2012, Zeljezic et al. 2006). In this case, the lowered biomarker response suggests an intensified genotoxic effect by PFOS.

When comparing the dose-response results of the three biomarkers, they displayed similar trend with respect to exposure concentration (Figure 4-7). As mentioned earlier, the Comet assay measures preliminary DNA damage, which is likely to lead to fixed genetic injuries such as apoptosis or the production of micronuclei. It has been previously suggested that DNA strand breaks is an apoptosis-inducing factor and also a contributor to MN induction (Parolini & Binelli 2012). It is possible that the hemocytes activate the apoptotic processes or produce micronuclei when serious DNA injuries occur. This helps to explain the similar pattern of the three biomarker results.

Genotoxicity potential of PFCs: In spite of similar response pattern, there are distinctions in the amount of adverse responses between the tested compounds. At the same exposure level (mainly 100 and 1000µg/L), the magnitude of toxic response normally followed the order of PFOA<PFNA<PFDA<PFOS. The integrated biomarker analysis of

the three biomarkers also shows that, in terms of the EIBR value, the relative genotoxicity potential followed the same order (Table 4-2, Figure 4-8). The EC₅₀ values and confidence interval (CI) calculated using probit analysis based on the integrative genotoxicity are 67 (61-73), 817 (790-838), 243 (229-251) and 118 (101-124) g/L for PFOS, PFOA, PFNA and PFDA respectively. PFOS was shown to have significantly higher response scores and lower EC₅₀ values compared to the other PFCs. The results suggest that PFOS has higher genotoxicity potential than the other perfluorinated carboxylates. Among the three PFCAs, the genotoxicity potential simply increases with their fluorinated chain length (Figure 4-9).

PFOS related genetic injuries have been reported in many studies (Guruge et al. 2009, Rosen et al. 2008, Yeung et al. 2007). The occurrence of DNA damage after exposure to PFOS has been observed in common carps (Hoff et al. 2003). Gene expression studies in zebrafish embryos revealed DNA damage mediated apoptosis after exposure to PFOS as well (Shi et al. 2008). In some other studies, examination of gene expressions suggested that the effect of PFC chain length is a more important factor than the functional group (Hagenaars et al. 2008, Nobels et al. 2010). However, Hagenaars et al. (2008) found that the effects at gene expression level were higher for the sulfonate than for the carboxylate. Biomarker results from the present study also show that the functional group of PFCs is the major factor that affects the compounds' interaction with genetic materials.

Table 4-2 EIBR values of genotoxicity biomarkers.

	PFOS	PFOA	PFNA	PFDA
Perfluorinated carbon no.	8	7	8	9
EIBR at 100 µg/L	0.64±0.04	0.10±0.03	0.30±0.04	0.45±0.04
EIBR at 1000 µg/L	0.86±0.02	0.25±0.04	0.55±0.04	0.72±0.04

EIBR values at each exposure level are statistically different from each other ($p < 0.01$, t -test).

Figure 4-8 Comparison of genotoxicity EIBR among tested PFCs.

There are also some inconsistencies in the genotoxicity data of PFOA. PFOA has been reported to induce apoptosis, strand breaks and MN in human HepG2 cells (Freire et al. 2008). PFOA induced apoptosis was also reported in Liu et al.'s study (2007) in fish cells. However, Kim et al. (2010) found no significant induction of DNA fragmentation from PFOA even at an elevated exposure concentration of 50000 g/L in carps (Kim et al. 2010). In the current study, there is also a lack of significant induction of apoptosis under PFOA exposure, while a notable response in DNA fragmentation was observed in PFOA exposed mussels. These contradictions could be possibly due to the fact that PFC induced genotoxic effects are species specific. In addition, exposure level, exposure duration and laboratory conditions may also be the causes of variability in the test results.

Figure 4-9 Comparison of EC₅₀ based on integrated genotoxicity evaluation.

Possible cause of genotoxicity: It has been suggested that one of the triggers of DNA damage is oxidative stress (Parolini et al. 2010). This may help to explain the observed adverse effects in the genotoxicity biomarkers in the present study as well. Previous study has demonstrated that PFCs can induce excessive production of ROS and impose oxidative stress in green mussels. The ROS was accused of interfering with DNA, leading to genetic injuries such as strands breaks (Freire et al. 2008). In addition, PFCs may also influence the homeostasis of DNA metabolism, as suggested in previous gene profiling studies (Arukwe & Mortensen 2011). A direct interaction with DNA molecules is also a possible toxic pathway. Besides the genetic damage measured by the above biomarkers, genotoxicity could also result from injuries such as double strand breaks, translocations and inversions, according to studies of other organic pollutants (Parolini et al. 2010).

Exposure time dependency: Other than the exposure concentration, exposure duration was also a significant factor influencing PFC induced genotoxicity on green mussels. The adverse effects showed both dose- and time-dependency (Figure 4-10). At the

Figure 4-10 Time dependency of genotoxicity biomarker responses and bioaccumulation in green mussels. Co and Cw are the organism and water concentration, respectively (Cw = 100 g/L).

same exposure level, the genotoxicity biomarker response increased with exposure time, as the organism burden of PFCs increased as a result of bioaccumulation. The amount of PFC bioaccumulation is also positively related with the exposure concentration, as discussed in previous chapter. Therefore, the inner PFC concentration may provide an important link to the observed dose-response and time-response relationship.

During the exposure period, biomarker responses increased with the accumulation of PFCs in the organism (Figure 4-10). This observation is in agreement with a previous conclusion that there could be a potential relationship between body burden of organic contaminants and DNA damage (Siu et al. 2008). During the depuration phase, PFCs are being eliminated from the organism. However, different response patterns among biomarkers were observed, where biomarkers showed both reversible and irreversible behavior after PFC exposure stopped, although for the same biomarker, there was not much variance in the response pattern of the tested PFCs.

For comet tail moment, toxic responses started decreasing with organism concentration of PFCs when the exposure stopped. As mentioned previously, the Comet assay measures only minor DNA fragmentation where the organism's self-repairing system is capable of fixing (Villela et al. 2006). This measured DNA damage is a net result of two concurrent processes: damage and repair (Siu et al. 2008). When PFCs started to accumulate in the body, the induced DNA fragmentation exceeded the capacity of the repair system and thus accumulated. When the exposure stopped, the stress reduced as PFCs were eliminated from the body, and the self-repairing system was capable of fixing most of the damaged DNA and the amount of DNA fragmentation decreased.

However, unlike the Comet assay, toxic effects measured by the Halo assay and MN test were not restored at the end of depuration. This could be because both assays measured permanent damage to genetic material and cell nucleus. Even though the inner concentration of PFCs decreased after exposure stopped, the damage could not be repaired and thus, the toxic effects are irreversible. Slight decreases in biomarker responses were still observed at the end of depuration. The minor reduction in the measured toxic effects could be caused by cellular renewal mechanism by which damaged cells are removed. The observed irreversibility of genotoxic effects suggest that injuries of genetic material caused at high PFC exposure level may not be recovered when exposure stops. Considering the far-reaching impacts of genotoxicity, as discussed earlier, even an accidental spill of the contaminants may have prolonged impact on the health of an exposed organism.

In summary, the genotoxicity potentials of tested PFCs were demonstrated in the present study. PFC induced genotoxic effects were shown to increase with both exposure concentration and time. The results also suggest that among the tested compounds, PFOS exhibits higher genotoxicity potential compared with other PFCAs. The fluorinated chain length has been proved in many studies to be the decisive factor influencing toxic activity of PFCs. However, results of the present study indicate that in terms of genotoxicity, the functional group plays a more important role. Although significant adverse effects were only detected at relatively high exposure concentration (approximately 100 g/L or higher), a short term exposure at elevated concentration could have prolonged effects on the health of organisms, since PFC induced damages to genetic materials could be irreversible and may lead to permanent health effects.

4.5 Immunotoxicity

At molecular level, PFCs were found to be able to activate/inhibit important enzymes activity, and cause damage to macromolecules such as lipid and DNA. In this section, cellular level effects of PFCs were studied by examining the immunotoxic biomarkers.

Organisms rely on their innate defence mechanism to identify and protect against foreign matter (Hannam et al. 2009). The ability of organisms to respond effectively against pollutants is important to their general health (Giannapas et al. 2012). Disruption of the immune system results in high health risks to the organism. Adverse effects from pollutants can be resulted from immune susceptibility even at sublethal level. Previous studies suggested that the immune system is a sensitive target of PFCs (Corsini et al. 2012). However, these studies targeted on human health effects and the data is limited to human cells or rodent species (Dewitt et al. 2012). To date there is little data available on the immunological effects of PFCs on aquatic invertebrates which represent more than 90% of the existing species and thus play an important role in the ecosystem functioning (Binelli et al. 2009a). Considering the widespread contamination of PFCs, it is necessary to extend the investigation to better understand their ecological impacts. For invertebrates, hemocytes are an important component of their immune system (Sheir & Handy 2010). Haemolymph has also been suggested to be the most appropriate tissue for biomonitoring, because of its direct exposure to environmental contaminants and its physiological role in transportation of toxic materials (Villela et al. 2006).

The aim of this study was to conduct an *in vivo* investigation on the immunotoxicity of PFCs in green mussels. Immunological effects of the four long chain PFCs (C7-C9) were examined by measuring biomarkers of the immune profile, which included Neutral Red retention, phagocytosis and spontaneous cytotoxicity. Mussels are often used as sentinel organisms for contamination monitoring (Luengen et al. 2004). The circulation system of mussels is continuously exposed to their living environment as well as the contaminants (Thiagarajan et al. 2006). The current study is the first to examine the effect of PFCs on the immune health of marine invertebrates. The investigation would reveal the robustness of organism in the presence of PFCs.

4.5.1 Materials and Methods

The chemicals, exposure and depuration experiment, mussel maintenance, sample preparation, chemical quantification and data analysis were described in Section 4.4.1. The integrated biomarker response (EIBR) calculation was described in Section 4.2.1.

Biomarkers

Trypan blue exclusion Trypan blue was employed to measure cell viability. The method has been described elsewhere (Liu et al. 2007). In brief, 0.4% trypan blue was added to the hemocytes suspension in 1:2.5 ratio (v/v). The cells were then examined under microscope at 400x magnification. An average of 150 cells was counted in 4 different fields per culture. Cell viability was evaluated based on the percentage of stained cells.

Neutral red retention time (NRRT) The haemolymph mixture was transferred to a poly-L-lysine coated microscope slide and the slides were immediately placed into a light-proof humidity chamber for 15 mins. After incubation, excess haemolymph mixture was removed. Neutral red working solution was added to the slides and examined under a light microscope using an x40 objective every 15 min. When not examined, the slides were kept in the humidity chamber. The time at which 50% of the cells showed stress was recorded as the retention time. The neutral red working solution was prepared by mixing neutral red stock solution (20mg/ml in DMSO) with physiological saline at 1:200 (v/v).

Spontaneous cytotoxicity The test method has been described previously (Sheir & Handy 2010). In brief, the sheep red blood cells were washed with PBS and resuspended in TBS-Ca to 2×10^6 cells/ml. The mussel hemocytes were washed with TBS and also resuspended in TBS-Ca to 2×10^6 cell/ml. 100 μ l hemocytes were then mixed with 100 μ l

sheep RBC and incubated at 25°C for 60 min on a shaker. The samples were centrifuged at 100xg for 5 min after the incubation. 100 μ l of the supernatant was transferred to 4 replicate wells in a microplate and the absorbance was read at 405nm. The specific cytotoxicity was calculated as a percentage relative to the maximum release (100 μ l sheep RBC in 100 μ l H₂O) and the minimum or the spontaneous release (100 μ l sheep RBC in 100 μ l TBS-Ca).

Phagocytosis The phagocytosis assay measures the ability of hemocytes to engulf foreign material which in this case are zymozan particles. The test method has been described previously (Sheir & Handy 2010). In brief, the zymozan particles were firstly stained with neutral red and resuspended in TBS at a final concentration of 1×10^7 particles/ml. Mussel haemolymph was diluted with TBS at 1:1 dilution. 50 μ l of each haemolymph samples was aliquoted to 4 replicate tubes followed by addition of 50 μ l of red-stained zymosan suspension. Zymosan with fixed hemocytes and zymozan in buffer were used as blank and negative control respectively. After 30min incubation at 10°C, 100 μ l Baker's formol calcium (with 2%NaCl) was added to stop the reaction. The tubes were centrifuged and supernatant was discarded. The sample was resuspended in 100 μ l TBS. The above procedures were repeated 6 times until there was no evidence of zymosan remaining in the negative controls. The hemocytes were solubilized by adding 100 μ l 1% acetic acid in 50% ethanol and incubated for 30 mins. They were then transferred to a microplate and read at 550nm.

4.5.2 Results and Discussion

4.5.2.1 Immunotoxicity biomarker results

The selected immunological biomarkers provide an assessment of the health status of the internal defence system of the organism. Biomarker results showed that exposure to PFCs resulted in measurable reductions in the immune fitness of green mussels, as indicated by the significant decrease in the biomarker response as the exposure level increases (Figure 4-11). The hemocyte cell viability was measured by trypan blue exclusion assay. Immune activity depends on viable cell-to-cell interaction. Trypan blue evaluates cell viability through measuring intact cellular membrane, which is closely related to the immune mechanism. The results show that the tested compounds can decrease cell viability in a dose-dependent manner (Figure 4-12). Some have suggested that the loss of cell viability could be mediated through ROS production (Liu et al. 2007c).

PFC induced loss in cell viability has also been reported in other species (Kraugerud et al. 2011, Slotkin et al. 2008, Watanabe et al. 2009).

Figure 4-11 Response of immunotoxicity biomarkers in green mussels under PFC exposure. Significant responses ($p < 0.05$) are marked with*. (a) NRRT, (b) Phagocytosis and (c) Spontaneous cytotoxicity

Figure 4-12 Hemocyte cell viability test result under PFC exposure. Significant responses ($p < 0.05$) are marked with*.

Lysosome is a cellular organelle that can engulf foreign substances and break down wastes and cell debris. They constitute the main sites of toxic metal and compound sequestration and detoxification (Dailianis et al. 2003, Giannapas et al. 2012). NRRT assesses lysosome membrane stability by measuring the Neutral Red retention time. In the current study, significant reduction of NRRT was observed at 100 g/L for most compounds and a further decrease at 1000 g/L (Figure 4-11). The results show that PFC exposure may lead to instability of lysosome membrane. Lysosomal membrane damage can be caused by excessive storage of PFCs in the lysosome, or direct interaction with the membrane. Previous studies showed that some PFCs can cause an increase in membrane fluidity through direct interference with the membrane (Hu et al. 2003). Also, PFC exposure can induce lipid peroxidation, which is the likely cause of damage to the lysosome membrane as well. The interaction with the organelle membrane could be the major pathway for PFCs to affect lysosome stability. However, only minor adverse response was observed in mussels under PFOS exposure compared with other PFCAs. As lysosomal instability is probably caused by the organelle membrane damage, this result may be explained by a previous finding that only minor inductions of cellular membrane damage were obtained from PFOS when compared with other PFCAs (Nobels et al. 2010)

Phagocytosis and spontaneous cytotoxicity are the major mechanisms in the immune system of invertebrates to suppress infection. Phagocytosis is the cellular process of engulfing solid particles. It is considered as an important mechanism to remove pathogens and bacteria. Inhibition of phagocytosis induced by pollutants could suppress the immune-competency of the organism. Previous studies have reported reductions in phagocytosis in marine bivalves exposed to oil-contaminated seawater and organic pollutants (Auffret et al. 2004, Hannam et al. 2009). The current study showed that most tested PFCs can also induce inhibition of phagocytosis, as demonstrated by the decreased biomarker response (Figure 4-11). This inhibition of phagocytosis could result from the reduced hemocyte cell viability under PFC exposure, as demonstrated by the trypan blue exclusion test. In addition, phagocytosis of bivalve hemocytes can be modulated by specific membrane receptors (Wang et al. 2011b), which in this case could be bonded to and deactivated by PFCs. Spontaneous cytotoxicity assay measures the ability of mussel hemocytes to recognize and kill foreign cells. Similar response patterns were observed for spontaneous cytotoxicity and phagocytosis (Figure 4-11). The reason for this could be that both immune

activities are closely related with cellular membrane viability, where functioning of these immune mechanisms rely on viable cell-to-cell contact between hemocytes and foreign cells (Hannam et al. 2009).

The PFC induced inhibition of these immunological biomarkers suggests a weakened internal defense system in the presence of the contaminants. As observed in the current study, statistically significant decreases in normal immune functionality were associated with elevated exposure concentration of PFCs, especially at 1000 g/L level, where the immune activities could be suppressed by up to 50% of their normal performance. These results indicate that PFCs have an immunotoxicity potential and will impose health risks to organisms at high exposure concentrations of the compounds, for example during an accident spill. Immune modulation has been reported in mussels from polluted sea water (Hannam et al. 2009), and also under the exposure of toxic metals and organic pollutants such as PAHs (Giannapas et al. 2012, Thiagarajan et al. 2006). PFCs have also been reported to modulate immune function in rodent and fish (Son et al. 2009, Yang 2010). In cultured human cells, PFCs were found to be able to directly affect immune cell activation and reduce cytokine production (Corsini et al. 2012). As the immune system protects organisms from foreign substances and pathogens, a weakened immune system implies that the organism becomes vulnerable to their living environment. Although some studies have shown that PFCs will not induce lethal toxic effects, the current results suggest that sublethal effects induced by PFCs could be equally potent: the health of an organism will be greatly compromised by a susceptible immune system, especially in the presence of other toxic pollutants or occurrence of disease. It is also worth noting that, in some cases, contaminants exposure could induce elevated immune responses to sequester potentially toxic contaminants, especially at low contamination levels (Hannam et al. 2009, Luengen et al. 2004). However, similar induction was not observed in the current study.

There are several hypotheses of the causes of the observed immunotoxicity under PFC exposure: 1) Gene regulation. As mentioned, the defence mechanism of phagocytosis and spontaneous cytotoxicity relies on effective cell attachment. Previous studies have demonstrated that PFCs can cause suppression of genes that are related to cell adhesion (Cui et al. 2009, Wei et al. 2009). 2) Binding with functional protein. The susceptibility in immune functions could also be due to alterations in cytoskeletal proteins which would then affect cell viability and functionality (Thiagarajan et al. 2006). The alterations could be caused by direct interaction with PFCs by binding with the cytoskeletal proteins, due to

their hydrophilic nature (Kelly et al. 2009). For example, the peroxisome proliferator-activated receptor (PPAR) proteins, which are one of the cytoskeletal proteins, were found to play an important role in PFCs immunotoxicity (Dewitt et al. 2012). The receptor proteins regulate important physiological process that impact homeostasis, inflammation, adipogenesis, wound healing and carcinogenesis (Corsini et al. 2011). PFCs may bind to and activate PPARs and lead to various effects and responses. 3) Oxidative stress. PFC induced oxidative stress has been reported in previous study. It has been suggested that immune susceptibility in hemocytes of bivalves exposed to organic toxicants is probably related to the depletion of non-enzymatic antioxidant molecules, such as GSH (Giannapas et al. 2012). Depletion of antioxidant components may cause depletion of antioxidant capacity and lead to oxidative stress. Studies on the antioxidant responses have demonstrated the reduction of GSH content under PFC exposure. Excess ROS and the associated oxidative stress could also be one of the causes of immunotoxicity.

4.5.2.2 Reversible responses and model

Exposure duration was another significant factor influencing the immune fitness of green mussels. At the same exposure level, adverse responses increased with exposure time (Figure 4-13). The magnitude of adverse response was found to be associated with the PFCs burden inside the organism. During the depuration phase, toxic responses started decreasing when exposure to PFCs stopped (Figure 4-13). The immune fitness shows the tendency to restore to its original healthy state during the depuration period. The recovery of immunotoxicity has been reported in studies of different environmental stresses (Wang et al. 2011b). Reversible effects of PFCs accompanied with depuration of organism burden of the compounds have also been reported in literature (Du et al. 2009, Hu et al. 2002, Stevenson et al. 2006). This could be a result of a reversible binding to integral membrane proteins and tissue proteins.

(a) Integrated immunotoxic response (EIBR) vs. time

(b) Organism concentration vs. time

Figure 4-13 Relationships between integrated immunotoxicity, organism concentration and time
($C_w=100\mu\text{g/L}$).

Immunotoxicity results suggest that the toxic effects are closely related to the concentration of PFCs in the internal environment of the organism. For organisms exposed to different levels of PFCs or with different exposure durations, approximately the same toxic response was obtained when the organism concentration of PFCs reached the same level. Correlation analysis was performed and the results further confirmed the correlations between organism PFC concentration and integrated toxic response (Figure 4-14). Models using organism PFC concentration and BAF as the independent variables were established accordingly to describe/quantify the integrated immunotoxicity as follows:

$$\log EIBR = a \log C_o + b \log BAF + c \quad (4.6)$$

$$n = 4, r^2 = 0.877, s = 0.142, F = 596, Q^2 = 0.872$$

where EIBR is the integrated immunotoxicity biomarker response value (Equation 4.3); C_o is the organism PFC concentration in ng/kg and BAF is the bioaccumulation factor in L/kg measured in previous bioaccumulation study. Parameters a, b and c are constant for all the tested compounds. Based on the current biomarker results, the values of these constant were found to be 0.983, -0.712 and -2.070 for a, b and c, respectively. Equation 4.6 provides a unified model to evaluated toxicity of different PFCs at various C_o by using a single set of a, b and c values. The model shows that for the same compound, immunotoxicity increases with the organism concentration of the compound. The toxic effects also varies among compounds as a factor of the bioaccumulation potential, where the larger the BAF, the more severe the exhibited immunotoxicity.

The dose-response relationship demonstrates that there is an effective exposure concentration, approximately 10-100 $\mu\text{g/L}$, from which significant response can be observed. Therefore results from exposure levels of 0.1 and 1 $\mu\text{g/L}$ were omitted for the model development.

Figure 4-14 Correlation analysis of immunotoxicity EIBR with organism concentration C_o . Dashed line represent 99% confidence interval.

The toxicity model provides a tool to evaluate and predict adverse effects induced in organism under PFC exposure when the organism concentration is known. However in reality, it is more conventional and easier to monitor the contaminants concentration in the water phase. In this case, a toxic response model using exposure concentration would be more convenient. In PFC bioaccumulation studies, a number of bioaccumulation models were established which can be used to transform the organism concentration C_o to exposure concentration C_w . For example in Chapter 3, it has been demonstrated that organism concentration of PFCs can be modeled through the following equation:

$$C_o = f(C_w, t) = \frac{nk_u C_w}{k_u C_w + k_e} \{1 - \exp[-(k_u C_w + k_e)t]\} \quad (4.7)$$

By substituting C_o in Equation 4.6, the immunotoxicity model could be further modified as a function of C_w , the exposure concentration:

$$EIBR = 10^c \cdot BAF^b \left(\frac{nk_u C_w}{k_u C_w + k_e} \{1 - \exp[-(k_u C_w + k_e)t]\} \right)^a \quad (4.8)$$

With this equation, toxicity of PFCs can also be estimated when the exposure level and exposure period are known. However it should be noted that the C_w based model may not accurately reflect the pollution status. This is because in the real environment, the contamination level could be fluctuating during the exposure period.

In summary, the immunotoxicity potential of selected PFCs was demonstrated and PFC induced inhibition on the organism immune mechanism was observed, which suggest a weakened internal defence system in the presence of these contaminants. The observed immunotoxicity was also found to be reversible and correlated with the inner concentration of the compounds. Based on this correlation, immunotoxicity models were established by which integrated immunotoxicity biomarker response can be evaluated when the inner concentration and BAF of compounds are known. For the tested compounds, their environmental levels are generally below the valid range of the immunotoxicity model, except for PFOA (50-240 ng/kg in Singapore coastal water), where the model indicates that current pollution level of PFOA may already affect the health, especially immunity health of marine organisms. Although not specified by the results of the current study, it should be noted that besides the low threshold, there might also be a high threshold where the immune system is totally destroyed by the compounds and is not able to recover. In either case, the proposed model may not be appropriate.

4.6 Effects on general well-being

In the previous part, PFCs was shown to be able to induce a number of adverse effects in green mussels in both bio-molecules and cell organelles. Besides molecular and cellular effects, contaminants can also affect the living function and general well-being of an organism. The effects are evaluated using bio-endpoints measuring an organism's physiological activities. Physiological biomarkers have been applied for biomonitoring purposes in many studies (Hagenaars et al. 2008, Okay & Karacik 2008), and provided useful information of the general health state of a target organism. Although lower level

biomarkers are usually more sensitive, physiological level biomarkers provide more ecologically relevant evaluation of an individual health. Effects on the general well-being of an organism and the effective concentration are thus more meaningful for environmental risk assessment. In order to investigate the effects on the general health of green mussels under PFC exposure, two physiological biomarkers were examined, namely filtration rate (FR) and relative condition factor (RCF). These two are common biomarkers in aquatic wildlife that assess the general well-being of the organisms.

4.6.1 Materials and Methods

The chemicals, mussel maintenance, exposure experiment, sample preparation and statistical analysis were described in Section 4.2.1. The exposure concentrations of individual PFC were 0.1, 1, 10, 100 and 1000 µg/L, and the exposure period was 7 days.

Biomarker analysis

Filtration rate The filtration rate was determined as described elsewhere (Okay and Karacik 2008) with modifications. The filtration rate was based on the filtration of microalgae by individual mussels in static systems. At the end of the exposure period, mussels were placed separately in 3L plastic tanks with magnetic stirrers. The tanks were filled with 2L of artificial seawater and 200 ± 1 dense algae were added to each tank. The concentration of algae in each tank was determined by a spectrophotometer (750 nm) at 10 min intervals for a total time period of 120 min. The filtration rate of mussels was evaluated as:

$$V \cdot \frac{dC}{dt} = Q \cdot C \quad (4.9)$$

$$Q = \frac{(\ln C_1 - \ln C_2) \cdot V}{t_1 - t_2} \quad (4.10)$$

where V = volume of the tank and Q = filtration rate in L/hour

Relative condition factor (RCF) The weight and shell length of mussels were measured at day 0 and 7 of the exposure experiment. The relative condition factor (RCF) was calculated as an indicator of the general well-being of the mussel:

$$RCF = \frac{W}{aL^b} \quad (4.11)$$

where W is total body weight (in g) and L is shell length (in cm). The parameters a and b were determined from the length-weight relationship ($W = aL^b$) of mussels at day 0 and were assumed constant for all individual mussels. The RCF is calculated as the ratio of measured body weight and calculated body weight. This procedure allows a comparison of the condition of each concentration group as well as the control group before and after the exposure.

4.6.2 Results and Discussion

The length-weight relationship ($W = aL^b$) assumes an important prerequisite in fishery biological investigations. Together with RCF, they have been applied in the monitoring of bivalves as indicators of general health state and suitability to the environment (Bradbury 2005). In the current study, an evident decrease in RCF value in green mussels was observed after exposure to most tested PFCs (Figure 4-15). This result is consistent with previous findings of the decrease in the body weight of mice and freshwater fish under PFC exposure (Hagenaars et al. 2008, Liu et al. 2009). Declines in both RCF and body weight could be directly equivalent to a decrease in the energy store of organisms. Microarray studies showed that PFC exposure could down-regulate energy metabolism related genes, leading to a lower energy uptake (Hagenaars et al. 2008, Wei et al. 2009). Dorts et al. (2011) in their proteomic analysis also demonstrated that PFC can induce decreases in proteins and enzymes related to energy metabolism, such as ATP biosynthesis. In fact, disruption of energy metabolism has often been associated with exposure to xenobiotics, since detoxification of xenobiotic is an energy consuming process. The increased energy expenditure will compromise processes such as growth and reproduction.

Figure 4-15 Responses of filtration rate (FR) and relative condition factor (RCF) in green mussels under PFC exposure. Significant responses ($p < 0.05$) are marked with *.

Filtration rate has been used in many studies as a component of scope for growth in bivalve mollusks as a sensitive indicator of pollutant exposure (Canty et al. 2009). It provides a measure of the fundamental living activity of the organism. In the current study, a decrease in the filtration rate was observed only at elevated exposure concentration

(Figure 4-15). Bivalves open their valves to facilitate the free circulation of water through gills, allowing them to respire and feed. It has been suggested that organic contaminants could alter the behavior of bivalves by changing the valve movement and reducing filtration rate (Faria et al. 2009). Filtration directly relates to food intake and thus energy intake. Decreasing filtration rate would most likely result in deficiency in energy store, which then affects the growth rate of the organism. It is also possible that valve movement was suppressed to a basic maintenance level due to the reduced energy store under PFC exposure. There is a lack of significant response to PFOA in both filtration rate and RCF. This could be due to the low accumulation of this compound in the organism, especially when these higher level biomarkers are generally less sensitive.

The observed responses in both RCF and filtration rate could both be attributed to the reduced energy store. There might also be a causal relationship between the two where the reduced filtration rate results in a reduced energy intake which then leads to a lowered RCF. It has been suggested that a decrease in the energy store could be attributed to DNA damage. As discussed earlier, DNA damage can cause modulation of genes, including those genes that are related to energy metabolism. Genotoxicity has been shown to be closely tied to the survival and development of organisms in other studies as well (Al-Subiai et al. 2011).

RCF and filtration rate are physiological level biomarkers. They provide an assessment of living function and general well-being of an organism. Contaminants could affect a biological system at many levels. Starting with bio-molecules, the effect can cascade through molecular, cellular, physiological and individual levels. Higher level effects are more closely related with individual health and are thus of more concerns. Results in the present study demonstrate that PFCs are able to induce adverse effects in green mussels at the physiological level, which implies a great health risk from these compounds.

4.7 Conclusion

The results of the environmental toxicity examination demonstrate that PFCs, at certain concentrations, could induce toxic responses across different biological levels (bio-molecular, cellular and physiological levels) of organism and through different toxic modes of actions. The PFC induced adverse effects indicate that these compounds have the

potential to cause oxidative stress and oxidative toxicity, DNA damage and genotoxicity, inhibition of xenobiotic metabolism, immunotoxicity and also deleterious consequences on the general well-being of the target organism. Significant toxic response was normally observed at exposure concentrations of 100-1000 µg/L, where the effective concentration is generally higher as the biological level of the measured bio-endpoint goes up. Compared with other environmental pollutants, the effective concentration of PFC is generally higher. In aquatic invertebrates, the effective concentrations of pharmaceuticals were found to be approximately 0.1-10 µg/L, and were around 10-100 µg/L for some heavy metals, which are very close to the environmental occurrence of these pollutants. Although significant toxic responses were normally observed at concentrations 3-4 orders of magnitude higher than the environmental levels of PFCs, it should be noted that low external dose does not necessarily mean low risks. Long term exposure to low contamination level may also lead to an internal dose which induces toxic responses. It should also be noted that in the current study, only water exposure is considered. However, in reality there may also be exposure through dietary, which will enhance the bioaccumulation from the environment and intensify the toxic effects at same exposure level.

Chapter Five Integrated assessment of ecotoxicity of PFCs

5.1 Introduction

The environmental toxicity of PFCs has been investigated through biomarker based toxicity testing in previous study. Due to the complexity of contaminants in the environment and the variety of responses that they may induce in organisms, it is essential that multiple biomarkers are employed (Brooks et al. 2009). Therefore, a series of biomarkers that correspond to different biological levels were employed. The results have demonstrated that various toxic effects can be induced by PFCs in the target organism. However, a great challenge is to combine these single toxicity test results for an integrated assessment, which includes correlations of different toxic effects and overall toxicity potentials.

In this chapter, biomarker results of various toxic modes of action were analyzed jointly. The correlations of these biomarkers were investigated to elucidate relationships between toxic responses at different biological levels, and more importantly, to examine the possible toxic pathways of the targeted compounds. The complex biomarker responses were also integrated by the Enhanced Integrated Biomarker Response (EIBR) system and transformed into a single value that was ecologically relevant and meaningful for environmental risk assessment. The EIBR enables comparison of PFCs in terms of integrative toxicity potential and toxic modes of action. Last but not least, the structure-activity relationships of these compounds were also examined based on the integrated biomarker results.

Six biomarkers were selected from the biomarker pool, which covered bio-endpoints of each measured toxic mode of action at the three biological levels (molecular, cellular and physiological levels). The selected biomarkers are the most representative ones that test the specific toxic effect. They are also the most commonly applied assays in environmental toxicity testing.

5.2 Examination of toxic pathways

A series of biomarkers corresponding to three different bio-organization levels, namely, molecular, cellular and physiological level, were tested previously. In order to elucidate relationships among toxic responses at various biological levels, correlation analysis was applied to selected biomarkers which included CAT, Comet Assay, EROD, NRRT, Filtration rate and RCF. The aim was to provide a comprehensive understanding of how PFCs could affect the target organism from biomolecules to general well-being. The biomarkers employed and their corresponding biological level and toxic mode of action are elaborated in the following table. In the correlation analysis, biomarker results of two compounds, PFOA and PFOS, were included to examine possible variations in the toxic pathways of the two groups of PFCs, where PFOA and PFOS represent perfluorinated carboxylate and perfluorinated sulfonate, respectively. Although the production of PFOS and PFOA has been phased out in the past decade, these two compounds are still the predominant species of PFCs detected in the environment and biota (Nobels et al. 2010). An indirect mode of toxic action was also observed, which is a distinctive feature from many well-studied POPs.

Table 5-1 Selected biomarkers and their corresponding biological levels.

Biomarker	Biological level	Toxic mode of action
Catalase (CAT) activity	Bio-molecular	Oxidative toxicity
Comet tail moment	Bio-molecular	DNA damage
7-ethoxy resorufin O-deethylase (EROD) activity	Bio-molecular	Xenobiotic metabolism
Neutral red retention time (NRRT)	Cellular	Immunotoxicity and cell viability
Filtration rate	Physiological	Energy consumption
Relative condition factor (RCF)	Physiological	General well-being

5.2.1 Materials and Methods

The chemicals, exposure experiment and biomarker analysis were described in

previous sections. The exposure period was 7 days and the exposure concentrations were 0.1, 1, 10, 100 and 1000 µg/L.

5.2.1.1 Data analysis

Multivariate statistical methods were utilized. The Pearson's correlation test was carried out to investigate possible correlations between biomarkers. All statistical analysis was performed using SPSS 19.

5.2.2 Results and discussion

The toxicity assay results of the selected biomarkers have been discussed in previous sections in Chapter 4.

5.2.2.1 Correlation analysis and toxic pathways

The correlation results show that CAT activity and Comet tail moment are positively correlated for both compounds (Table 5-2). As discussed previously, as an antioxidant enzyme, induction of CAT is an adaptive response to oxidative stress imposed by ROS. Increased CAT activity indicates an increase in the amount of ROS in the cells. On the other hand, ROS has been accused to be responsible for DNA damage in many studies (Al-Subiai et al. 2011, Binelli et al. 2009b). Therefore, the excess ROS production induced by PFC exposure and the resulting oxidative stress provide important links to the antioxidant enzyme activity and the damage to DNA integrity.

For PFOA, the NRRT was found to negatively correlate with CAT activity (Table 5-2). The negative correlations between the NRRT and CAT activity and with Comet tail moment are in line with previous studies of other xenobiotics (Binelli et al. 2009b, Parolini et al. 2010). It was suggested that lysosomal membrane destabilization is most likely to be attributed to the overproduction of free radicals, which is one of the possible pathways in which PFC exposure can induce membrane damage, as discussed earlier. However, similar correlations were not observed for PFOS, which indicate a difference in the modes of action of the two compounds.

Good correlations have also been demonstrated between DNA damage and physiological responses for PFOS. A negative correlation (Table 5-2) was observed between RCF and Comet tail moment. This suggests that a decrease in the energy store could be attributed to DNA damage. As discussed earlier, DNA damage can cause

modulation of genes including the genes that are related to energy metabolism. Genotoxicity has been shown to be closely tied to the survival and development of organisms in other studies as well (Al-Subiai et al. 2011). The low correlation for PFOA could be due to the lack of responses in physiological biomarkers for this compound.

Table 5-2 Correlations of biomarker responses to PFOS and PFOA.

		CAT	EROD	Comet	NRRT	FR	RCF
PFOS	CAT		-0.31	0.85**	-0.07	-0.31	-0.79*
	EROD			-0.20	0.25	0.23	0.34
	Comet				-0.14	-0.47	-0.81*
	NRRT					0.51	0.21
	FR						0.71
PFOA	CAT		-0.30	0.83**	-0.82*	-0.37	-0.30
	EROD			-0.25	0.51	0.04	0.15
	Comet				-0.78*	-0.63	-0.43
	NRRT					0.52	0.24
	FR						0.73

*CAT: CAT activity, EROD: EROD activity, Comet: comet tail moment, NRRT: neural red retention time, FR: filtration rate and RCF: relative condition factor. ** $p < 0.01$, * $p < 0.05$*

Based on the correlation results and reports from other studies, it is reasonable to assume that the induction of excess ROS production could be one of the main pathways of PFCs to induce adverse effects in the tested organisms. This deduction is also in accordance with the results of a gene profiling study in *E. coli* (Nobels et al. 2010). Correlation and biomarker results show that ROS can directly interact with cell organelles or even DNA molecules and leads to changes or even damages in them. DNA damage, in turn, provides important links between the molecular level adverse effects to higher level responses. Although not shown by the current study, cellular damage might also lead to effects at physiological level. For example, apoptosis of cells in the fish tail area can cause malformation of the tails through alterations of muscle fiber (Hagenaars et al. 2011). The possible toxic pathways of the compounds based on the correlation analysis are shown in Figure 5-1.

Interestingly, the correlation between RCF and filtration rate was lower than expected. As discussed earlier, responses in the two biomarkers could both be attributed to the reduced energy store. Alternatively, a causal relationship between the two is also possible where reduced filtration rate results in a reduced energy intake and finally leads to a lower RCF. The weak correlation is most likely due to the low toxic responses in these physiological level biomarkers, where small fluctuations at low exposure could greatly affect the correlation analysis.

Figure 5-1 Possible toxic pathways of PFCs based on correlation results.

In fact, the reactions and interactions within organisms are complex and interrelated. The response for each single biomarker is normally a result of a number of bio-reactions and factors. Although the proposed mechanism only provides one possibility based on the biomarker results, by correlating bio-endpoints at different biological levels, the current study provides a new perspective of the environmental and ecological consequences of PFCs. However, further studies are still needed to test some of the hypotheses that were deducted based on the current results. Those studies may include genomic and proteomic analyses that examine gene and protein expression related in responses to toxicant exposures.

5.2.2.2 *Implication of indirect toxic effects*

In contrast to the well-studied POPs, adverse impacts imposed by PFCs could be caused indirectly. This may be one of the reasons, besides their inert chemical properties and trace environmental level, that toxicity of PFCs has been underestimated from the beginning (Giesy et al. 2010). Stevenson et al. (2006) in their study brought up the hypothesis of indirect inhibition of a transporter protein by perfluorononanoic acid (PFNA). Indirect adverse effects have been indicated in the current study as well.

Unlike PFC induction of ROS, which can directly cause oxidative stress, some of the adverse impacts could be the secondary effects of PFC exposure. For example, PFCs are likely to inhibit EROD activity at elevated concentrations. This inhibition per se will not harm the organism. However, the inhibition could lead to suppression of the MFO system, which then cannot perform normally in the detoxification process when other toxic xenobiotics, such as PAHs and PCBs, are present. Likewise, PFC induced disruption in energy metabolism may not affect the basic maintenance and harm the organism directly. However, it could lead to a reduced energy store, which then may compromise the growth and reproduction. Moreover, although the membrane damage by PFCs is a direct adverse effect to the cellular integrity, it also induces severe secondary effect. The loss in membrane selectivity resulting from the damage potentially poses greater threat when other exogenous toxic substances are present. Considering these indirect effects, exposure to PFCs are likely to cause injuries to organisms in the long term by rendering the organisms vulnerable in their living environment, especially when PFCs and other toxic pollutant co-exist. Due to the unique properties of these compounds, the toxic actions of PFCs appear to be more complicated than conventional POPs. Further investigations are needed to better understand the modes of action of PFCs.

The joint analysis of biomarkers from multiple biological levels brings a comprehensive understanding of how PFC can influence the health of the organism. Although correlation analysis revealed that for both compounds, the excess production of ROS is suspected to be one of the major toxic pathways, it is noteworthy that differences existed between PFOS and PFOA in both biomarkers and correlation results, which indicate different modes of action of the two. It is most likely that structural differences between the two are the reasons for variations in the interactions and biochemical reactions, which will subsequently affect the higher level expressions. Studies of structure-activity relationship would help to shed some light on the toxic mechanisms of these compounds.

5.3 Integrated biomarker assessment

In the present study, the environmental toxicity of the four PFCs (PFOA, PFNA, PFDA and PFOS) was evaluated using an integrated biomarker approach. A new and improved integrated biomarker index approach was proposed. As mentioned earlier, biomarkers are measureable bio-endpoints at different biological organization levels. To date, multi-biomarker assessment of PFCs is limited, and an integration of biomarkers from multiple biological levels has not been attempted. In the present study, complex biomarker responses from 3 biological levels were integrated by the Enhanced Integrated Biomarker Response (EIBR) system and transformed into a value that was ecologically relevant and meaningful for environmental risk assessment. The EIBR also enables comparison of PFCs in terms of integrative toxicity potential and toxic modes of action.

5.3.1 Materials and Methods

The chemicals, exposure experiment, biomarker analysis and statistical analysis were described in previous sections.

Enhanced Integrated Biomarker Response (EIBR) and star plot

The integrative index approach has already been used for biomonitoring purposes (Hagger et al. 2008). While most indices place integrative toxicity evaluation into broad categories, the Integrated Biomarker Response (IBR) system (Beliaeff & Burgeot 2002) generates meaningful and explicit results that can differentiate among compounds and different exposure levels, and therefore, has been applied in environmental toxicity studies in laboratories (Damians et al. 2007, Kim et al. 2010). However, the conventional IBR is flawed and shows deficiencies in assessing PFCs toxicity. Hence, an improved EIBR system is accordingly proposed and applied in the present study, which is improved from the IBR system. Biomarker response data were first standardized to allow direct comparison between compounds and different exposure concentrations. The standardized response was calculated as:

$$Y_i = \frac{X_i - m_i}{s_i} \quad (5.1)$$

where Y_i is the standardized biomarker response; X_i is the response value of each biomarker; m_i and s_i are the mean and the standard deviation of the biomarker, respectively

(Kim et al. 2010). The minimum value ($\min_i$) for each biomarker was also calculated from the standardized response value, and the score of each biomarker response (B_i) was calculated as:

$$B_i = Y_i + |\min_i| \quad (5.2)$$

To visualize the biomarker results, star plots were performed, where each radius coordinate represents the score of a given biomarker response (B_i). The star plot was obtained by connecting adjacent radius coordinates.

In the conventional IBR calculation, B_i and B_{i+1} are assigned to 2 consecutive scores, and area A_i is obtained by connecting the i th and the $(i+1)$ th radius coordinates. The IBR value is calculated to be the total star area:

$$IBR(conventional) = \sum_{i=1}^n A_i \quad (5.3)$$

In the proposed method, the sum of the product of each biomarker score and its weighting is used to calculate the EIBR value:

$$EIBR(proposed) = \sum_{i=1}^n B_i \times W_i / \sum_{i=1}^n W_i \quad (5.4)$$

where W is the weighting of each biomarker. The rationale of EIBR will be explained in the Results and discussion section.

5.3.2 Results and discussion

5.3.2.1 The integrated biomarker assessment

Multiple biomarkers are often necessary to assess the toxicity of emerging contaminants because of their unknown toxic mode of action (Brooks et al. 2009). This is especially true for PFCs considering their unique physical-chemical properties and thus, unpredictable behavior. Scoring and integration of multiple biomarkers provide direct understanding of the relative toxicity of pollutants. This information could be important for evaluation of risks and aid the decision making process.

The proposed EIBR. In the EIBR system, we propose to calculate the integrative toxicity index by adding up the weighted response score of each biomarker (Equation 5.4). The star plot, instead of being a representation of integrated response values, was used to visualize the toxicity patterns of compounds and was shown to be useful in the

interpretation of toxic modes of action of different PFCs. Details of the EIBR system are discussed in the following part.

1) *Integrative index calculation.* Defects exist in the integrative toxicity index calculation and star plot scoring process in the conventional IBR. As discussed in the Method section, in the conventional IBR, each radius coordinate of the star plot represents the score of a biomarker response, and the IBR value is calculated as the cumulative area after connecting the radii on the star plot (Equation 5.3). Even though the star area calculation was later modified by the Sine Theorem (Damiens et al. 2007), this method is still problematic. For instance, in a triangular star plot, if 2 biomarkers give zero response, no matter how intense the third biomarker is, the star area and resulting IBR value is zero (Figure 5-2a). In addition, the IBR value given by the star area is not necessarily unique: for example in a tetragonal plot, if there are only 2 responsive biomarkers, the total star area depends on the arrangement of the biomarker on each vertex (Figure 5-2b). The above examples clearly demonstrate that the conventional IBR calculation is inappropriate. Moreover, even though the star area may provide direct visualization, it does not have a comprehensible physical meaning with respect to an integrated toxicity value. In contrast, the proposed method of summing up weighted individual biomarker response scores provides an overall measure of responses deviating from the normal healthy state with weight-of-evidence. The resulting EIBR value is, therefore, unique and meaningful as an integrative toxicity evaluation.

(a) One biomarker has response but star area is zero.

(b) Biomarker position and star plot area.

Figure 5-2 Illustration of problems with conventional IBR calculation.

2) *Weighting of biomarker*. As mentioned previously, biomarkers employed in the present study cover multiple biological organization levels. It is necessary to specify the significance of bio-endpoints at different levels. Pollutants can affect a biological system at many levels. Starting with interaction with biomolecules, the effects can cascade through molecular → cellular → physiological → individual levels (Newman 2009), and the impacts of alterations on the health of organism normally increase through these bio-organization levels. Changes at low bio-organization level do not necessarily lead to damage at high levels. High level responses, on the other hand, are more ecologically meaningful, because they are more closely related to the health of individuals. Thus, adverse effects at higher levels convey more important information about the ecological impacts of the compounds than those at lower levels. With this in mind, our proposed EIBR considers the significance of bio-responses and biomarkers are weighted according to their bio-organization levels where molecular, cellular and physiological biomarkers are weighted as 1, 2 and 3 respectively. Similar weighting of bio-endpoints has also been used in pollution monitoring programs in Europe (Hagger et al. 2008). The final EIBR value is calculated as the sum of the product of biomarker weighting and biomarker score (Equation 5.4). This approach provides important weight-of-evidence to support an integrated assessment of biomarkers from multiple biological levels. Without weighting, the results could be biased and even misleading.

EIBR results of PFCs. The EIBR values and biomarker response star plots of each compound at various concentrations are shown in Figure 5-3.

Figure 5-3 The Enhanced Integrated Biomarker Response (EIBR) values and response star plot of each PFC compound.

1) *Different exposure levels.* The EIBR values clearly display concentration dependency for all tested compounds where the resulting integrative toxicity, in terms of EIBR value, increases with exposure concentration for all compounds with linear relationships observed at certain concentration ranges (in most cases 10-1000 g/l) (Figure 5-4). Similar observations were reported by Kim et al. (2010) where integrated response

values of several molecular level biomarkers were positively related to the concentration of PFOA and PFOS. The current results demonstrate that the contamination level of PFCs is a decisive factor on their adverse impacts on the organism, or in other words, the integrative toxic effects are a consequence of the contaminants concentration. This toxicological information is especially important for PFCs which show high bioaccumulation potential in many studied organisms (Houde et al. 2011, Liu et al. 2011).

Figure 5-4 Comparison of Enhanced Integrated Biomarker Response (EIBR) values at different exposure concentrations.

2) *Different PFCs*. The integrative toxicity of PFCs was also found to relate to and increase with fluorinated chain length. It can be seen from Figure 5-5 that, the EIBR values increase with fluorinated chain length in a linear fashion. This is also true for the sulfonate, PFOS. With an 8 carbon fluorinated chain, its integrated response value is in between that of PFOA (C7) and PFDA (C9), and is very close with its C8 corresponding PFNA. This demonstrates that the fluorinated chain length of PFCs has a significant impact on the overall toxicity potential. Compounds with the same fluorinated chain but different functional groups possess similar toxicity potential in terms of their integrative toxicity.

Figure 5-5 Comparison of Enhanced Integrated Biomarker Response (EIBR) values among tested PFCs (at 1000 g/L).

It is also very interesting to note that the weighting of biomarkers changes the results of relative toxicity status of the two C8 compounds, PFOS and PFNA. Figure 5-6 shows that without weighting the biomarkers, the integrative toxicity of PFOS is higher than PFNA. However their difference in EIBR values diminished when weighting is included.

Figure 5-6 Total biomarker scores of tested PFCs without weighting (at 1000 g/L).

Table 5-3 summarizes the biomarker scores for PFOS and PFNA. It shows that PFOS has a much higher score for the molecular biomarkers, suggesting that it imposes more severe adverse effects at the biomolecular level. This actually helps to explain the statement from previous studies, that PFSAAs have higher toxicity potential than the corresponding PFCAs (Nobels et al. 2010), since these studies measured mainly molecular level bio-endpoints. In the present study, the relative integrative toxicity changed when higher level biomarkers were included. As shown by the table, PFNA has higher toxic response score at cellular level. More importantly, the effect of this cellular biomarker on final EIBR value is magnified by the weighting of the biomarker, resulting in an EIBR very close to the one for PFOS. The similar EIBR values indicate that PFNA can be as equally potent as PFOS in terms of integrative toxicity. Without biomarker weighting, PFOS will give a higher EIBR and show higher environmental toxicity potential due to its stronger effects at the molecular level. However, in such cases, the result will be somewhat misleading: as discussed earlier, low level biomarkers are more sensitive but normally have lower ecological significance. By providing weight-of-evidence, our proposed EIBR brings a new perspective to the risk assessment and provides an ecologically relevant evaluation of environmental impacts of PFCs.

Table 5-3 The effect of biomarker weighting on the relative integrated toxicity of PFOS and PFNA.

PFOS	Molecular	Cellular	Physiological
Sum of score at each level ^a	70.01	3.13	27.40
Score at each level (with weighting) ^b	6.36	0.57	7.47
Score at each level (no weighting) ^c	11.67	0.52	4.57
PFNA	Molecular	Cellular	Physiological
Sum of score at each level ^a	52.57	10.44	27.34
Score at each level (with weighting) ^b	4.78	1.90	7.46
Score at each level (no weighting) ^c	8.76	1.74	4.56

Scores are from biomarker response at 1000µg/L.

^a $\overline{X}_{ij} = \sum_{k=1}^n X_{ijk} / n$ at each level.

^b $\overline{X}_{ij}^2 = \sum_{k=1}^n X_{ijk}^2 / n$ at each level.

^c $\overline{X}_{ij}^2 = \sum_{k=1}^n X_{ijk}^2 / n$ at each level.

3) *Star plot vs. toxic pattern.* Although PFOS and PFNA have similar integrative toxicity, the response patterns of the two are quite different, as demonstrated by the star plot (Figure 5-3). From the star plot, differences in the sensitivity of each biomarker can be observed by the magnitude of the response score on the star plot (Figure 5-3). The most sensitive biomarkers have the most acute pointing angle. Moreover, the distinct star plots among compounds also help to identify the differences in their toxic patterns and modes of action. For example, there is a large vertex angle pointing at CAT, which suggests that oxidative stress and oxidative damage could be a toxic mechanism for PFCs. Moreover, the star plot for PFOS has a distinguishing vertex angle pointing at the Comet tail moment compared with other compounds. This implies that compromising DNA integrity could be an important pathway of damage for organism exposure to PFOS. The star plot for PFOA has a relatively prominent vertex angle pointing at the NRRT, indicating a stronger disturbance of lysosomal membrane stability compared with other compounds.

It should be noted that in the present study, the assigned weighting in EIBR was heuristic under the common assumption that higher level alterations have greater impacts on organism health. The values are subject to the choice of biomarkers and target of the assessment. An optimized weighting scheme should be based on the measured significance of impacts of each specific biomarker on individual health. However, it is difficult to identify how a single bio-endpoint may be linked to individual health, since to induce and examine one biomarker without provoking the others is almost impractical. A possible way to estimate this significance could be to measure biomarker responses at a critical dosage (e.g. LD₅₀), and weigh the biomarkers according to this value, with the assumption that the biomarker with higher response at the critical dosage has higher tolerance and thus, is less significant. However, there will still be a lack of direct links between biomarker tolerance and impacts on individual health. The optimum weighting strategy may still need to be refined to improve the quality of toxicity evaluation and risk assessment.

5.4 Structure activity relationships

PFCs are differentiated by their chain structure and functional group. It has been shown that the physicochemical properties of PFCs vary according to their structural differences (Conder et al. 2008, Liu et al. 2011). Mechanistic information is also urgently needed for identification of the potential risks of these contaminants. Understanding structure-activity relationships is important to predict the toxic behavior of compounds in the group, where toxicity screening of many individual PFC is not practical. However, the study of structure-activity relationships of PFCs is very limited so far. With this in mind, the structure-activity relationships of these compounds were examined based on the biomarker and EIBR results.

5.4.1 Materials and Methods

The chemicals, exposure experiment, biomarker analysis and statistical analysis were described in previous sections.

5.4.2 Results and discussion

The tested PFCs gave varying performances in the selected biomarkers, and these toxic responses were shown to be affected by various structural effects.

5.4.2.1 Chain length

Results show that toxic responses generally increased with fluorinated chain length for most biomarkers, e.g. for CAT activity at 1000 g/L, the magnitude of toxic response follows the order: PFOA (C7) < PFNA (C8) < PFOS (C8) < PFDA (C9) (Table 5-4), indicating an increase in oxidative stress induced by the exposure. The relationship between toxic effects and fluorinated chain length has been reported in other studies as well (Latala et al. 2009). As discussed previously, the integrated response value of PFCs was also found to be positively related with fluorinated chain length (Figure 5-5 in section 5.3.2). In fact, chain length is the most frequently observed structural effect on PFCs activity. Increasing chain length directly impacts the physical and chemical properties of PFCs, resulting in lower aqueous solubility and higher bioaccumulation potential (Conder et al. 2008, Stahl et al. 2011). As adverse effects are mostly a consequence of concentration in target organs or tissues in organisms (Newman 2009), the increase in bioaccumulation

potential and thus, the high internal organism concentration, helps to explain the increased toxic responses with chain length.

Table 5-4 Comparison of biomarker scores for 4 PFCs at different biological levels.

	Molecular level			Cellular level	Physiological level	
	CAT	EROD	Comet	NRRT	FR	RCF
PFOS (C8)	37.87±1.33	6.93±0.84	25.21±1.87	3.13±0.62	6.26±1.57	21.14±2.31
PFOA (C7)	24.45±2.16	4.22±1.03	6.68±1.59	11.49±1.01	3.89±2.13	6.73±0.62
PFNA (C8)	33.21±2.47	7.84±0.63	11.52±1.70	10.44±0.76	8.12±1.42	19.22±1.30
PFDA (C9)	40.71±2.48	9.05±0.99	18.16±1.90	8.36±1.14	10.16±1.98	32.19±0.62

Biomarker score $\bar{x} = \frac{\sum_{i=1}^n x_i}{n} + \left| \frac{\sum_{i=1}^n x_i^2}{n} \right|$, at exposure concentration 1000µg/L. Data are provided with standard error ($\pm SE$). The presented biomarker scores of all four compounds are statistically different from each other ($p < 0.05$, t test). CAT=catalase activity, EROD=7-ethoxy resorufin O-deethylase activity, Comet=Comet tail moment, NRRT=neutral red retention time, FR=filtration rate, RCF=relative condition factor.

5.4.2.2 Functional group

Besides the fluorinated chain length effect on toxicity, it is also noticed that in some cases the two groups of PFCs, i.e. PFCAs and PFSA, can have distinct modes of action. As mentioned earlier, for the Comet assay, PFOS produces a much stronger adverse response compared with the other compounds. The magnitude of toxic response follows the order: PFOA (C7) < PFNA (C8) < PFDA (C9) < PFOS (C8) (Table 5-4). This suggests that PFOS can induce a higher level of DNA damage and thus has a higher genotoxicity potential than the PFCAs. The DNA damage of PFOS has also been documented in various species (Hoff et al. 2003, Shi et al. 2008). It is possible that compared with the carboxylate group, the sulfonate group has stronger chemical interactions with DNA molecules or selected genes that lead to DNA damage. Therefore, in the case of DNA damage, the functional group is probably the most decisive factor rather than chain length. It is also noted that current results in green mussels are in contradiction to previous findings in bacterial profiling assays, where in terms of induction of changes in gene expression, the effect of chain length is stronger than the effect of functional group (Nobels et al. 2010). This contradiction may imply that the structure-activity relationship of PFCs can be species sensitive.

In addition to the Comet assay, the sulfonate and the carboxylates also performed differently in the NRRT. Reduction in the NRRT indicates damage in lysosomal membrane and a stressed condition of the environment. It is interesting to note that in NRRT, PFOS exposure did not induce a significant response when compared with other PFCAs (Table 5-4). This result, however, supports a previous finding in bacteria where high levels of membrane damage were restricted to only PFCAs, while only minor inductions were obtained from PFOS (Nobels et al. 2010). Membrane damage can be induced by direct interaction with the compounds, for example, binding with membrane proteins or interruption of the lipids (Hu et al. 2003). The current results imply that interactions with the carboxylate group rather than the sulfonate group are more likely to cause membrane instability. It is also noteworthy that in the case of NRRT, the adverse responses follow the order: PFDA<PFNA<PFOA among the PFCAs, which decrease with fluorinated chain length. It is possible that besides the functional group effects, the molecular weight or the size of the compounds also play an important role in the membrane interaction, where the smaller the size, the easier for compounds to be incorporated into and disrupt the biological membranes.

5.4.2.3 Comparison between C8 compounds

As mentioned earlier, it was previously concluded that for PFCs with the same fluorinated chain length, the toxicity induced by sulfonate is higher than the corresponding carboxylate. However, the present study results show some contradictions to this statement. Between the two compounds with 8 fluorinated carbon, PFOS did not always display higher toxicity potential than PFNA.

For CAT activity and RCF, PFOS indeed induced slightly higher toxic responses than PFNA (Table 5-4). Previously, we demonstrated that in green mussels PFOS has a greater bioaccumulation potential than PFNA (Conder et al. 2008, Liu et al. 2011). A higher accumulation in organisms could be the reason for PFOS to display higher toxicity potential in these bio-endpoints that are susceptible to the internal concentration of contaminants. As discussed earlier, CAT activity reflects the oxidative stress caused by reactive oxygen species (ROS), where ROS production was proved to be concentration dependent under PFC exposure (Freire et al. 2008). On the other hand, RCF is linked to the energy store of organisms, and energy expenditure for detoxification of xenobiotics also largely depends on the quantity of the contaminants. Therefore, a larger amount of

contaminants accumulated inside organisms could lead to stronger responses in CAT activity and RCF.

In contrast, for the EROD and FR bioassays, the toxic responses to PFNA were higher than those to PFOS (Table 5-4). A previous study suggests that toxicity of PFCs can be hydrophobicity dependent, and is positively related to K_{ow} which describes the hydrophobicity of the compounds (Wang et al. 2011a). Studies showed that the values of modeled K_{ow} follow the order of PFOA<PFOS<PFNA<PFDA, where PFNA was more hydrophobic than PFOS (Kelly et al. 2009). It is possible that for EROD activity and FR, hydrophobic interactions govern the modes of action of these compounds. Hence PFNA induced stronger toxic responses than PFOS. Although the direct link between hydrophobicity and toxicity displayed in these bio-endpoints is lacking, it is conceivable that hydrophobicity could affect the binding and interaction with proteins and enzymes that are involved in the above mentioned biomarkers.

While multiple structural effects on the toxicity of PFCs were observed from the biomarker results, any adverse effect on the organism is likely to be the result of a combination of various factors. For different bio-reactions and interactions, a predominant factor that governs the structure-activity relationship and mode of action of the compound usually emerges.

5.5 EC₅₀ based on integrative toxicity

The EIBR based dose-response relationships can be established from the integrated biomarker results (Figure 5-4 in Section 5.3.2). The integrated toxicity of PFCs, as evaluated by the EIBR values, showed similar dose-response relationships for individual compounds. The EC₅₀ values and confidence interval (CI) based on the integrative toxicity were calculated using probit analysis. For PFOS, PFOA, PFNA and PFDA, the EC₅₀ values are 99 (93-105), 635 (620-647), 129 (118-136) and 42 (38-45) g/L, respectively. The EC₅₀ values were found to be negatively correlated with both fluorinated chain length and the bioaccumulation factor of the compounds (Figure 5-7), which indicates the importance of the chain length and organism burden of PFCs in the overall toxicity potential of the compounds. EC₅₀ are often used as an indicator of the toxicity of a compound to the environment. The EC₅₀ value based on the integrated toxic response provides an evaluation of the potency of these contaminants, and thus, criterion for environmental regulation and

guidelines. The results could also be used to predict the integrative toxicity of other PFCs when bioaccumulation factor or chemical structure is known. Besides EC₅₀, the no observed effect concentration (NOEC) can also be derived from the low end of dose-response curve, which can provide reference for environmental safe level of the compounds.

Figure 5-7 Relationship between EIBR based EC₅₀ and bioaccumulation.

5.6 Conclusion

The integrated biomarker assessment and structure-activity analysis revealed the diverse toxicity patterns of PFCs. Besides the commonly recognized chain length and functional group effects, several structural factors are also involved in the toxic action of PFCs. EIBR analysis demonstrate that it is the fluorinated chain length that governs the integrative toxicity of the compounds. To our knowledge, this is the first environmental toxicity study of PFCs where biomarkers from multiple biological levels are concurrently assessed and integrated using a new and improved integrated biomarker index approach. The resulting evaluation of integrative toxicity and toxic pattern provides a new perspective of environmental consequences of these pollutants. The observed structure-activity relationships also help to elucidate the gaps in understanding the mechanisms

behind PFCs toxicity. PFCs are a group of compounds with similar structures. The findings of the present study can be applied to predict the toxic behavior of different compounds in this group. The application of integration of biomarkers from multiple biological levels by EIBR enables an ecological relevant evaluation of environmental toxicity, and therefore, is a promising tool for risk assessment.

The estimated EC_{50} values based on the current study results were higher than the environmental concentrations so far detected in the Singapore marine waters. The results imply that current contamination levels may still be safe for marine organisms. However, the results of the current study proved the toxicity potential of PFCs and their capability to cause various adverse effects. It is also worth noting that the toxicity evaluation was based on acute toxicity tests. It is possible that with prolonged exposure and the bioaccumulation effect, these contaminants could induce severe long-term toxic effects even at low exposure levels.

Chapter Six Conclusions and recommendation

6.1 Conclusions

The bioaccumulation and ecotoxicity of four common PFCs were investigated in indigenous green mussels, *Perna viridis*. The major findings and conclusions are summarized as follows:

1. Bioaccumulation of the tested PFCs was shown to be concentration dependent. The bioaccumulation factors (BAF) were found to range from 15 to 839 L/kg and from 12 to 464 L/kg at exposure concentrations of 1 and 10 g/L, respectively. The bioaccumulation potential was found to increase with fluorinated chain length. For all compounds, the BAF was larger at the lower dosage. The sensitivity of BAF to exposure concentration was found to be positively related to both fluorinated chain length and the binding affinity of the compounds. A kinetic model based on an adsorption mechanism was proposed, which provides a more accurate description of the bioaccumulation process of PFCs. The investigation of bioaccumulation enhances the understanding of the partitioning process from environment to organism and the concentration profile of the contaminants inside the organism.
2. Several toxic modes of action have been investigated and the results demonstrate that PFCs were able to induce a series of adverse effects and toxicity at different biological levels. Examination of antioxidant enzyme activity demonstrates that PFCs can impose severe oxidative stress to the organism and cause significant induction/inhibition of antioxidant enzyme activities. As metabolic inert compounds, PFCs minimally go through any biotransformation. However, they were found to inhibit xenobiotic metabolizing enzymes that play an important role in detoxification of organic pollutants. Besides enzymes and proteins, the integrity of other bio-molecules, such as lipid and DNA, were also found to be affected by these contaminants. Lipid peroxidation, DNA fragmentation and apoptosis were observed after exposure to PFCs. At cellular level, PFC exposure was shown to

result in adverse effects on cell viability, cellular functions and integrity of cell organelles, and directly suppressed the immune activity thus placing the health of the organism in jeopardy. At physiological level, PFC exposure exhibited influences on energy producing activities such as filtration. Some of the tested compounds also caused significant weight loss and affected the general well-being of the organism.

3. Correlation analysis revealed the links between toxic responses at different biological levels. PFC induced excessive production of ROS was shown to be one of the major pathways for the compounds to cause adverse effects in the tested organisms. ROS can directly interact with cell organelles and genetic materials and cause alterations and even damage. DNA damage and alterations in gene expression, in turn, provide important links between effects at the molecular level to higher level adverse responses. Cellular damage might also directly lead to effects at physiological level.
4. Toxic mechanisms of PFCs were examined. The toxicity of PFCs demonstrates both exposure concentration and exposure time dependency. Adverse effects measured by biomarkers normally increased with exposure concentration and time. Exposure concentration was found to be one of the most important factors that affected the magnitude of toxic effects of PFCs. A similar pattern and trend were observed in dose-response and time-response relationships where the organism concentration of PFCs provides an important link. For some toxic modes of action, the amount of bioaccumulation was found to be the decisive factor of the magnitude of toxicity (which is related to the higher internal dose and activity of the more bioaccumulative compounds.), for example, the oxidative toxicity. Both toxic response models and structure activity models were established to better describe the underlying mechanisms.
5. The structure-activity relationship of PFCs was examined and the results shows that a number of structural factors could affect the toxic activity of the compounds. The fluorinated chain length was found to be the one of the governing factors. The functional group, due to its effect on polarity and hydrophobicity, was also shown to play an important role in many cases including bioaccumulation, oxidative toxicity and especially genotoxicity, where the sulfonate was found to be more potent than the corresponding carboxylate. Any adverse effect is likely to be the

result of a combination of various factors, where there is usually a predominant factor that governs the structure-activity relationship and mode of action of the compound.

6. An enhanced Integrated Biomarker Response (EIBR) system was proposed to assess the integrative toxicity potential of PFCs. The tested compounds exhibited different toxicity potential in individual toxic modes of action. It is the EIBR that provides an overall evaluation of the potency of the compounds. The results shows that among the tested compounds, toxicity potential followed the order of PFDA > PFOS > PFNA > PFOA. With the same fluorinated chain, the overall toxicity of PFOS and PFNA were found to be close to each other.

The present study provides important ecotoxicological data and information that have been missing in previous investigations. These include 1) Ecotoxicity assessment of commonly detected PFCs besides PFOS and PFOA, 2) PFC induced ecological impacts on marine invertebrates, 3) A systematic examinations of different toxic modes of action at different biological levels, 4) Both qualitative and quantitative structure-activity analysis of PFC induced toxicity, which facilitates the understanding of the toxic mechanism and prediction of behavior of other compounds in the group, and 5) Mathematical models for both bioaccumulation and ecotoxicity evaluations. The results of this study have brought a better understanding of the environmental and ecological consequences of these newly emerging pollutants. The bioaccumulation and ecotoxicity data and model will facilitate the risk assessment and environmental management of these contaminants.

6.2 Recommendation

The following work is recommended for future study:

1. Chronic toxicity examination. In the present study, the applied biomarkers measured only acute and sublethal toxic effects. Chronic effects under prolonged exposure should be examined in future studies to gain a better understanding of the long-term impacts of the compounds.

Extended examination of more PFCs. In the current study, four commonly detected PFCs were investigated. Toxicity data of other compounds may provide additional information regarding structure-activity relationship of the group and help to optimize the mathematical

models of bioaccumulation and toxicity. In the current laboratory study, only toxic effects from individual compounds were examined. In real environment, PFCs exist in a mixture form. As the results of bioaccumulation study suggested, distribution of PFCs highly depends on the number of binding sites, and thus competitions and interactions between PFCs are expected. This is also true for their toxic activities. Therefore, investigation of mixture bioaccumulation behavior and mixture toxicity will provide useful information.

2. Isomer specific effects. Environmentally detected PFCs do not all exist in linear form. For example, prior to its recent phase-out, PFOS was produced by electrochemical fluorination processes, which yielded mixtures composed of linear and branched isomers. Recent studies have shown that the isomer proportions in PFC sources, environment and biota are different, which suggest a possible isomer-specific behavior of PFCs. The current study only investigated the linear PFCs, and hence in future work, isomer-specific bioaccumulation and toxicity should also be examined.

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Appendix

Section A

Table A. Nomenclature, formulas, and molecular structures of target PFCs.

Name		Chemical structure	Formula	Molecular weight	Fluorinated chain length
<i>Perfluorinated carboxylate</i>					
PFOA	perfluorooctanoic acid		$C_8HF_{15}O_2$	414.07 g/mol	C7
PFNA	perfluorononanoic acid		$C_9HF_{17}O_2$	464.08 g/mol	C8
PFDA	perfluorodecanoic acid		$C_{10}HF_{19}O_2$	514.09 g/mol	C9
<i>Perfluorinated sulfonate</i>					
PFOS	perfluorooctane sulfonate		$C_8HF_{17}O_3S$	500.13 g/mol	C8

Appendix

Section B

Table B1 Measured exposure concentration corresponding to the nominal concentration.

Nominal conc. (µg/L)	Control	0.1	1	10	100	1000
<i>PFOS</i>	nd	0.12 ± 0.02	1.1 ± 0.1	9.6 ± 0.5	106 ± 10	968 ± 86
<i>PFOA</i>	nd	0.08 ± 0.01	1.2 ± 0.05	11.4 ± 0.6	99 ± 8	1120 ± 46
<i>PFNA</i>	nd	0.11 ± 0.02	1.1 ± 0.1	10.4 ± 0.4	117 ± 7	1072 ± 27
<i>PFDA</i>	nd	0.11 ± 0.03	0.9 ± 0.2	9.9 ± 0.3	89 ± 4	983 ± 10

Values represent the mean ± standard error.

Table B2 PFC concentration in mussel tissues after 7 days exposure (g/Kg).

Exposure conc. (µg/L)	Control	0.1	1	10	100	1000
<i>PFOS</i>	nd	13±0.4	124 ± 5	1092±37	3464±25	4186±34
<i>PFOA</i>	nd	0.7 ± 0.1	6.5 ± 0.3	58±8	151±12	202±14
<i>PFNA</i>	nd	6.4 ± 0.5	64 ± 2	559±19	1758±10	2583±27
<i>PFDA</i>	nd	24±0.7	243 ± 11	2106±48	5183±33	6891±101

Values represent the mean ± standard error.