DETECTION OF PERFLUORINATED COMPOUNDS IN THE ENVIRONMENT AND THEIR EFFECT ON CELLULAR ORGANISMS

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety under the supervision of Professor Li Fong Yau Sam, Chemistry Department, National University of Singapore, between 02/08/2010 and 31/03/2016.

I have duly acknowledged all sources of information which have been used in the thesis.

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

The content of the thesis has not been published yet and manuscripts are in preparation.

LEE SI NI		24 March 2017
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Summary

Perfluorinated compounds (PFCs) are detected worldwide, even in regions such as the Arctic where production and application of PFCs are not found. Due to this alarming realization of PFCs' persistent nature, poor elimination and ability to bioaccumulate in the environment, research studies on this group of persistent organic pollutants (POPs) have been focused mainly on the determination of its quantity and corresponding effects in the environment.

Perfluorooctanoic acid (PFOA) and pefluorooctanesulfonic acid (PFOS) are the two most commonly studied PFCs due to the fact that they are the ultimate breakdown products of most PFCs. These compounds, even at low concentrations, are highly toxic. To illustrate their toxicity, several animal models and a few human studies have been applied in toxicological studies and results indicate that PFCs are likely to cause adverse effects to human health, wildlife and the environment.

The main route of exposure of PFCs in human is through dietary consumption. Aquatic habitat, being the major exposure source of PFCs to human, is of utmost concern as it ties up with our water and food source. The two aquatic organisms employed in this thesis are green microalgae, *Chlorella vulgaris*, and water flea, *Daphnia magna*. *Chlorella vulgaris* is employed primarily due to it being the key source in the aquatic food chain while for *Daphnia magna*; it is a common test organism for aquatic toxicological studies. Furthermore, both aquatic species have not been extensively studied in the environmental metabolomics field.

Chapter two of this thesis aims to study the bioaccumulation of PFCs in green microalgae *Chlorella vulgaris* and use it to better understand the effect of trophic magnification of PFCs in the aquatic environment. Solid phase extraction and liquid chromatography tandem mass spectrometry (LC-MS/MS) is used in the determination of PFCs in *Chlorella vulgaris* and culture medium. UV-Vis spectroscopy and

microscopic images were obtained to better illustrate the effect of algae cell growth inhibition and cell aggregations and clumps upon acute and chronic PFCs exposure.

In chapters three and four, environmental metabolomics is applied to understand the detailed effects of PFCs on aquatic organisms: green microalgae *Chlorella vulgaris* and water flea *Daphnia magna*. Liquid chromatography tandem mass spectrometry (LC-MS/MS) was used in untargeted metabolomics mode to investigate the metabolome of these aquatic organisms upon the acute stress exposure of PFOA and PFOS. Furthermore, the use of *Daphnia magna* as an aquatic test organism in toxicological and metabolomics studies is applied to PFCs and leachate eco-toxicity determination.

In summary, the aim of this thesis is to explore and utilize different techniques of analytical determinations, toxicity studies and environmental metabolomics together to better understand the mechanism of bioaccumulation of PFCs and other related compounds and how this can impact the aquatic environment and in turn affect humans.

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List of Abbreviations and Symbols

^{1}H	Proton
^{13}C	Carbon-13
^{15}N	Nitrogen-15
^{19}F	Fluorine-19
^{31}P	Phosphorus-31
μ	Micro
APCI	Atmospheric Pressure Chemical Ionization
BBM	Bold’s Basal Medium
CE	Capillary Electrophoresis
CE	Collision Energy
DSS	4,4-dimethyl-4-silapentane-1-sulfonic acid
DIMS	Direct Infusion Mass Spectrometry
EC_{50}	Half Maximal Effective Concentration
EI	Electron Impact
ESI	Electrospray Ionization
EPA	Environmental Protection Agency
FTIR	Fourier Transform Infrared Spectroscopy
FTOH	Fluorotelomer alcohol

LIST OF ABBREVIATIONS AND SYMBOLS

GC	Gas Chromatography
HILIC	Hydrophilic Interaction Liquid Chromatography
HPLC	High Performance Liquid Chromatography
IS	Internal Standard
K_{OC}	Partition coefficient between organic carbon and water
LC	Liquid Chromatography
LC_{50}	Lethal Concentration required to kill 50% of the population
LCQTOFMS	Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry
LCQTRAPMS	Liquid Chromatography Quadrupole Linear Ion Trap Mass Spectrometry
LOEC	Lowest Observable Effect Concentration
m	milli
m/z	Mass-to-Charge
MALDI	Matrix Assisted Laser Desorption Ionization
MRM	Multiple-Reaction-Monitoring
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MVA	Multivariate Analysis
n	nano

LIST OF ABBREVIATIONS AND SYMBOLS

NMR	Nuclear Magnetic Resonance
NOEC	No-Observable-Effect Concentration
OPLS-DA	Orthogonal Projection to Latent Structures – Discriminant Analysis
ppm	parts per million
pK_a	Acid dissociation constant
PCA	Principal Component Analysis
PFCs	Perfluorinated Compounds
PFCA	Perfluorinated Carboxylic Acids
PFHxA	Perfluorohexane Sulfonic Acid
PFOA	Perfluorooctanoic Acid
PFOS	Perfluorooctane Sulfonic Acid
PFSA	Perfluorinated Sulfonic Acids
PLS-DA	Projection to Latent Structures – Discriminant Analysis
POPs	Persistent Organic Pollutants
Q^2	Prediction Goodness
QC	Quality Control
QTRAP	Quadrupole-Ion Trap Mass Spectrometer
QQQ	Triple Quadrupole Mass Spectrometer
RPLC	Reverse Phase Liquid Chromatography
R^2	Goodness of Fit

LIST OF ABBREVIATIONS AND SYMBOLS

SIM	Selected Ion Monitoring
SIMCA	Soft Independent Modeling of Class Analogy
SPE	Solid Phase Extraction
SRM	Selected-Reaction-Monitoring
TOF-MS	Time-of-Flight Mass Spectrometer
UPLC	Ultra Performance Liquid Chromatography
UV	Unit Variance
UV-Vis	Ultraviolet-Visible Light Spectroscopy

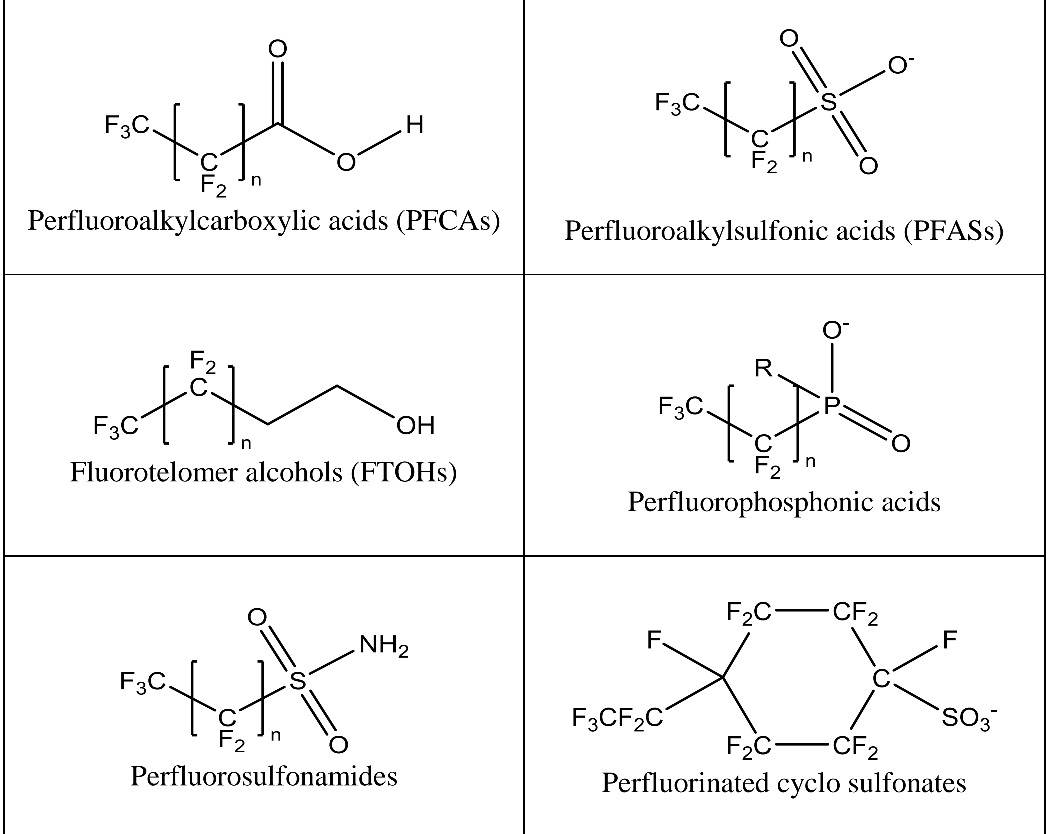
CHAPTER ONE

1. Introduction

1.1 Perfluorinated compounds (PFCs)

PFCs are chemical compounds that contain mainly carbon-fluoride (C–F) and carbon-carbon (C–C) bonds. These fully-fluorinated compounds can be attached to other hetero-atoms or functional groups and it contains very few or no carbon-hydrogen (C–H) bonds. There are several groups classified under PFCs and they usually differ based on the functional groups attached to the carbon-fluoride backbone chains. Some of the most commonly found groups of PFCs and its precursors are listed in Table 1.1[1]. Perfluoroalkylcarboxylic acids (PFCAs) and perfluoroalkylsulfonic acids (PFASs) are the 2 most commonly studied groups of PFCs due to the fact that most PFCs ultimately break down to form these compounds.

Table 1.1 Structures of compounds classified under PFCs [1]



Perfluorosulfonamidoethanols	Fluorotelomer phosphate esters
$F_3C-[CF_2]_n-S(=O)_2-N(CH_2CH_2OH)(CH_2CH_3)$	$F_3C-[CF_2]_n-CH_2-CH_2-O-P(=O)(OH)-R$

These fully-fluorinated chains in PFCs gave it several unique physical and chemical properties. In particular, PFCs are inert towards very stable to many chemicals even at high temperatures (e.g. acid, alkali, oxidation and reduction) even at high temperatures and are able to reduce friction and repel both oil and water. These properties make it very suitable for industrial and consumer applications. Since early 1950s, PFCs have been synthesized and applied in several industries such as clothing, food, aerospace and electronics[1,2]. A well-noted application of PFCs is found in the stain-resistant coatings in non-stick cooking pans and food packaging.

After years of using PFCs, organic fluorine was first detected in human serum samples in the late 1960s[1,3,4]. Taves et al utilized nuclear magnetic resonance (NMR) spectroscopy techniques and speculated that the organic fluorine found is closely related to a fully-fluorinated carbon molecule[3,4]. For fear that PFCs are the main cause of concern; research on these toxic compounds had been conducted using human biological samples. Due to the limitations of the analytical techniques at that time, detections of PFCs were only evident in very concentrated samples such as in the serum of occupationally-exposed adults and findings were not entirely conclusive. It was until early 2000s, where liquid chromatography – mass spectrometry (LC-MS) were introduced; detection of low concentration PFCs was improved and made possible for many environmental samples. It was then realized that these anthropogenic chemicals were existed in water, air and soil[5,6], wildlife such as fishes[5,7,8] and birds[9,10] and humans (children and adults)[11,12]. Alarmingly, they were also found in areas where production and application of PFCs are not based, such as

the Arctic[7,10,13,14]. With these conclusive findings based on different environment samples in several regions, it is evident that PFCs are capable of being transported from the source of production and application to all parts of the world without breaking down which is also known as long-range transport. In addition, these persistent compounds, even at low concentrations, are found to be highly toxic to the environment.

As a result of this particular property that they possess, they have the ability to cause adverse effects to human health, wildlife and the environment. With its persistent nature and its ability to bioaccumulate through the food web, PFCs such as perfluorooctane sulfonic acid (PFOS) and its salt were classified as persistent organic pollutants (POPs) in May 2009[15,16]. In addition, long-chain PFCs, which are described as compounds which consist of 8 or more carbons for PFCAs and 6 or more carbons for PFASs, are among the main groups of hazardous PFCs.

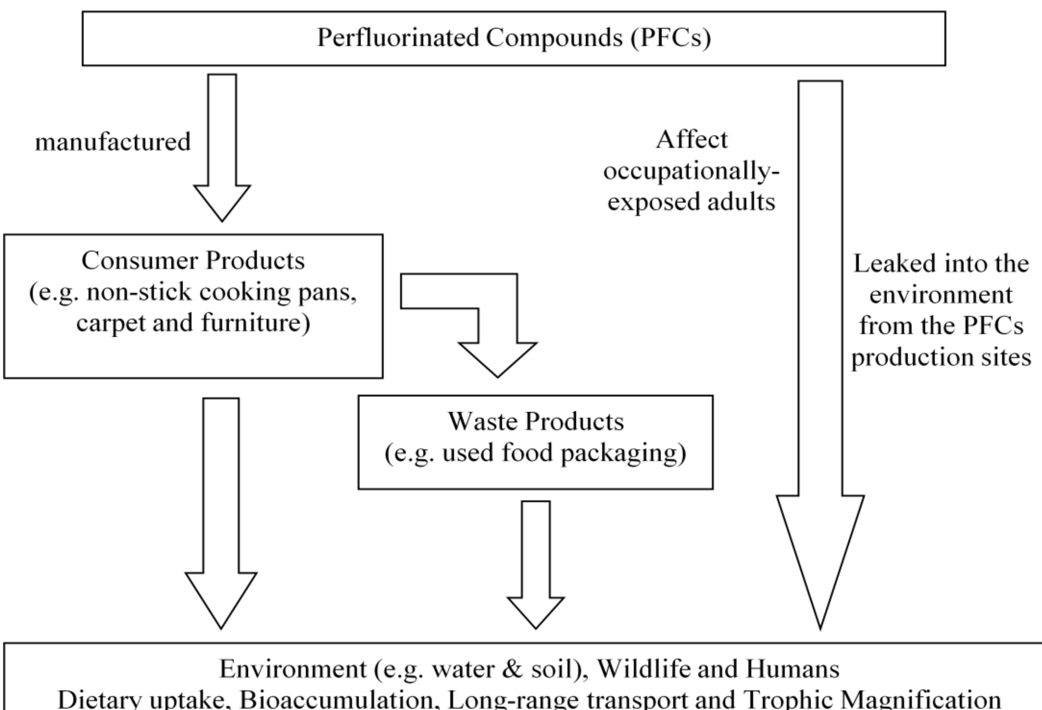


Figure 1.1 Route of exposure of PFCs to the environment

With these routes of exposure for PFCs as shown in Figure 1.1, emissions of PFCs posed great concern and necessary actions need to be taken. PFCs posed a global issue and some regulations have been issued to solve and prevent any further environmental problems and consequences. There were several countries who took action to mitigate the PFCs issue, namely United States and Canada[17]. To eventually assist the transition of reducing and eliminating the use of PFCs, United States Environmental Protection Agency (US EPA) came up with 2 strategies. One of which is the PFOA Stewardship Program where participating companies work towards reducing and ultimately eliminating the use of PFOA and related chemicals by this year 2015[18]. Major companies such as DuPont and 3M are involved in this voluntary program. As indicated in the 2014 company progress report for 2010/2015 PFOA Stewardship Program, 3M had successfully eliminated the use of PFOA in their production system as early as in 2000 and have already begin finding alternatives for any precursor that may have a chance to degrade to PFOA and PFOS or similar compounds[18]. The production and use of perfluorinated compounds with 8 carbons have been minimized and reduced in these companies. Moreover, apart from PFOA, US EPA regulated and restricted the use of long-chain PFCs in December 2009. With the phasing out of long-chain PFCs in some of these major companies, and with more action taken by the industries, the concentrations of PFCs found in humans are dropping[19]. Concentrations of PFOS and PFOA in human blood were reported to be reduced by 32% and 25% respectively[18].

However, this trend may have limited success. The measures discussed were mainly voluntary and participated by only 8 major companies in the world. Despite efforts from these companies, there are others which do not follow, persistently continue and even started to increase the use of such toxic chemicals in their productions[20-23]. In addition, with insufficiently strong evidences that PFCs are detrimental to the environment and wildlife, international environment bodies do not

feel the urgent need to limit and stop the use of PFCs. So with the continued emission of PFCs from other companies who are not involved in any regulation and programs, PFCs may still be detected and transported globally in the near future.

For companies who have already withdrawn the use of long-chain PFCs in their production chain, alternatives are proposed and introduced to replace these toxic compounds[18]. Still, majority of these alternatives have similar chemical and physical properties as long chains PFCs with only slight changes[16,18]. They are used without any substantial evidence of being environmentally-safe[24,25]. In fact, some researchers have questioned about the safety and use of these alternatives and have researched and looked into greater details regarding these substitutes[24,25].

In particular, short-chain PFCs are used as substitutes for their longer chain counterparts. Although short-chain PFCs theoretically have a shorter half-life and less harmful toxic properties as it is less-persistent compared to longer chains PFCs, it is often overlooked that these compounds still possess similar properties and its ultimate impact on the environment is still unknown. In like manner, Eschauzier et al. determined that shorter chains PFCs are expected to be harder to remove in water treatment processes and can ultimately end up in our drinking water[26]. Recent studies have also shown that these short-chain PFCs may cause environmental effects as drastic as compared to PFOA[25].

Consequently, with no strict regulations and restrictions imposed on these PFCs worldwide, the ongoing battle with these persistent compounds, its precursors and substitutes will continue. Therefore there is still a constant need to monitor and understand the underlying theory and ultimate effects and impacts of these compounds to the environment.

1.2 *Environmental effects of PFCs*

PFCs can be detected in the environment worldwide and not just near the source of production and application. Based on its persistent nature and the different routes of exposures (Figure 1.1), PFCs can enter and be absorbed into different organisms. Once it enters the body, it is not metabolized and it stays in the organism without being secreted out immediately[27,28]. As a result of this poor and limited elimination of PFCs, bioaccumulation of PFCs in organisms occurs. Furthermore, the half-lives of these compounds in humans are reported to be about 2.5–3.8 years and 5.4 years for PFOA and PFOS respectively[29,30]. This results in a bioaccumulation effect of PFCs and it may cause harmful effects to the organism and the environment.

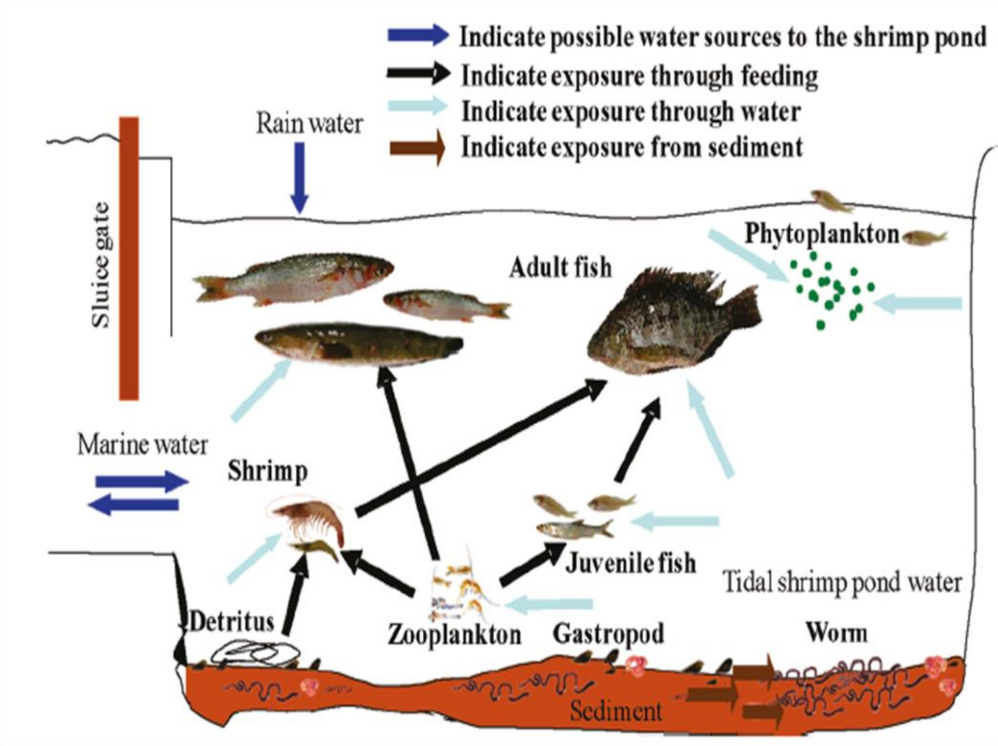


Figure 1.2 An example of a food web magnification in aquatic environment[31]

The eventual fate of these chemicals may also be carried forward through a food chain magnification as shown in Figure 1.2. This food chain or trophic

magnification had been observed and is very evident for long-chains PFASs, including PFOS[7,8,31,32]. Long-chain PFCs tend to have a longer half-life and is more persistent in the environment. In view of this, Kannan et al investigated the fate of PFOS in the Great Lakes benthic food web and concluded that PFOS in the benthic invertebrates were a thousand times greater than the concentration found in the water[8]. PFOS were generally found in the blood and liver of these organisms. Furthermore, PFOS is reported to have bigger biomagnifications potential than PFOA[8]. In another research, Ahrens et al have also collated the concentration of PFOA and PFOS in wildlife from the aquatic environment. Several different aquatic species were analyzed which includes fishes, birds and mammals. Similarly, from the PFOS quantities detected, an increasing PFOS concentration is observed as the organism goes higher in the food chain and this clearly indicates that bioaccumulation of PFOS is observed[33].

From these findings of PFCs biomagnifications and detections in the environment and wildlife, another key point to note is the toxicity of these compounds. Due to the presence of PFCs in different environments and the risk of it being able to affect so many different organisms, toxicity studies were performed on several different models such as rodents, fishes, algae, cells and rotifers[34]. These studies are used to infer more about the impact of PFCs in the various environments. As a result from these studies, PFCs are reportedly toxic to several laboratory-tested organisms and animals and adverse effects are mainly observed in the reproductive and developmental systems[17,35]. This can be seen by the delayed eyes developments and growth defects in PFOA-exposed mice[36]. In addition, it is reported that PFOA and PFOS causes liver, pancreatic, testicular and mammary gland tumors in laboratory animals[37]. Similarly, based on several bioaccumulation studies of PFCs in animals, their ultimate breakdown products (namely PFCAs and PFASs) were found in the liver, serum and kidney. Therefore, it is possible to deduce that PFCs not only

accumulate at specific organs but also affect the function and health of these organisms. In addition, Johansson et al studied the effect of PFCs on 10 days-old mice and confirmed that PFOS and PFOA are developmental neurotoxicants through their behavior and activity[38]. It is evident that inflammatory and immune responses and cytokines production are affected in PFCs-exposed animal models[39].

As for the case in humans, PFCAs and PFASs, have been detected in several human samples, for instance serum, breast milk and several organs (liver, kidney, etc.) worldwide[11,40,41]. Testing of PFCs in human serum samples are the most commonly-found studies as the samples were easily obtained. In several countries, such as China, PFOA and PFOS had already been detected in human serum and tissue from several occupationally exposed workers and general public[42]. As early as 1996, Gilliland et al have investigated the effect of PFOA on occupationally exposed workers by analyzing several health parameters such as enzymes, cholesterol and lipoproteins. However, results were not conclusive and factors other than the exposure to PFCs could contribute to the results[43].

Apart from just detecting PFCs, there are a few human toxicological studies especially on the postnatal, reproduction and developmental aspects. In particular, Barry et al investigated the occurrence of cancer in a PFOA-exposed population. A survey and statistical analyses were conducted to give positive results that showed association between kidney and testicular cancer and PFOA exposure[37]. In several other research studies, slow growth of newborns and delay of puberty of children were observed upon the mothers' prenatal exposure to long-chain PFCs[44-47]. In short, PFCs do have a detrimental effect to the environment, wildlife and humans in the long run.

In another study conducted by Genius et al, it demonstrates and establishes evidences that PFCs bioaccumulates in human beings. This group of researchers

focuses on the elimination of PFCs in the human body by analyzing PFCs in human excretion samples such as urine, stools and perspiration[48]. From their findings, only PFOA was detected in the urine while PFOA, PFOS and PFHxS were detected in stool after the volunteer consumed some saponin compounds. The most compelling evidence is that these quantities detected were noted to be small compared to the levels of PFCs detected in the human serum. It can be concluded that PFCs are not easily excreted out of the human body partly due to enterohepatic circulation (Figure 1.3)[48]. In order words, PFCs are highly suspected to be reabsorbed into the body and not eliminated out of the human body easily through our urine, stools and perspiration.

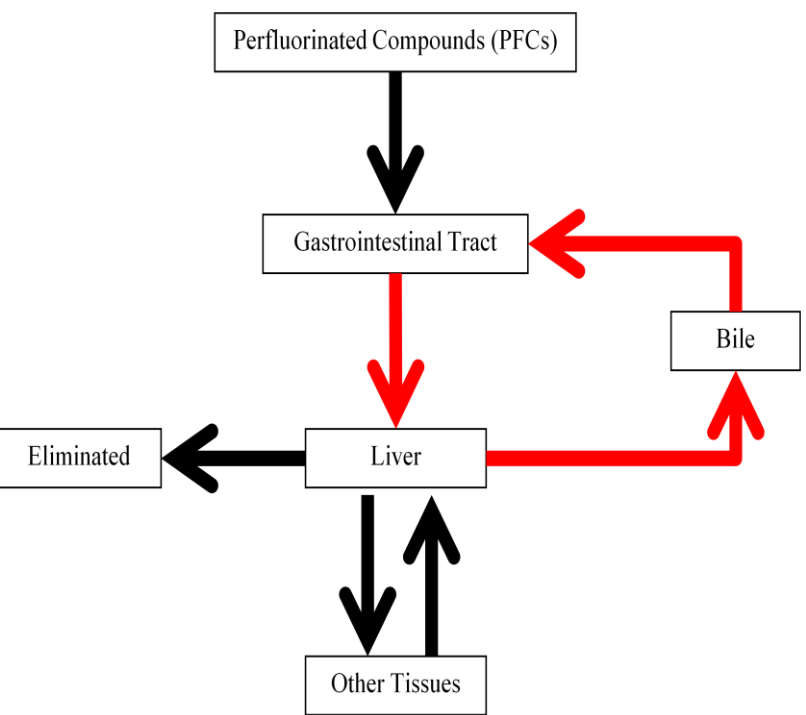


Figure 1.3 Possible routes of PFCs in the human body – enterohepatic circulation (Red arrows)[48]

Unlike animal studies, the effect of PFCs on human are not covered in much detail due to the lack of samples for laboratory tests. In addition, human studies are not

controlled in such a way like laboratory animal such that PFCs is the only main variable. As a result, conclusive results were not obtained and there is insufficient strong evidence to conclude that PFCs pose a major risk to human health. Still, toxicity tests for PFCs conducted on numerous animal models should not be overlooked[25,39,49-51]. It should be taken into serious consideration and used to indicate relation and potential risk to human health. At present, PFOA and PFOS are only reported to be likely human carcinogens[50,52]. It is crucial to note that the limiting factor of categorizing PFCs as a hazardous chemical to humans is due to the lack of samples and studies. In the recent years, there are more reports and findings of the impacts of PFCs on humans.

It is important to realize that the bioaccumulations of PFCs in the environment and wildlife organisms are still ongoing and the consequences of this bioaccumulation and biomagnification are still largely unclear and very concerning. In due time, the continued exposure of PFCs, even at low concentration, may be a potential threat to the environment and health of animals and humans.

1.3 Conventional methods for the detection of PFCs in the environment

Due to the wide range of samples that PFCs can be detected in, there are numerous sample preparation and analysis methods available. These environmental samples have different states (solid, liquid, and gas) and can even exist in mixtures. Some examples of samples tested for PFCs are mussels, sludge, popcorn, serum, breast milk, earthworms, cells, algae and even umbilical cord. The planning and the specific and optimized method for each sample will not be discussed in this thesis but a general workflow for the analysis and determination of PFCs is as illustrated in Figure 1.4.

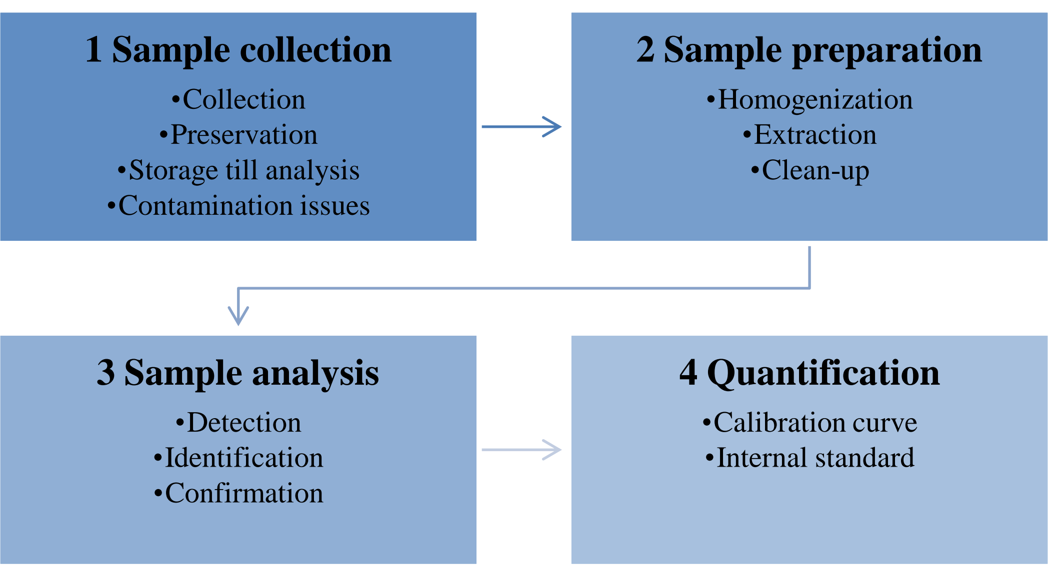


Figure 1.4 Workflow for analysis of PFCs[53]

The first stage of PFCs analysis is sample collection. Proper sample collection techniques need to be performed to prevent any biased samples and contamination. Upon sample collection, the sample is usually preserved adequately, prepared and analyzed within a few days of collection to prevent any change in the sample quality and changes to the precursors and end-products of PFCs. It is important to note that all laboratory equipment and consumables used should be checked and free of any possible fluorinated contaminants before use. Furthermore, glassware should also be avoided as PFCs tend to adsorbed on these surfaces.

Due to the complexity of sample matrices and the concentrations of PFCs, sample preparation is usually required and it involves several steps which consist of homogenization, extraction and clean-up. Depending on the sample type, sample preparation varies. Typically for most samples, liquid-liquid extraction is used followed by solid phase extraction (SPE) for a clean-up step. It is noted that, pressurized liquid extraction is applied by Zafeiraki et al to extract PFCs in food packing materials[54]. Due to PFCs' good chemical stability, this method proved to be faster and more efficient method for PFCs detection. Another commonly used method which involves an weak anion exchange interaction for PFCs extraction is SPE[55]. It

utilizes both the reverse-phase and ion-exchange mechanism in the SPE sorbent to extract PFCs out from the samples. Zabeleta et al compared the use of several SPE cartridges for extracting PFCs in fish, vegetables and soil samples[56].

As for the analytical method, liquid chromatography – tandem mass spectrometry (LC-MS/MS) is most preferred due to the combined sensitivity and ability to simultaneously detect several PFCs in a single injection. Without any derivatization, PFCs can be separated to obtain good peaks with the use of suitable LC column type and composition of mobile phase (pH and type).

Other techniques apart from LC are utilized. One example is the use of gas chromatography (GC). Perfluorinated carboxylic acids undergo pretreatment via benzyl esterification and were quantified in composite dietary samples by GC-MS[57]. However this technique is generally not preferred for the analysis of PFCs as PFCs are involatile and they are not easily derivatized to produce volatile analytes[58]. Also, PFCs are not chromophores and the lack of standards lead to easier techniques such as HPLC-UV being not suitable or accurate enough for the quantification of PFCs[59-61]. A summary of possible analytical techniques for PFCs are shown in Table 1.2.

With the advances in tandem mass spectrometry, multiple-reaction-monitoring (MRM) is employed to easily identify the different PFCs based on its parent and daughter ions. Furthermore, trace analysis is made easier as indicated by Llorca et al where they compared three different liquid chromatography-tandem mass spectrometry systems for trace analysis of several PFCs in aquatic species[62]. Isotopically-labeled perfluorinated compounds are used as internal standards to give higher selectivity and sensitivity results.

The general direction of PFCs analysis is towards developing a fast, high-throughput, automated and sensitive detection method for a wider range of PFCs and its precursors. Improvements were made to quantify as many PFCs compounds in the

shortest possible analysis time as shown by Chung et al where a UPLC-ESI-MS/MS method was developed for analyzing seventeen PFCs in twelve minutes and it have been successfully incorporated in routine analysis of PFCs in food[63]. Generally for PFCs analyses, current analytical instruments combined with sample preparation techniques, it has definitely reached low ng/L, low ng/g and pg/m^3 for environmental samples such as water, soil and air respectively[64-69]. Table 1.2 shows a summary of analytical techniques used for the analysis of PFCs in various samples types. Thekey features, advantages and disadvantages of some of these analytical techniques are elaborated in Figure 1.7 and Table 1.3. The most preferred techniques for the analysis of PFCs are still the use of LC-MS/MS due to the sensitivity and ease of operation.

Table 1.2 Summary of techniques used for PFCs analysis

Analytical Techniques for PFCs analysis	References
Attenuated Total Reflected Fourier Transform Infrared Spectroscopy	70
^{19}F Nuclear Magnetic Resonance	71
Gas Chromatography Mass Spectrometry	57,58,72-74
Ion-Exchange Chromatography	75
LC - Conductimetric Detection	76
LCMS and LC-MS/MS Includes techniques such as the direct-injection and also the different interface between LC and MS.	62,77-82

1.3.1 Determination using analytical instruments alone is insufficient

In order to better understand the impacts of PFCs in the environment and also the effects of bioaccumulation, it is critical to not only determine the quantity of PFCs in the environment using analytical methods. Chemicals in the environment are able to cause an impact by different routes and means. To put it in another way, quantitation alone is insufficient to determine the toxicity of the chemical in the environment. It is necessary to take into consideration that in the environment, any contaminants do not exist just by itself; environmental pollutants are mixtures of chemicals or toxicants. In this situation, compounds in a mixture may exist and cause

a synergistic or antagonistic effect. Therefore quantitation using analytical techniques alone may not be a sufficiently good gauge to evaluate the toxicity effect of PFCs in the environment. Hence, not only quantitation but also toxicity studies have to be performed to better determine whether PFCs pollutants are toxic to the environment and organisms.

1.4 Toxicological studies

Toxicological studies are usually vary based on the subject-of-interest, medium, duration of the study (acute and chronic), concentration of the toxicants and the type of exposure (ingestion, inhalation or adsorption) and several other factors. In fact, PFCs toxicity tests have been conducted on several animals, for instance, monkeys, rats, rabbits and aquatic animals with its LC_{50} (lethal concentration when 50% of population dies) determined[83]. In addition, the effects of PFCs on these test subjects are determined by several parameters such as its mortality, behavior, activity and mobility. Several biological health parameters were also measured and observations in its physical state such as its growth and developments such as length and weight and also in specific areas such as reproductive, pregnancy and tumor generation can be determined. As described in the earlier sections, PFCs are known to cause reproductive and development toxicity, neurotoxicity and hepatoxicity in animals[48]. It is also suspected to be carcinogenic. With only a handful of PFCs toxicity tests or studies on human, animals' studies are used as much as possible to infer and predict the possible potential human effects of PFCs.

PFCs toxicological study coupled with metabolomics research in rats has begin as early as in 1996[84]. In this study, GoeckeFlora et al investigated the effect of long chains PFCAs to rats' liver by using ^{19}F and ^{31}P NMR spectroscopy. In addition from the ^{31}P NMR results, phosphocholine was detected in significantly higher

concentrations when the rats are exposed to long chain PFCAs. From this study, it can be summarized that long chains PFCAs causes significant toxicity to the liver as shown by the inhibition of phospholipid metabolism, glucose uptake and glycogen deposition.

In another study, Lankadurai et al tested the 48 hour exposure of PFOA and PFOS to earthworm *Eisenia fetida* and have observed increase in succinate, 2-hexyl-5-ethyl-3-furansulfonate, glutamate and decrease in several sugars, amino acids and ATP which signify an increase in fatty acid oxidation and reduction in ATP generation[85,86]. In general, many organisms showed similar extent of toxic responses and mode of actions due to PFOA and PFOS exposure.

As discussed, contaminants exist in mixtures in the environment and not as individual by itself. With the techniques of analytical determination, toxicity studies and metabolomics, it is possible to determine the effect of mixtures of PFCs in the environment. With several toxicological information and results, it is definitely more conclusive than just analytical determination of PFCs. Therefore to better understand the whole situation and effects of PFCs in the environment; it is best to incorporate both analytical determinations and toxicological studies.

1.5 Introduction of metabolomics

Metabolomics is one of the 'Omics' techniques used to study small molecules, also known as metabolites. These molecules are present in a selected biological system which can be a cell, tissue, bio-fluid and even the whole organism. Upon interaction with a stressor, the metabolites level may change and directly affect its biochemical activities which express a change in phenotypes[87,88]. Apart from metabolomics, there are other 'Omics' techniques available such as genomics, transcriptomics and proteomics. The main difference among these techniques is the molecules of interest that they monitor which are genes, ribonucleic acid (RNA) and proteins respectively.

Recently, metabolites are of increasing interest compared to the other 'Omics' approaches as it is a direct method of studying the biological system when a change in the expression of phenotype is indicated. For the case of genomics and proteomics, it is not as direct and straightforward and the functions of genes and proteins can be affected by other cell activities. As a result, metabolomics is one of the rapid developing techniques and there are many different applications, for example biomedical, food, clinical, plant and environmental metabolomics. In the next section, one of the trending applications in metabolomics[89] - environmental metabolomics will be introduced in greater detail.

In general, there are two different approaches for metabolomics study: targeted and untargeted metabolomics. For targeted metabolomics, a set of specific metabolites are of interest and are analyzed and quantified during the study. This approach is useful if the metabolites of interest for the studied species and the related pathway are known. On the contrary, for untargeted analysis, all metabolites are taken into consideration and there is no singling out of any metabolites. Performing untargeted metabolomics, which is also known as metabolite profiling, the whole metabolome is being used and simultaneously analyzed, the different types of

metabolites can be determined and new biomarkers, cellular pathways and biological mechanisms can be discovered to better understand the system and effects[87]. Therefore, depending on the research goals, different approaches of metabolomics such as untargeted and targeted techniques can be used.

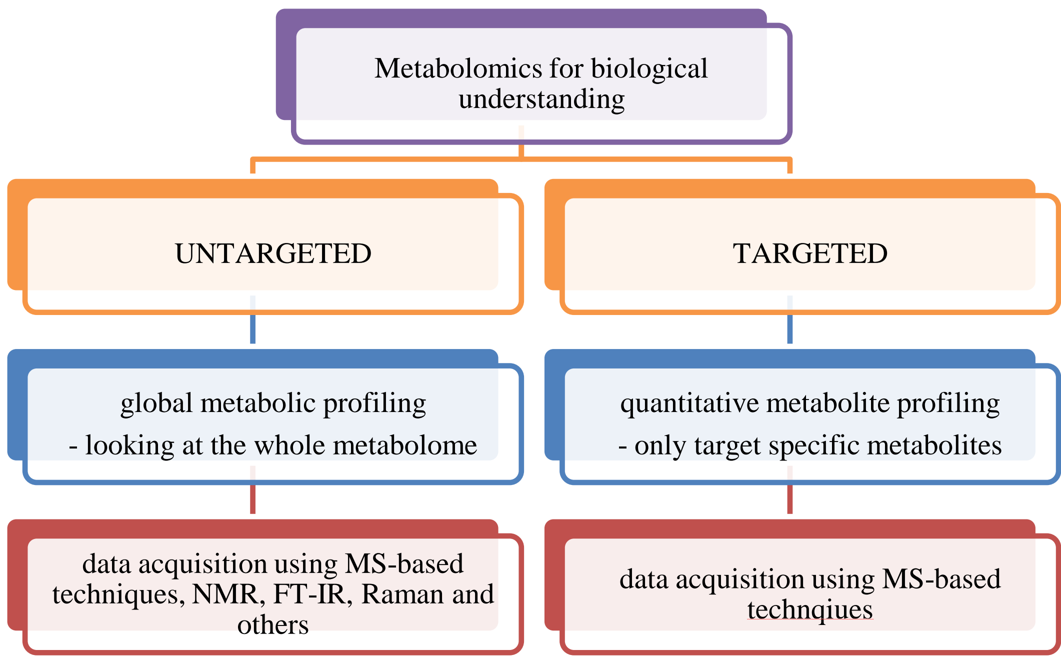


Figure 1.5 Targeted and untargeted metabolomics

The workflow for targeted and non-targeted metabolomics differs[87]. As shown in Figure 1.5, for untargeted metabolomics, the sample preparation steps generally require it to be able to extract as much metabolites from the sample as possible and all of these metabolites will undergo data acquisition in an instrument with higher mass resolution and range and a global metabolite profile is obtained for that specific sample. The sample preparation procedure will be a general method where as much metabolites can be extracted out without any bias. On the other hand, for targeted approach, the sample preparation steps generally favors these specific sets of standard metabolites of interest and quantification of these metabolites will be performed using a very sensitive MS with selected reaction monitoring (SRM).

1.5.1 Environmental metabolomics

Environmental metabolomics focuses on the interaction of the organisms to the natural or man-made stressors of the environment and the related metabolic responses obtained from it[85,90]. It reveals how stresses or changes in the environment can cause differences in the metabolites of the organism. The results are then used to determine any variations in the biological functions by figuring out the different mechanisms and pathways of how these stressors affect these organisms in the environment[91]. Abiotic environmental stressors include climate change, air quality (haze), nutritional values[92,93], temperature, light, salinity and pollution in the environment[94]. They can exist individually and in even worse and complicated conditions, several stressors at the same time. For instance, Simpson and Mckelvie demonstrated the use of environmental metabolomics to investigate earthworm responses to contaminated soil[95]. Metabolic profiling of these earthworms which are exposed to toxic chemicals was obtained using proton ^{1}H NMR spectroscopy. It was revealed that environmental metabolomics and the use of earthworms have the potential to be applied as a direct monitoring and measuring tool for contaminant bioavailability.

Similarly, metabolomics will be applied in this study to understand better the metabolome upon environmental stress and this technique can also be used simultaneously as a water quality indicator. In this study, environmental metabolomics in terms of toxic compounds present in the aquatic environment is applied to two different aquatic organisms, *chlorella vulgaris* and *daphnia magna*. This technique enable the better understanding of how these toxic compounds are going to act on these aquatic organisms and their mode of action and their resulting effects. There will be 2 different groups: the control and the exposed. The control where the studied organism with no stress exposed will be compared with those with the ones exposed to the specific stress. The metabolic composition and profile of the

organisms will be analyzed and through the use of environmental metabolomics, a better understanding of the organisms and its habitat can be achieved[94].

In order for metabolomics to proceed further and be applied more extensively, particularly environmental metabolomics, Miller et al have presented and discussed the all possible strengths, weaknesses, opportunities and threats that environmental metabolomics faced[96]. To highlight some weaknesses and challenges of metabolomics, the main concern is the lack of databases and software for the easy identification and quantitation of the metabolites found. Most of the metabolites found were not being identified resulting in a lot of valuable information lost. In addition, its concept is still not widely accepted for regulatory authorities and of any real world application. However, the use of environmental metabolomics is still important as these small molecules give lots of insights on the environment. Metabolic changes can be used to predict and identify any environmental disturbances before it potentially occurs. As a result, it can act like a bio-indicator and biomarkers can be identified upon exposure and effect of environmental disturbances. Furthermore, these analytical instruments are already in use in most of the laboratories which makes analysis easier. Therefore to have a better future in environmental metabolomics, steps have to be taken to minimize weaknesses and threats to fully utilize the potential of metabolomics.

1.6 Workflow of metabolomics studies

A typical metabolomics workflow consists of several steps which include experimental design to sample collection, preparation and analysis to lastly, data processing and interpretation. With this in mind, certain procedures and steps may be altered or substituted to better cater to the specific goals for the study (e.g. targeted or untargeted approaches). Figure 1.6 illustrates a typical workflow of a metabolomics study where part A illustrates the planning, design, sample collection and preparation while part B focuses on instrumental analysis, data analysis and interpretation.

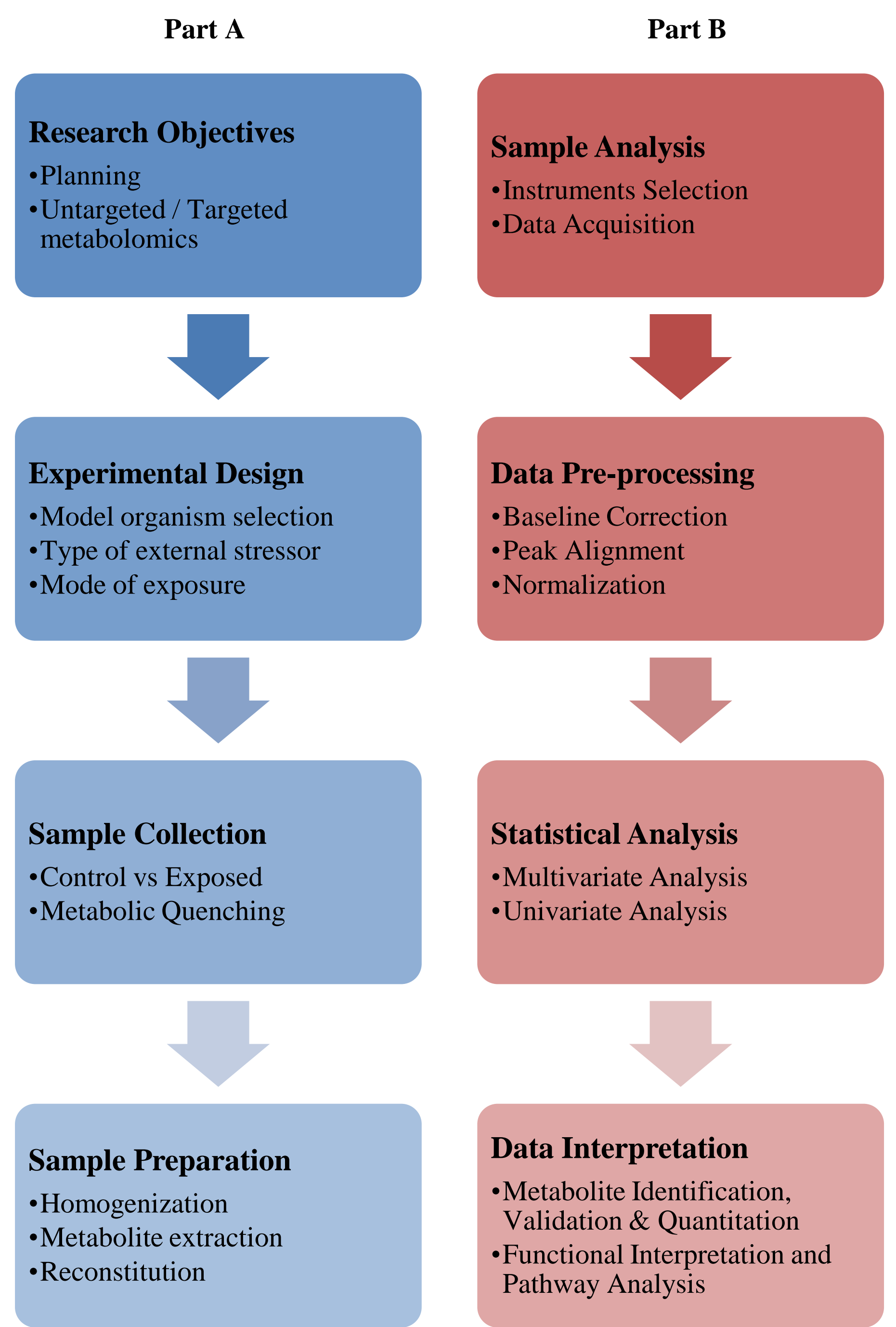


Figure 1.6 Typical workflow of a metabolomics study[97]

In general, the experimental design and planning section is the most critical. With regards to environmental metabolomics, it is important to consider the type of study, the organisms and pollutants of interest and the mode of exposure (in terms of the type, duration and concentration) and what type of data/results are required for this particular experiment. There are other factors which may be critical and of interest depending on the objectives and type of studies.

The first step after planning is sample collection. For laboratory-studies, where the situation is slightly more controlled, it is essential to minimize and prevent metabolic variability and ensure that only stress-induced metabolic changes are recorded and obtained[98]. As such, experimental design and sample collection needs to be planned and executed correctly. Randomization of study samples and homogenization of cultures is critical to prevent any bias occurring in the sample selection process. Quenching of the metabolic response at the right time point is critical and is usually performed by flash freezing using liquid nitrogen. Samples were then lyophilized completely and stored at -80°C till extraction. There are other quenching techniques which involves the use of acid or organic solvents such as methanol and acetonitrile[85].

Currently, there are no single instruments able to detect all the different types of metabolites. Depending on the objectives of the study, different instruments are employed to obtain a comprehensive analysis of the whole metabolome. In addition, the type of metabolites-of-interest, the type of study and the data quality dictates the sample preparation procedure and also the sample analysis techniques (instruments etc.). For instance, gas chromatography – mass spectrometry (GC-MS) generally favors the less polar compounds while more polar compounds are suitable for LC-MS analysis. MS-based analyses and nuclear magnetic resonance (NMR) analyses differs in their sample preparation procedures too.

In the event where there are sufficient biomasses for comprehensive analyses, samples undergo specific sample extraction and preparation unique for that particular analysis platform. By doing so, this enables higher extraction efficiency and specificity for that particular group of metabolites. However, this is not always the case and samples are quite limited. A universal protocol, where the sample preparation is optimized for all analysis methods, is usually used. An example is a mixture of chloroform: methanol and water in a 2:1:1 ratio where it is a solvent

commonly used for sample preparation for both the hydrophilic and lipophilic fractions[99]. This extraction mixture is not only the most reproducible but also boasts the highest recovery for both fractions[85]. Therefore it is critical to plan properly and cover every single aspect before proceeding on with the experiments and sample preparation.

As can be seen in Figure 1.6, part A of the workflow of a metabolomics studies generally consists of planning, experimental design, sample collection, preparation and extraction. The detailed discussion for this section is discussed in the individual chapters of the thesis. Part B of the workflow consists of data acquisition, processing and interpretation which are discussed in greater detail in the following sections.

1.6.1 *Data acquisition*

NMR spectroscopy and MS-related techniques are two of the most commonly-used platforms in metabolomics studies[85,97]. Additionally, there are several other analytical approaches for metabolomics, such as Fourier Transform infrared (FTIR) and Raman spectroscopy. The ideal choice of techniques will largely depend on the type of study and the metabolites of interest (type and concentration).

NMR spectroscopy has played a main role and is still widely used in metabolomics due to its non-selectivity and simplicity in data acquisition and sample preparation[85]. Its principle of use consists of the spin properties of certain atomic nuclei where there are an unequal number of protons and neutrons such as proton (^{1}H), carbon-13 (^{13}C), fluorine-19 (^{19}F) and phosphorus-31 (^{31}P). This technique has been applied in both untargeted and targeted metabolomics due to its strength of having easy sample preparation and being non-destructive. Apart from the chemical shift values that are obtained from a NMR spectrum, the intensity obtained for each chemical shift can be used for quantification which will reveal additional useful

information. One of the many examples of using NMR spectroscopy for environmental metabolomics is the study of the toxicity effect of an insecticide lambda-cyhalothrin in goldfish[100]. In this study, Li et al used ^{1}H NMR to demonstrate the effects of this insecticide on different tissues of the goldfish and discovered biomarkers for pesticide pollution in aquatic environment.

Another analytical technique commonly found in metabolomics study is chromatography. It is critical for separating complex matrices and numerous metabolites for analysis. It enables clear separation before detection and an increased in its sensitivity and specificity is expected. A short description of each of the techniques of chromatography will be discussed: Gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE).

GC is an analytical technique which separates analytes based on its volatility and retention on the stationary phase. Compounds that are more volatile and soluble are being carried first by the inert carrier gas to the detector. As a result, these compounds are eluted and detected first. It has always been the method to go for volatile organic compounds due to the ease and simplicity of the instrument. However, it requires analytes to be volatile and thermally stable in order for analysis to be carried out accurately. Derivatization is otherwise required to assist in the volatility of the analyte. Due to these compatibility issues and thermolabile property of some metabolites for GC analysis, LC is the alternative chromatography technique for metabolomics studies.

LC separates compounds based on the analytes' interaction with the mobile and stationary phase. Analytes having a higher affinity to a certain polar or non-polar phase will retain there until a more favored condition occurs. During a LC run, based on the different affinities, separation and elution of the analytes occurs when the mobile phase composition changes. Reverse-phase LC is one of the more frequently

applied techniques in the separation of metabolites due to its robustness and wide range applicability. It consists of a non-polar stationary phase column (e.g. C_{18} non-polar) and an initial polar mobile phase. In reverse-phase LC, the more polar compounds will be eluted first followed by the more non-polar compounds. Apart from the usual reverse phase LC, there is a complementary technique available which is the hydrophilic interaction liquid chromatography (HILIC). It works on the same concept as the reverse phase LC just that the mechanism of separation is based on the hydrophilic interaction of the analytes with that of the hydrophilic column phase and the increasing aqueous mobile phase. With advancements in LC, higher resolution, better separation efficiency and faster run times were obtained by using high performance liquid chromatography (HPLC) and even ultra-performance liquid chromatography (UPLC).

CE is the newest addition to the separation technique for metabolomics. It separates charged analytes based on its electrophoretic mobility and electro-osmotic force generated upon an applied voltage. Ions separated based on their electrophoretic mobility which is dependent on the charges and size. This extremely sensitive technique, which boasts of faster run time and higher separation resolution, requires very small sample sizes and buffers for separation. Advances have been made to the interface to connect CE and MS and made it more applicable and robust for routine metabolomics studies. In general, chromatography is an essential tool for metabolomics, especially suitable for samples with lots of compounds and contaminants as it is able to separate metabolites before detecting it to improve sensitivity and accuracy. In fact hyphenated techniques which couples chromatography with MS provide another emerging method of analysis for metabolomics. Chromatography coupled with MS techniques have even advanced to a two dimension chromatography platform where better separation, resolution, sensitivity can be achieved[101-106]. Together with the advances of several new and

upcoming mass analyzers, the analysis of metabolites moves into a much higher sensitivity and specificity level.

Mass spectrometry (MS) can be applied in metabolomics studies by itself as a single technique (e.g. direct infusion mass spectrometry (DIMS)) or in a hyphenated technique. MS is an instrument where it measures the mass to charge (m/z) ratio and also the abundance of the ions. To put it simply, analytes are first introduced into the MS and then converted to ions in the gaseous phase through ionization.

These MS ionization techniques can be separated into hard or soft ionization methods based on the energy required and the extent of fragmentation. Hard ionization methods uses electron impact (EI) or chemical ionization (CI) to ionize gaseous analytes into ions which is commonly seen in GC-MS application. On the other hand, soft ionization methods usually converts liquid samples into gaseous ions by electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photo-ionization (APPI) or matrix-assisted laser desorption ionization (MALDI). These methods assist in the generation of gaseous ions without high energy involved. These gaseous ions are then separated based on the mass to charge ratio (m/z) in the mass analyzers. The principles of how the various mass analyzers work may be different and some of the commonly used mass analyzer and the separating principle are shown in Figure 1.7. Upon separation, the ions passed through a detector and the abundances of the respective m/z ions are detected and recorded.

Quadrupole Mass Filter

- Radiofrequency (RF) and direct current (DC) voltages are applied on the quadrupole such that only ions of selected m/z ratio can passed through. The other ions will be unstable and hit the quadrupole rods.
- Strengths: Good reproducibility, small and relatively low in cost.
- Limitations: limited mass range and ion transmission, not suitable for pulsed ionization.

Time-of-Flight (TOF)

- It separates based on the different time it takes for the different m/z ions to travel from the ion source to the detector.
- Strengths: Fast and well-suited for pulsed ionization, high mass resolution and widest mass range.
- Limitations: limited precursor selectivity for tandem MS.

Fourier Transform - Ion Cyclotron Resonance (FT-ICR)

- Ions are trapped and move in a circular motion in a cell. It is only released if the radiofrequency matches the ion cyclotron resonance of the ions. ICR frequency is dependent on the mass.
- Strength: highest mass resolution, high mass accuracy, non-destructive
- Limitations: limited dynamic range and expensive

Magnetic Sector Mass Analyzer

- As ions accelerate through an applied magnetic field , it separates based on the Lorentz force law and its m/z ratio.
- Strengths: High reproducibility, resolution, sensitivity and dynamic range and able to do MS/MS in same analyzer.
- Limitations: Larger in size, not suitable for pulsed ionization, limited MS/MS selections and resolutions.

Figure 1.7 Principles, strengths and limitations of mass analyzers

Different mass analyzers have its advantages and disadvantages listed out in Figure 1.7. Apart from full scanning in just a single mass spectrometer or a selected-ion-monitoring mode (SIM), tandem mass spectrometers (MS/MS) are used to give

more sensitive analysis through the use of selected-reaction-monitoring (SRM) and multiple-reaction-monitoring (MRM). Basically for SRM and MRM modes, a selected parent or precursor ion will be isolated out through the first MS, and undergo a fragmentation with optimized parameters to produce several fragments or daughter ions. These fragments, which can act as quantifiers and qualifiers, will then be measured and detected, MRM mode of the tandem MS is able to monitor several of such reactions (precursor ion → fragment ions) for the analysis to give highly sensitive and selective quantification results. These techniques are gaining popularity and can be applied to targeted metabolomics and also for quantitative methods for determining pollutants.

Direct injection-MS (DIMS) which is another alternative for metabolomics study is not commonly used due to the complexity of the sample matrix and other matrix and ion effects. Instead, for this study, liquid chromatography coupled with quadrupole-ion trap MS (Q-TRAP-MS) and quadrupole time-of-flight (Q-TOF-MS) are used respectively due to its better sensitivity for accurate quantitation analysis and its large mass range and high mass resolution for untargeted metabolomics approach.

As discussed earlier, the two most commonly used analytical platforms are NMR and chromatography-coupled MS[85]. The main strengths and weaknesses of both techniques are listed in Table 1.3. Within these GC-MS, LC-MS and CE-MS related techniques, there are already many strengths and limitations. One such example will be the presence of reliable and reproducible database for GCMS techniques but for LCMS and CEMS, these techniques require more time for comparison, identification and validation. All in all, different techniques have its strengths and limitations. Depending on the objectives of the metabolomics study, different analytical techniques can be used individually or together to ultimately achieve and understand the subject of interest better.

Table 1.3 Strengths and limitations of NMR and chromatography-coupled MS

	NMR	Chromatography-coupled MS
Advantages	• Rapid • Applicable to ^{1}H, ^{13}C & other nuclei • Able to do 2 dimensional NMR to improve spectral resolution • Non-destructive • Easier sample preparation • Non-selective • Not sample-biased • High throughput • Good stability • Quantitative • Comparable between different NMR of identical field strength	• Range of chromatography (GC/LC/CE) techniques with MS and tandem MS • Able to separate and provide qualification and quantification results or direct infusion mass spectrometry (DIMS) • Higher sensitivity – able to detect trace levels of metabolites • Different ionization techniques available • Quantitative and qualitative • Able to provide M/Z information, perform fragmentation and identification
Disadvantages	• Low sensitivity (µmol/l) • Requires external isolation of metabolites • Overlapping chemical shifts • Solvent peaks • Hard to identify • Requires database • Requires chemical shift reference	• Time consuming • Laborious • Elaborate procedures • Stability of instruments performance • Sample preparation dictates metabolites obtained • May be biased in sample preparation • Requires database
Comments	New technologies to increase sensitivity - Coupling with LC - High resolution magic angle spinning NMR	Advances in chromatography systems and tandem MS - 2 dimensional chromatography systems (e.g. GCxGC, LCxLC) - MS/MS

1.6.2 Data processing

Generally, metabolomics studies have to deal with huge volumes of data from several samples obtained from various analytical instruments, for instance NMR and MS-based techniques. NMR results for each sample run generates a list of chemical shift values with their corresponding intensity while for MS-based techniques, each sample run will obtain a list of retention time with the corresponding intensity and

mass spectrum or m/z value. In order to make full use of these results and to obtain qualitative and quantitative information about these metabolites, data pre-processing have to be conducted first, followed by statistical analysis and lastly data analysis and interpretation[107-109].

Firstly, data pre-processing steps for these different analytical techniques differ slightly but generally follow the same guidelines and give similar outputs where a tabulation of values is recorded at different variables for each sample. For NMR data pre-processing, it consists of phase and baseline correction, accounting for chemical shift reference, solvent peak identification or deletion, integration and peak picking. Furthermore, data binning can be included so that variations in the chemical shifts across samples can be minimized and lessen the complexity of the data collected. Likewise for MS-based techniques which generate massive volume of data, it involves retention time correction, alignment, feature detection, peaking picking, deconvolution and normalization. Each of these sub-steps itself may have several different parameters to choose from and may be complicated if chosen wrongly or not performed consistently. For instance, normalization is commonly performed for MS-based data to reduce any variability in the MS-runs[110,111]. There are several different normalization techniques such as total area normalization, median normalization and also normalization using internal standards. If performed inconsistently on the samples in the same metabolomics study, more variability will be carelessly introduced. For easier compilation and recording, software is usually used in data pre-processing. MS vendors have their own MS pre-processing software and there are other free online software available for use[112], for instant XCMS online[113-116], MZmine[112,117,118] and MetAlign[119,120]. Currently, there is no universal method for pre-processing and it may differ depending on the instrument used, experiment performed, parameters set and the type of software used[121]. Detailed data pre-processing steps needs to be recorded as different pre-processing methods will give

different final results. Pre-processing steps may be unique for that particular metabolomics study and several trial and errors have to be performed initially to fully understand the different pre-processing parameters and to choose the most suitable step.

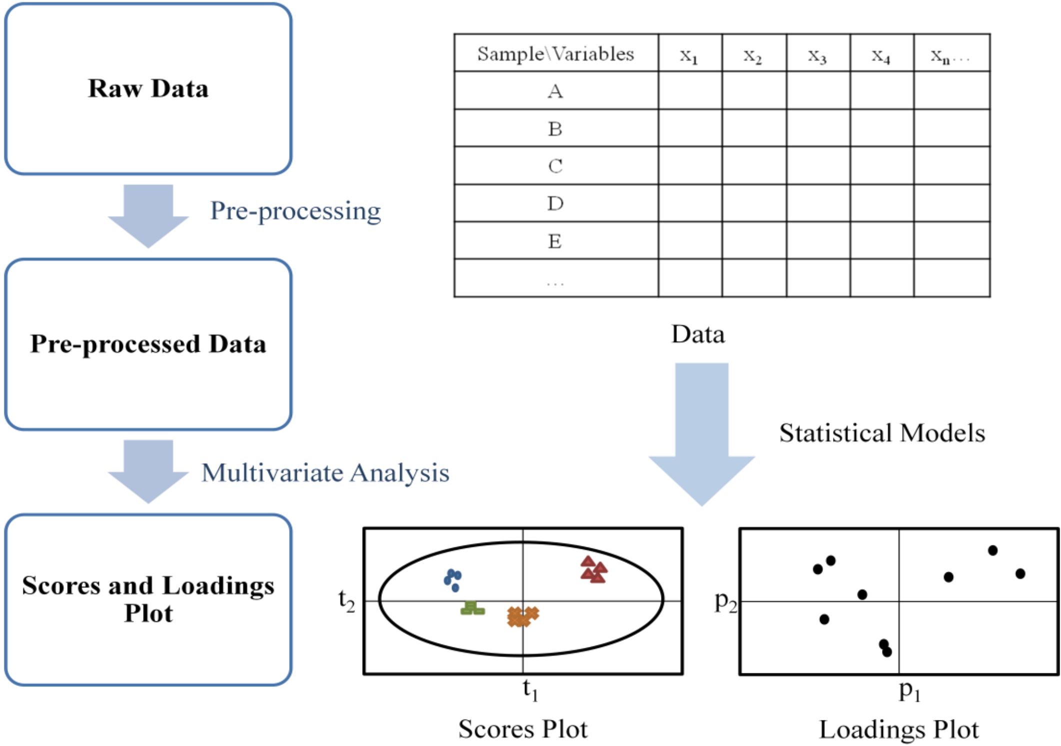


Figure 1.8 General workflow for multivariate analyses (MVA)

After data pre-processing, statistical analyses such as univariate, bivariate and multivariate analyses are performed. Univariate analyses focus on only one variable for that comparison among different samples while multivariate analyses (MVA) focus on all variables included and are compared at the same time. T-test and analysis of variance (ANOVA) are commonly used univariate and bivariate analyses in metabolomics to indicate whether sample groups are significantly different or otherwise. For MVA, where massive amount of data are involved, chemometric techniques are usually utilized to facilitate processing, allow easier understanding and generation of possible associations, relationships and patterns. So with MVA (Figure 1.8), these huge volumes of data that is incomprehensible by researchers are made

simpler by statistical models which converts data to easily understandable graphical visual outputs (e.g. score and loadings plots)[108].

It should be noted that processing in multivariate analyses (MVA) is critical such as the selection of scaling techniques. The selection of unit-variance (UV), Pareto or mean-centered scaling depends on the intensity of the results for the different variables. Scaling is performed on the data to ensure that equal weightage are given for all variables and not causing any biased representation.

There are several different statistical models (e.g. unsupervised and supervised) used in metabolomics. Principal Component Analysis (PCA) is a widely used unsupervised form of analysis[122]. It works by reducing hundreds and thousands of variables present in the data set to a few uncorrelated variables known as principal components. The first principal component will thus have the largest variation followed by the second then third and so on. The first principal component illustrates the greatest variability in the data set. The second principal component which is independent of and orthogonal to the first is the second best in explaining the variability of the data set. As shown in Figure 1.8, upon undergoing such processing, scores and loadings plot are generated. Each point on the score plot represents a specific metabolic profile. It indicates the relationships between each point. The closer the points are located on the score plot, the closer they are in their profiles. Depending on the study, there may be clustering of points and also scattering or dispersing of some points. Score plots will group and place the samples based on the measured metabolic responses by the NMR or chromatography-MS techniques. This often reveals possible trends and associations. In order to obtain more information, the loading plots are then used to determine the metabolites responsible for a certain clustering or discrimination of samples. From the loadings plot, the further the points are away from the origin, the more significant the correlation is observed. The different variables are then studied in greater detail using the loadings plot.

For supervised MVA, there are soft independent modeling of class analogy (SIMCA), projection to latent structures discriminant analysis (PLS-DA), orthogonal projection to latent structures discriminant analysis (OPLS-DA) and OPLS-regression analysis. These methods are conducted where the grouping of the samples are known and have been classified to allow greater differentiation between the sample groups. The main difference between PLS-DA and OPLS-DA is the presence of an additional predictive component used to separate out the variables related to between-class and within-class variations. Apart from the scores and loadings plot, the Variables Important to Projection (VIP) list can be generated and used to identify the significantly different variables. As these methods are supervised, these datasets may be forced to fit into these specific arrangements. In order to ensure unbiased and accurate data, statistical analysis and validation methods are required.

With the increased in interest and application in metabolomics, there are several metabolomics software available and some even with free access. As described earlier, XCMS online[113-116] is one of the free online platform which comes with pre-processing and also metabolomics MVA.

1.6.3 Data interpretation and metabolite identification

After data processing and statistical analyses, the compiled results need to be interpreted to identify any possible metabolites and biomarkers. An absolute confirmation method, commonly employed in targeted metabolomics, is the use of commercially available pure standard metabolites to identify and validate. It is possible as only a selected group of metabolites are monitored. However, in the case of untargeted metabolomics, it is expensive and laborious to purchase hundreds of commercially available pure standards just for identification. Therefore we can only make use of information that these instruments provide. NMR spectroscopy gives

information such as the chemical shift values, intensity and peak multiplicity. On the other hand, MS-based techniques provide information such as m/z, intensity and tandem MS (MS/MS) spectrums. In addition, with the coupling of chromatography techniques, retention time and intensity are included. Together with these MS and spectral information, metabolites can be searched and compared using literature and online databases such as NIST[123], HMDB[124,125], METLIN[126-128] and MassBank[129,130] depending on the organism studied.

For techniques such as NMR[131-135] and GC-MS, they possess commercially available database which aids in the identification of metabolites significantly. However based on the capabilities of other MS-based techniques (e.g. LCMS and CEMS) and the lack in comprehensive databases currently, it is time-consuming and not possible to identify all the possible metabolites obtained or extracted. Therefore one of the challenges of metabolomics still lies in the metabolites database. Hopefully, with the inclusion of widely available tools and databases, universal databases can be compiled and established which can lead to metabolites being identified easily.

Apart from metabolite identification, it is possible to move on further and understand the mechanism and theory behind these significantly changing metabolites upon exposure to any stress factors. Upon isolating several metabolites, associations can be determined and possible mechanisms can be hypothesized. There are databases which provide information about the different pathways in the studied organism such as MetaboAnalyst[136,137].

1.7 Scope of research

Due to the emerging concern of PFCs' persistent nature and ability to bioaccumulate in the environment, this thesis will be dedicated to exploring further in detail about this group of POPs and its effects mainly to the aquatic environment. It will explore and utilize different techniques of analytical determinations, toxicity studies and environmental metabolomics together to better understand the mechanism of bioaccumulation of PFCs and other related compounds and how this can impact the aquatic environment and in turn affect humans. The two aquatic organisms employed in this research work are green microalgae, *Chlorella vulgaris*, and water flea *Daphnia magna*. Both aquatic species chosen due to it being critical in the aquatic food chain and also being relatively new in this field and have not been extensively studied in the environmental metabolomics field. Chapter 1 of this thesis comprises mainly the introduction of PFCs, environmental metabolomics and the importance and significance of this research.

Chapter 2 aims to study the bioaccumulation pattern and its fate of PFCs in green microalgae *Chlorella vulgaris* and use it to better understand the effect of trophic magnification of PFCs in the aquatic environment. Techniques such as solid phase extraction (SPE) and liquid chromatography tandem mass spectrometry (LC-MS/MS) are used in the determination of PFCs in the aquatic medium and *Chlorella vulgaris*.

Following which, chapter 3 will build upon the results obtained and environmental metabolomics will be applied to understand the detailed effects of PFCs on these green microalgae. Both nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography tandem mass spectrometry (LC-MS/MS) is used in untargeted metabolomics mode to investigate the metabolome of this green microalga upon the acute stress exposure of PFOA and PFOS.

Water fleas are commonly used species in aquatic toxicity study and are recently gaining interest in for metabolomics studies. Chapter 4 will investigate the use of water flea from different age groups for environmental metabolomics study and applied it for eco-toxicity determination of PFCs and bottom ash leachates. Furthermore, MS-based metabolomics approach will be used to determine the possible contents present in these bottom ash leachates. This technique of using *Daphnia magna* will indicate water quality quickly and adequately and also determine possible acute environmental effects. The final chapter will conclude and summarize the work done in this thesis. The future work and perspectives on PFCs trends and research will also be discussed.

2 Investigation of the fate of perfluorinated compounds upon acute exposure to green microalgae (*Chlorella vulgaris*)

2.1 Introduction

Perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) have been studied in great detail compared to other groups of PFCs due to its harmful and toxic effects. They are classified as long-chains PFCs and research on these 2 toxic compounds has been mainly focused on determining its concentration, toxicity, removal and impacts on the environment. They are very resistant and tend to accumulate in the environment. Their structures and properties are listed in Table 2.1. To understand the eventual fate of PFCs in the environment, values such as K_{OC} and acid dissociation constant (pK_a) are commonly used. Depending on the techniques used for calculating pK_a and the various models, considerations and assumptions, there are several values out in the in the literature resulting in the true uncertainties in the actual pKa value for PFOA. Factors such as aggregation (complete or partial aggregation) have been taken into consideration resulting in a range of pK_a values being calculated for PFOA. However, with evidence of having a ratio of less than 10^{-7} for the protonated to un-protonated form of PFOA at pH 8.1, most of the researchers have largely confirmed that PFOA exist in the anionic form in aquatic oceanic environments[138-140]. There has been less of an issue of uncertainty of pK_a for PFOS. In anionic form, these PFCs are water soluble and can be transported long range which results in their high persistency in the environment.

Table 2.1 Structure and properties of PFOA and PFOS

Properties	Perfluorooctanoic Acid (PFOA)	Perfluorooctane Sulfonic Acid (PFOS)
Molecular Weight	414.1	500.1
Melting Point	45-50°C	>400°C
Boiling Point	189 – 192°C	Not measureable

log K_{OC}*	1.47[141]	2.10[141]
Vapor Pressure	4.17Pa (at 25°C)[142]	3.31 x 10^{-4}Pa (at 25°C)[143]
Water Solubility	4340mg/l (at 24.1°C)[144]	570mg/l[143]
pK_a (equilibrium acid dissociation constant)	0 – 3.8 0.5 (experimental determination) 1.01 (complete aggregate) 2.8 (partial aggregate) 3.8 (determined using a standard water-methanol mixed solvent approach) [138-140]	-3.3 (calculated value for acid) or 0 (dominant form in environment is anion)
US EPA Provisional Health Advisory Value	400ng/l[16,18]	200ng/l[16,18]
Structure of PFOA		
Structure of PFOS		

Apart from being detected near the source, PFCs are also detected in several aquatic organisms, mammals, birds and even humans[55,69,145-150]. Based on the persistency and fate of PFCs, it will ultimately end up in the environment which consists of the atmosphere, water, soil and sediments[67,69,146,148,149]. These PFCs will exist for years in the environment till they dissociate further to form smaller products.

* K_{OC} is the partition coefficient between organic carbon (soil) and water. It refers to the concentration ratio of the chemical that is adsorbed in the soil to that in water. It is used to predict the mobility of the organic contaminants; higher the log K_{OC} values correlate to less mobile organic chemicals.

Under these circumstances, accumulated PFCs will eventually affect the terrestrial and aquatic ecosystems to quite a large extent. This is being illustrated by the biomagnification and bioaccumulation trends that can occur in the environment. Therefore to completely understand the effects of the contamination or toxicity of PFCs in our environment, it is necessary to begin from a single key food source or organism. In addition, dietary uptake, in terms of liquid and food intake, is still the main source of exposure of PFCs in human[83]. In this chapter, PFOA and PFOS are chosen as the main compounds of research interest. The accumulation, impacts and fate of both compounds in the aquatic environment are investigated in greater detail using the aquatic microorganism – green microalgae, *Chlorella vulgaris*

Another equally important research focus is to find a method to ultimately remove these toxic pollutants. In order to do so, one such method is to use an organism which these compounds will accumulate in and can be disposed easily. The concept of bioaccumulation and bioremediation can then be utilized and applied in this context by using *Chlorella vulgaris*. In addition, this aquatic organism can served as a bio-indicator or a bio-monitoring tool for PFCs detection in the environment.

2.1.1 Introduction of green microalgae (Chlorella vulgaris)

Chlorella vulgaris is a freshwater unicellular microalga. As shown in the microscopic image (Figure 2.1), it is green, spherical in shape and measure about 2 to 10μm in diameter. The chemical composition of this green microalga consists mainly of protein, followed by lipids, carbohydrates, nucleic acids and other components[151-153]. For the cell structure, *Chlorella* species either have (a) a single microfibrillar layer or (b) two microfibrillar layers and a mono or trilaminar outer layer. *Chlorella vulgaris* has very similar structural components compared to a plant cell and it consists of a single electron-dense algae cell wall layer and it grows to be sturdier and thicker as it matures. Its rigid cell wall basically serves to protect its internal components from extreme conditions. In the exponential growth phase, the cells tend

to disperse (Figure 2.1) due to the negatively charged surface on the algae cells[154]. *Chlorella vulgaris* have been cultivated in laboratories and also commercially for many purposes such as biofuels[155-160], wastewater treatment[155,157,161,162] nutritional and pharmaceutical purposes[155,163] and also for cosmetic applications[153,154,158].

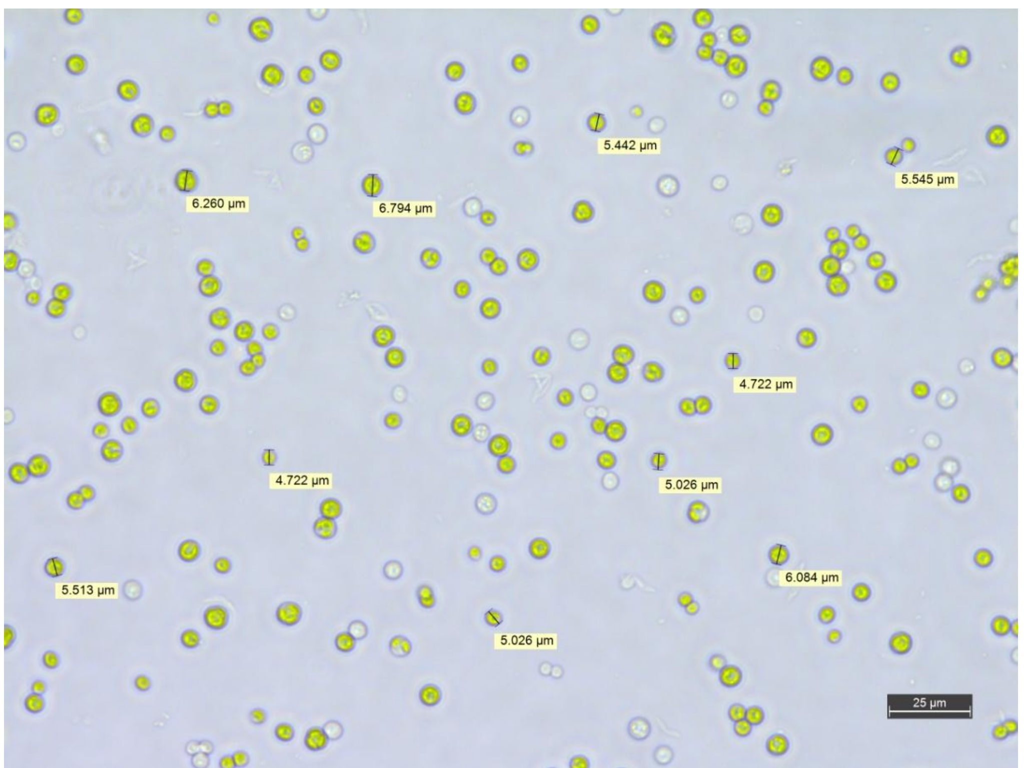


Figure 2.1 Microscopic image of green microalgae *Chlorella vulgaris*

This species of green freshwater microalgae, Chlorella *vulgaris* is selected as the biological model for this project, primarily due to it being the key trophic source of the aquatic food chain. Zooplanktons and rotifers such as *Daphnia magna* feed on *Chlorella vulgaris*, while bigger aquatic organisms feed on these zooplanktons and rotifers[164]. Apart from being a key source in the aquatic food chain, *Chlorella vulgaris* also has the ability to remove toxins from their own biological body[162,165,166]. In addition, it is known to be able to bioaccumulate and eliminate heavy metals, hydrocarbons, insecticides and pesticides in the environment[167-170]. Another positive factor of *Chlorella vulgaris* is that it can be cultivated easily and can multiply at a

rapid rate by autosporulation. There are many commercial methods to cultivate this microalga such as in ponds and in bioreactors. In addition, it can be used as a biomonitoring device and act as a bioindicator for several pollutants. The fate and biomagnification patterns of PFCs in freshwater and aquatic eco-systems are not well-studied[171]. Therefore to aid in this investigation, microalgae *Chlorella vulgaris* is used.

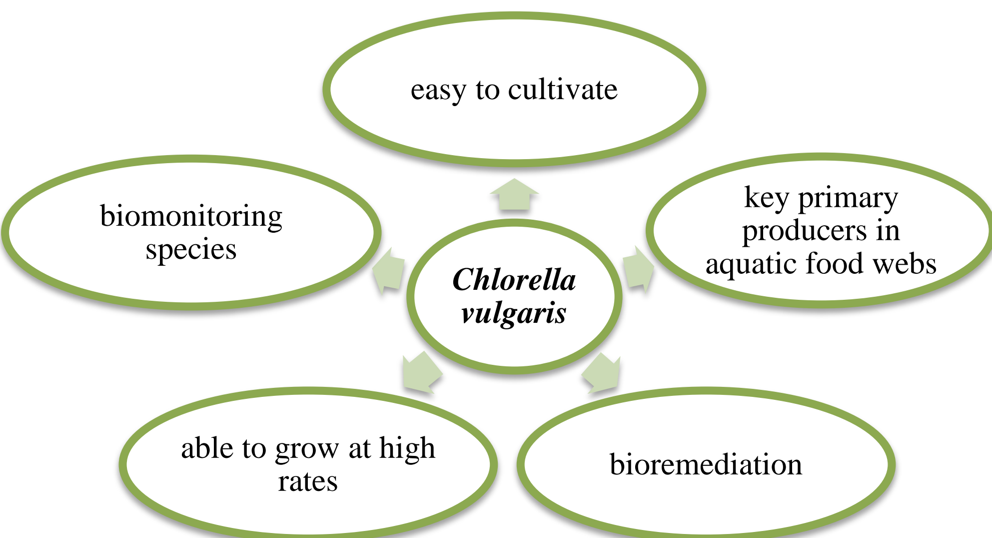


Figure 2.2 Positive characteristics of *Chlorella vulgaris*

2.1.2 Toxicity of PFCs in green microalgae

Presently, research on the fate of PFCs and how it is going to affect the aquatic environment (fresh and seawater conditions) is somewhat limited, inconclusive and not well-studied enough. To start off this research direction, toxicity tests are usually conducted to actually determine whether these compounds do cause significant harmful impacts or not. Further tests can then be conducted to investigate the toxic effects and their implications in depth.

There are several toxicological studies performed on aquatic microalgae. In particular, Boudreau et al investigated the toxic effects of PFOS on *Chlorella vulgaris* by performing acute algae growth inhibition tests and obtained LC_{50} and no observable effect concentration (NOEC) values (Table 2.2)[172]. Based on the results

obtained by Boudreau et al, cell density and chlorophyll analysis can be used to determine the toxicity of pollutants to *Chlorella vulgaris*.

Table 2.2 PFOS toxicity test values for *Chlorella vulgaris*[172]

Endpoint	EC_{50} or LC_{50} (mg/l) (Confidence interval)	NOEC value (mg/l) (Confidence interval)
Cell Density	81.6 (69.6, 98.6)	8.2 (6.4, 13.0)
Chlorophyll	88.1 (71.2, 104)	9.6 (7.6, 16.5)

Other microalgae and aquatic invertebrates were also tested for PFOS toxicity. In another study in 2009, Latala et al investigated the acute toxicity effect of PFCAs (from 7 to 9 carbons) to three microalgae of the Baltic Sea. EC_{50} (also can be known as LC_{50} in this context) is the effective/lethal concentration where 50% of the studied population is dead after specified test duration. The smaller these values, the more toxic these contaminants are. These values were all obtained for the individual PFCAs for all 3 microalgae, including *Chlorella vulgaris*. It indicated that the longer the carbon chains for PFCAs, the smaller the EC_{50} values for these algae are.

Pablos and her research group have recently investigated the acute and chronic toxicity effect of PFCs and other contaminants in mixtures in *Chlorella vulgaris* and *Daphnia magna*. Toxicity tests on these aquatic organisms (e.g. growth inhibition, immobilization, reproduction and feeding) have been carried out. However, more results need to be obtained to draw conclusions for synergistic effects for PFOS, if any. Other than *Chlorella vulgaris*, other research groups have tested PFCs toxicity on other aquatic organisms. Ding at el has studied the toxicity of several PFCs and their effects on aquatic plants (lettuce) and aquatic green algae *Pseudokirchneriella subcapitata*. It was reported that long-chains PFCs cause more significant toxic impacts on the growth of the roots of the lettuce and the photosynthetic ability of the green algae, with the exception of perfluorobutanoic acid[173]. Similarly, the longer the carbon chains are in PFCs, the smaller the EC_{50} values for this aquatic plants are, with the exception of perfluorobutanoic acid.

In another study, the toxicity of PFCs have been examined on freshwater rotifer *Brachionus calyciflorus*[34]. An acute toxicity test using short-chain PFCAs was performed with this freshwater organism. On the contrary to results that long-chains PFCs are more toxic, Wang et al recorded the growth and also the morphological results upon a 3-day exposure and concluded that the shorter the chain of PFCAs, the toxicity increases.

In summary, it is evident that PFCs does accumulate, cause retardations in growth and toxic impacts to several aquatic plants and organisms. The toxic effect will depend on the lengths of the chains of PFCs and it may vary depending on the organisms and the parameters and studies performed. As a result, the toxicological studies of PFCs in the aquatic environment need to be studied in greater detail to eventually determine the fate and environmental impacts of PFCs.

2.1.3 Aim of study

In this study, the fate of PFOA and PFOS when exposed to green microalgae *Chlorella vulgaris* during a 4 day acute exposure will be investigated in greater detail. A range of concentrations of PFOA and PFOS will be spiked individually into the algae culture. Upon sample collection and extraction, LC-MS/MS techniques will be utilized to determine and quantify the amount of PFCs in the algae and culture medium. It is hope that these results will provide a deeper understand of how the PFCs affect aquatic organisms and serve as a starting point for further studies and applications.

2.2 Materials and methods

2.2.1 Chemicals and reagents

Perfluorooctanoic acid, PFOA (95% purity) and heptadecafluorooctane sulfonic acid potassium salt, PFOS (98% purity) were purchased from Alfa Aesar (Ward Hill, MA, USA). Isotope-labelled PFOA and PFOS internal standards, perfluoro-n-[1,2,3,4-$^{13}C_8$]octanoic acid (MPFOA) and sodium perfluoro-1-[1,2,3,4-$^{13}C_4$]-octanesulfonate (MPFOS) were both purchased from Wellington Laboratories (Guelph, ON, Canada). Individual stock solutions of PFOA and PFOS are prepared using 96:4% (vol/vol) methanol (LCMS grade): ultrapure water. All PFCs stock solutions were freshly prepared and stored at -20°C in polypropylene tubes. Further dilutions of individual stock solutions and mixture solutions were prepared from the individual stock solutions and diluted with ultrapure water (Smart2Pure, Thermo Scientific, TKA). All PFCs standard solutions were prepared freshly when required and stored at 4°C in polypropylene bottles and tubes.

Chemicals used in formulating the algae growth medium – Bold's Basal Medium (BBM) as described by Andersen[166,174,175] were purchased from Sigma

Aldrich (St. Louis, MO, USA). Composition of BBM was followed closely with only slight modifications to fit the cultures in the laboratories. Deionized water (Smart2Pure, Thermo Scientific, TKA) was used to prepare all nutrient solutions and growth culture medium. BBM was first sterilized by autoclaving before use. pH and conductivity were all measured.

Ammonium acetate (≥ 98%) and ammonium formate (≥ 97%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Ultrapure water (Smart2Pure, Thermo Scientific, TKA) was used to prepare the mobile phases used in liquid chromatography-mass spectrometry. Methanol, dichloromethane and acetonitrile, both of LC-MS grades, were purchased from Sigma Aldrich (St. Louis, MO, USA). For pH adjustment, hydrochloric acid (37%) and sodium hydroxide (≥ 97%) from Sigma Aldrich (St Louis, MO, USA) were used.

2.2.2 *Microalgae Chlorella vulgaris cell culture*

Figure 2.3 *Chlorella vulgaris* algae batch culture in Bold's Basal Medium (BBM)

Bacteria-free *Chlorella vulgaris* was obtained from Carolina Biological Supply Company (North Carolina, USA). This freshwater green alga was transferred and cultured in sterile Bold's Basic Medium (BBM) at 24°C and pH 7.0 with a photoperiod of 18:6 hours in a lighting incubator GPX-350BP (Huike Electronics Co. Ltd., Yang Zhou, China)[166,174,175]. All glassware, materials and culture medium were sterilized before use. Air was pumped in to the culture through a 0.2μm filter and a sterile pipette to prevent algae sedimentation as shown in Figure 2.3. In addition, swirling of cultures was done twice daily to ensure homogenous culture conditions. Cultures were allowed to grow to required cell density before further use. For bulk volume/quantity usage, algae were cultured in individual bottles first and mixed together before experiment to ensure homogeneous and unbiased cultures.

2.2.3 Determination of growth phase, UV-vis and cell count method

Cell densities of *Chlorella vulgaris* were determined by two different methods which are by manual cell counting and by UV-Vis optical density procedure. In this study, manual cell counting of *Chlorella vulgaris* was performed using a microscope and a Glass-Superior Marienfeld Hemocytometer of Neubauer chamber.

Algae cell samples were first fixed with a small volume of formaldehyde then mixed and diluted to the appropriate value so as to have about 20-40 cells in each hemocytometer counting chamber. This is to ensure that there are no overcrowding and also sufficient numbers of cells to give a reliable data and they do not overlap each other. Detailed procedure for manual cell counting using a hemocytometer is described by Lavens and Sorgeloos[176]. In order to test variability of the method, a technical replicate is performed in which the cell counts were repeated until two values within ± 5 were obtained. Biological replicates, where multiple samples of the same types of cells were tested using the same method, were conducted.

Due to the time-consuming and laborious manual counting technique, UV-Vis optical density method was proposed where it utilized the wavelength of chlorophyll in alga. Using BBM as a reference, a diluted *Chlorella vulgaris* culture was scanned from 190-1100nm using a Shimadzu UV 1800 spectrophotometer. Two wavelengths of highest intensity (at 442.5nm and 682.5nm – mainly that of chlorophyll) were selected for quantification (cell density) of *Chlorella vulgaris*. Several *Chlorella vulgaris* cell densities obtained by manual counting were correlated with the values obtained from UV-Vis optical spectrometer.

A total of nine *Chlorella vulgaris* suspensions of different cell densities and a blank, each with 3 biological replicates were prepared and measured by manual cell counting and UV-Vis spectrometer. A calibration curve of UV-Vis reading at 442.5nm and 682.5nm against cell density was obtained. From the results obtained, the relationship between the UV-Vis optical values and the cell density obtained through manual cell counting, within the working range, is linear. Therefore UV-Vis optical density method at 442.5nm and 682.5nm was adopted for the fast and reasonably accurate method to determine cell density. Cell density of *Chlorella vulgaris* have to be in the range of that in the standard curve. In order to do so, it was diluted to the required range by diluting it with BBM. However, it is important to take note that viewing the cells through a microscopy is still necessary to determine the condition of the cells which are not observable by UV-Vis spectrometer.

2.2.4 Experimental design and workflow

The overview of the workflow for this study is as shown in the figure below:

1 **Experimental design & execution**

- *Chlorella vulgaris* algae cultures
- PFCs (PFOA & PFOS) was spiked into cultures separately
- Concentration & time study
  - 4 day acute exposure PFOA/PFOS study
  - [PFOA]: 0-200ppm
  - [PFOS]: 0-50ppm

2 **Sample collection & preparation**

- Collection of algae
- Collection of medium
- Extraction of PFCs

3 **Sample analysis & results interpretation**

- Analysis of PFOA and PFOS in algae and medium after a 4 days acute exposure study
  - using LC-QTRAP-MS (MRM)
- To determine the fate of PFCs

Figure 2.4 Overview of workflow to investigate fate of PFCs in *Chlorella vulgaris*

Chlorella vulgaris was first cultured in six similar 2-litres bottles to the required cell density with the setup as described in section 2.2.2. Cell density was determined by UV-Vis optical density procedure and also by cell count before proceeding on further. *Chlorella vulgaris* was mixed thoroughly and distributed equally in the test bottles labeled A - F. Individual PFOA and PFOS concentrations were then spiked into these algae growth cultures separately as shown in Tables 2.3 and 2.4. pH of the growth medium of the control (Bottle A) and spiked PFC solutions (Bottles B – F) before algae inoculation were determined to be in the range of 6.6 – 7.

With careful planning and execution, pH is kept constant to ensure that it is not the factor contributing to any subsequent effect observed (e.g. toxicity).

Table 2.3 Concentrations of PFOA spiked in the respective treatments

Bottle/Treatment	A	B	C	D	E	F
Concentration of PFOA (ppm)	0	10	25	50	100	200

Table 2.4 Concentrations of PFOS spiked in the respective treatments

Bottle/Treatment	A	B	C	D	E	F
Concentration of PFOS potassium salt (ppm)	0	1	5	10	25	50

A 4-day concentration-time study was planned such that the effect of concentration and also time on the exposure of PFCs to *Chlorella vulgaris* can be investigated. 500ml of algae will be collected at the following time point of 0 (immediately at time of exposure), 24, 48, 72 and 96 hours for all treatments or concentrations. In total, there are 30 different samples of *Chlorella vulgaris* exposed to each PFOA and PFOS as shown in Table 2.5 and 2.6 respectively. The whole set of experiment was repeated thrice for both PFOA and PFOS.

Table 2.5 List of samples for PFOA-spiked *Chlorella vulgaris*

0ppm / 0hr	0ppm / 24hr	0ppm / 48hr	0ppm / 72hr	0ppm / 96hr
10ppm / 0hr	10ppm / 24hr	10ppm / 48hr	10ppm / 72hr	10ppm / 96hr
25ppm / 0hr	25ppm / 24hr	25ppm / 48hr	25ppm / 72hr	25ppm / 96hr
50ppm / 0hr	50ppm / 24hr	50ppm / 48hr	50ppm / 72hr	50ppm / 96hr
100ppm / 0hr	100ppm / 24hr	100ppm / 48hr	100ppm / 72hr	100ppm / 96hr
200ppm / 0hr	200ppm / 24hr	200ppm / 48hr	200ppm / 72hr	200ppm / 96hr

Table 2.6 List of samples for PFOS-spiked *Chlorella vulgaris*

0ppm / 0hr	0ppm / 24hr	0ppm / 48hr	0ppm / 72hr	0ppm / 96hr
1ppm / 0hr	1ppm / 24hr	1ppm / 48hr	1ppm / 72hr	1ppm / 96hr
5ppm / 0hr	5ppm / 24hr	5ppm / 48hr	5ppm / 72hr	5ppm / 96hr
10ppm / 0hr	10ppm / 24hr	10ppm / 48hr	10ppm / 72hr	10ppm / 96hr
25ppm / 0hr	25ppm / 24hr	25ppm / 48hr	25ppm / 72hr	25ppm / 96hr
50ppm / 0hr	50ppm / 24hr	50ppm / 48hr	50ppm / 72hr	50ppm / 96hr

UV-Vis measurements at 442.5nm and 682.5nm were conducted for all the samples to enable fast determination of any cell death or cell growth retardation. In

the following sections 2.2.5 to 2.2.7, the sample collection, preparation and analysis steps are described in detail.

2.2.5 Sample collection

At the respective time points, 500ml of algae sample were isolated from each treatment. The algae biomass and liquid medium were then separated by centrifuging at 3000g at 4°C for 10 minutes. The liquid medium was then poured into another centrifuging tube and underwent a more vigorous centrifuging at 8000g at 4°C for another 10 minutes. The aqueous medium was then kept in 4°C fridge till PFCs extraction as described in section 2.2.6.2. Algae biomass obtained were then washed with ultrapure water and undergo the same centrifuging procedure twice. The algae biomass were flash froze by liquid nitrogen before placing it in a freeze dryer overnight (Labconco, Freezone 4.5 – 105°C). Dried algae biomasses were then stored in -30°C freezer (Sanyo, Biomedical Freezer, MDF-U5312) till weighing and sample extraction.

2.2.6 Extraction of PFCs

2.2.6.1 Extraction of PFCs in green microalgae

Dried algae sample was first homogenized and mixed thoroughly. 2.0ml of each dichloromethane and methanol was added to 10mg of homogenized algae biomass. The algae mixture was vortexed for a minute and sonicated for 10 minutes in ice. The mixture is then centrifuged at 10 000g for 10 minutes at 4°C. The supernatant was then pipette and placed into a new polypropylene tube. The extraction procedure was repeated and the combined supernatant was centrifuged twice at 19 000g for 5 minutes at 4°C. The required volume of supernatant was added with internal standard and then evaporated to dryness using nitrogen gas. The extract is reconstituted using 96:4% (vol/vol) methanol:water and is ready for injection to HPLC-QTRAP-MS for analysis.

2.2.6.2 Extraction of PFCs in aqueous culture medium

10ml of methanol was added to a fixed volume of culture medium. The liquid mixture was vortexed, sonicated in ice then centrifuged at 5000rpm for 5 minutes. 0.1ml of methanol extracts and internal standard were then evaporated to dryness using nitrogen gas. The extracts were then reconstituted using an appropriate volume of 96:4% (vol/vol) methanol:water and injected to HPLC-QTRAP-MS for quantification. To ensure accurate determination, the aqueous culture medium was prepared and analyzed for PFCs within 2 weeks upon sample collection.

2.2.7 Quantification using HPLC-QTRAP-MS

Liquid chromatographic separation was performed using a 2.1mm x 150m with particle size 3.5μm Agilent C_{18} Eclipse XDB column on a Dionex Ultimate 3000 HPLC system. The mobile phase consisted of (A) 2.5mM ammonium acetate and (B) methanol. The LC gradient program used was as indicated in Figure 2.5.

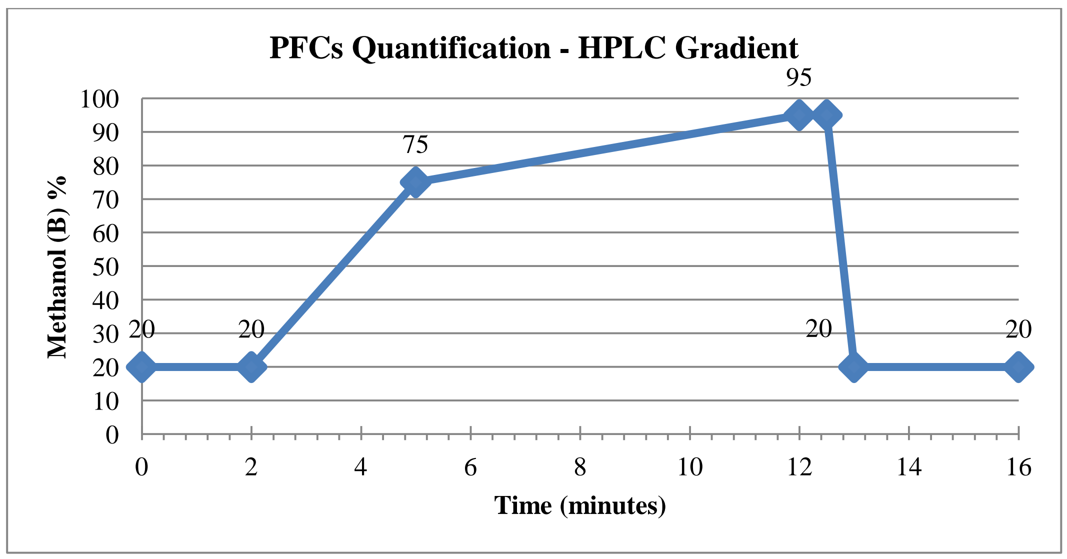


Figure 2.5 Liquid chromatography gradient program for PFCs quantification

The flow rate was set to be 0.3ml/min with the sample injection volume to be 10μl. The temperature of the column oven and the auto-sampler was programmed to be at 35°C and 20°C respectively. Reagent blanks and prepared blanks were analyzed

together with all standards and samples. They were analyzed randomly and blanks were included to ensure an unbiased sample analysis.

Mass spectrometry was performed using the AB Sciex QTRAP® 5500 system in the multi-reaction monitoring (MRM) scans – negative mode. Both PFOA and PFOS MS analyses were performed at the optimized source and compound parameters. The optimized source parameters used were 30psi for nebulizer (GS1), 55psi for heater (GS2) gas, 30psi for curtain gas (CUR), heater temperature (TEM) at 550°C and ion spray voltage (ISVF) at -4500V for negative mode. Compound optimization was performed in order to obtain the m/z values of the parent and daughter ions (quantifier and qualifier ions) and the optimal MRM parameters which consist of dwell time, collision energy (CE), declustering potential (DP), entrance potential (EP) and collision cell exit potential (CXP). These parameters were all optimized specifically for each analyte.

Samples were analyzed randomly and blanks were included to ensure an unbiased sample analysis. The MRM mass chromatogram peaks of the PFCs were viewed, integrated and extracted using the PeakView or MultiQuant AB Sciex program software.

2.2.8 Microscopic images of Chlorella vulgaris

10µl of formalin was added to each 190µl C*hlorella vulgaris* sample. 20µl of well-mixed algae sample was then placed on a microscopic plate for imaging. The Leica DM IL LED microscope at 40x magnification and digital camera Leica DFC 450C was used. The microscopic images of *Chlorella vulgaris* were obtained at Day 0, 4 and 21 for both PFOA and PFOS-spiked algae.

2.3 Results and discussion

2.3.1 Relating cell density using UV-Vis optical density method and manual hemocytometer cell count – constructing calibration curve

A UV-visible absorbance scan of a *Chlorella vulgaris* suspension in BBM shows the 2 highest absorbance values at 442.5nm and 682.5nm. This is mainly due to the chlorophyll present in *Chlorella vulgaris* which also have similar absorbance values of chlorophyll a. A total of 10 *Chlorella vulgaris* suspensions of different cell densities including a blank, each with 3 biological replicates were prepared and measured by manual cell counting and UV-Vis spectrometer at 442.5nm and 682.5nm. The UV-Vis absorbance values were plotted against the corresponding cell density obtained through manual cell counting in Figure 2.6 (442.5nm) and Figure 2.8 (682.5nm). Based on the curves shown, saturation of this method is observed to be starting at about $30x10^6$cells/ml for both wavelengths. It is clearly indicated by the red data points. Therefore only 8 points were obtained for the constructing of the linear optical density calibration curve.

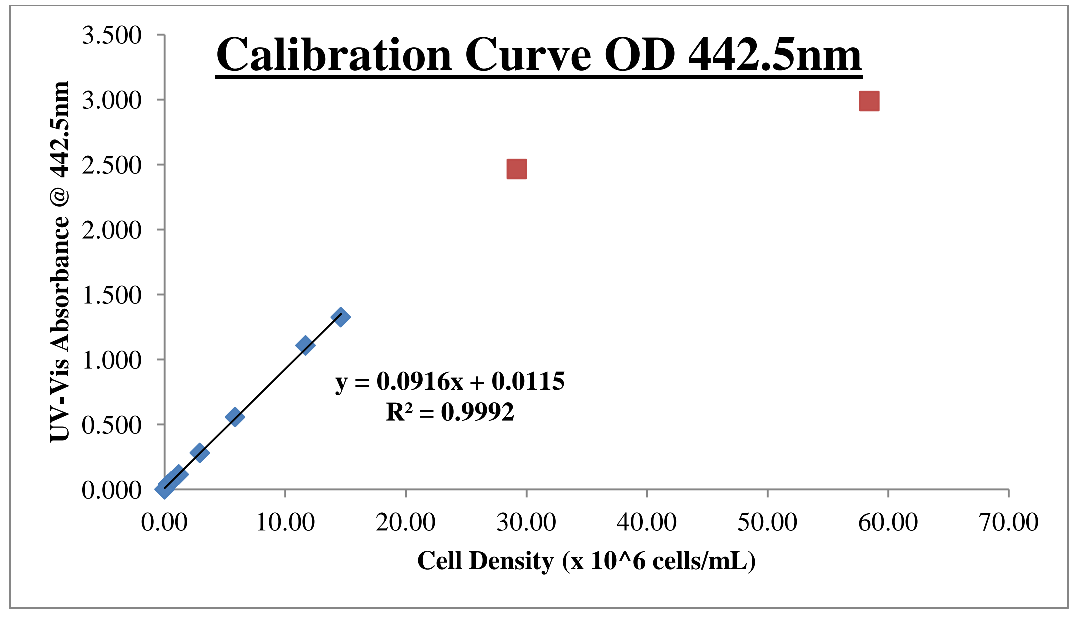


Figure 2.6 Graph of UV-Vis absorbance values at 442.5nm against the corresponding cell density of *Chlorella vulgaris*

The finalized calibration curves and equations used for 442.5nm and 682.5nm are shown in Figure 2.7 and 2.9 respectively.

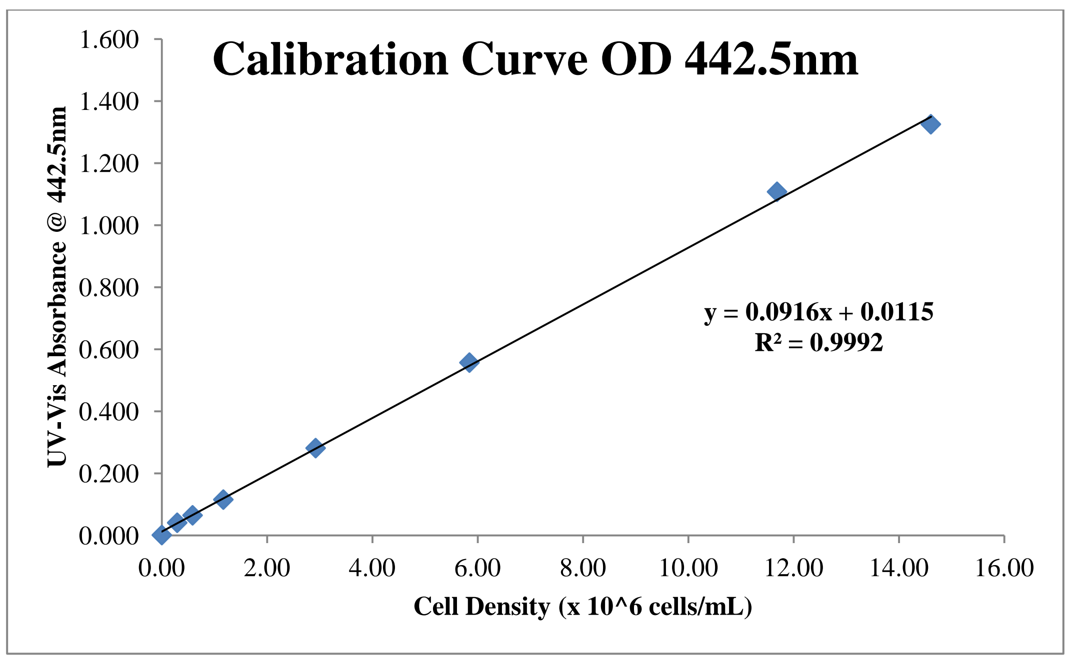


Figure 2.7 Optical density calibration curve of *Chlorella vulgaris* at 442.5nm

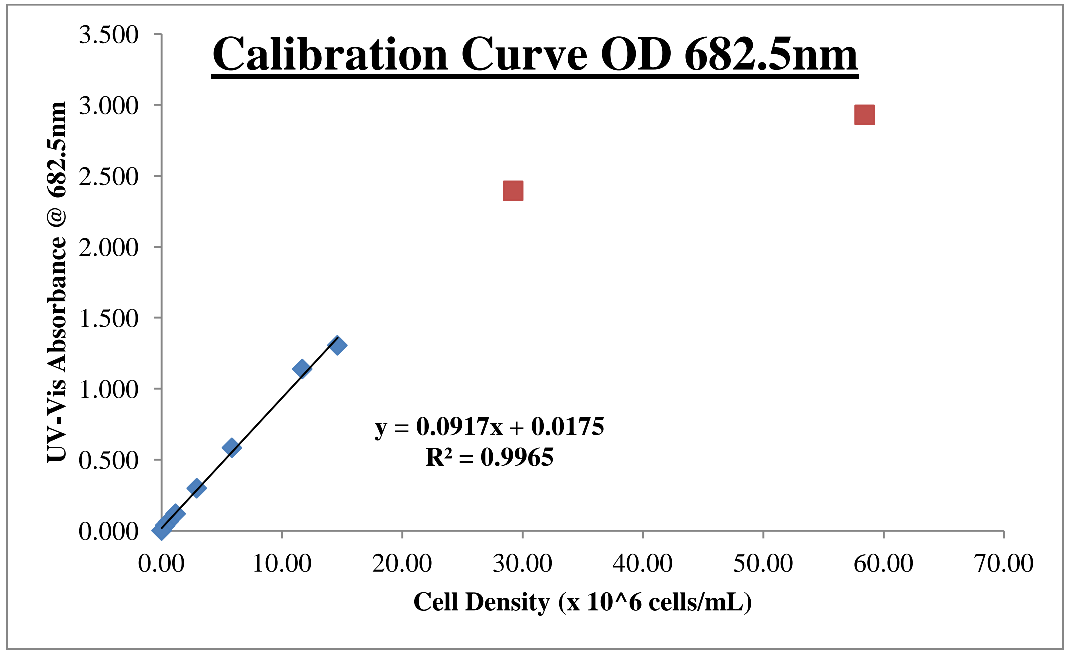


Figure 2.8 Graph of UV-Vis absorbance values at 682.5nm against the corresponding cell density of *Chlorella vulgaris*

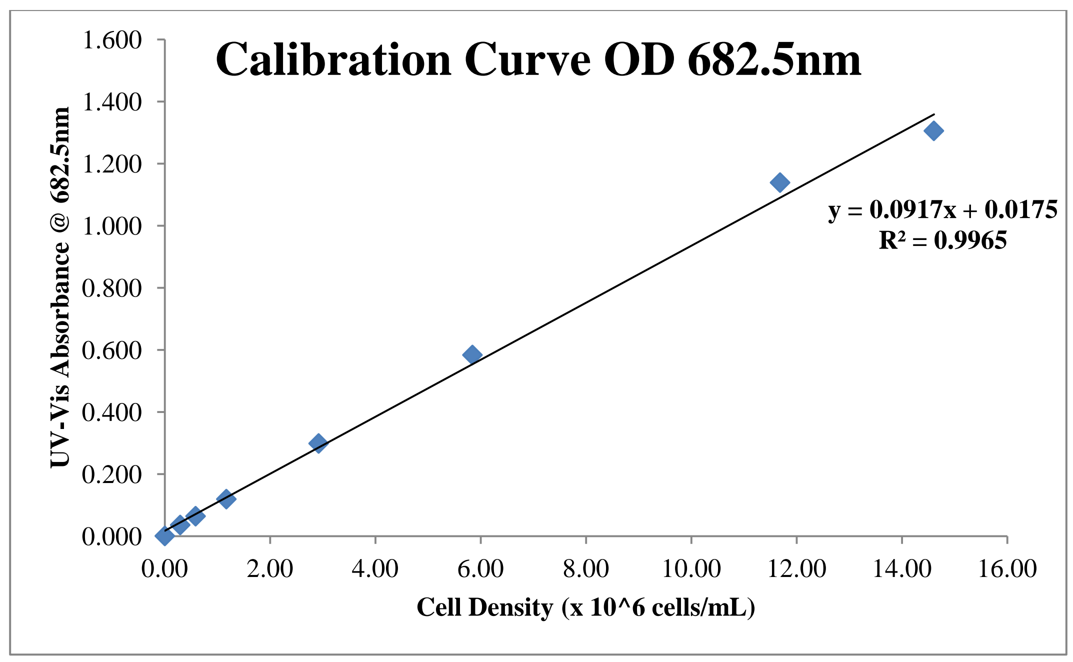


Figure 2.9 Optical density calibration curve of *Chlorella vulgaris* at 682.5nm

The error bars are all included in the calibration curves but are not prominent due to the small values obtained. Therefore UV-Vis optical density method at 442.5nm and 682.5nm was adopted for the fast and reasonably accurate method to determine cell density of *Chlorella vulgaris*.

2.3.2 Determination of growth phase to use

Chlorella vulgaris was cultured based on the conditions stated in Section 2.2.2. Its growth dynamics and cell density was monitored using the UV-Vis optical density method described in Section 2.3.1 for faster, easier and accurate measurements. Following which, a graph of cell density against age of culture was plotted in Figure 2.9. The cell density of this specific alga was cultured 3 times separately in identical conditions. Each of the cultures' growth dynamics resembles are similar and resembles the growth phase as indicated by Lavens et al[176]. This growth dynamics phase for microalgae such as *Chlorella vulgaris* generally begins with (1) induction, followed by (2) exponential, (3) declining growth rate and finally (4) stationary[176].

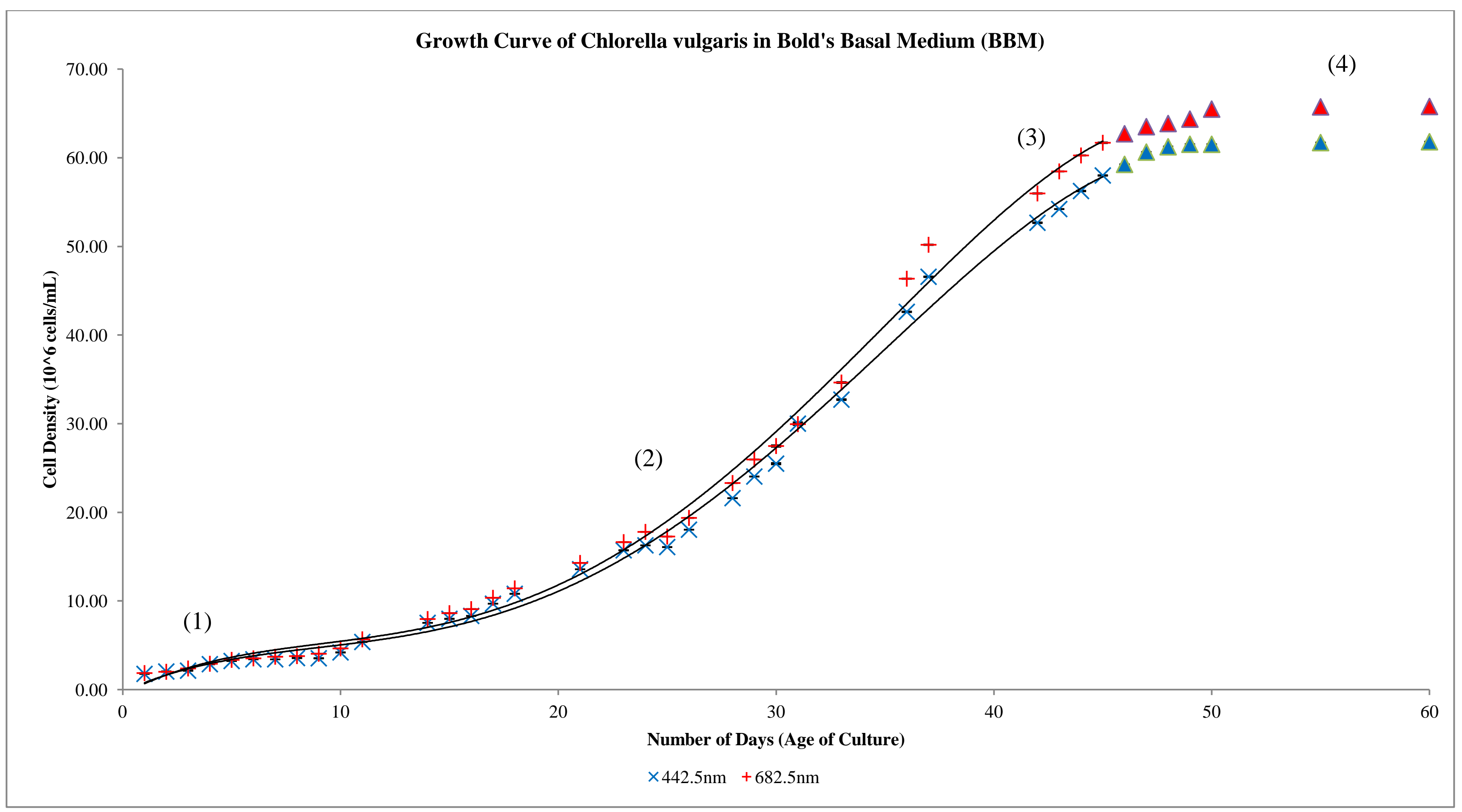


Figure 2.10 Growth dynamics for *Chlorella vulgaris* – different phases, (1) Induction, (2) Exponential, (3) Declining and (4) Stationary

The growth phase depicted in Figure 2.10 is slightly longer than the typical growth period in the literature, with an entire growth period of about 30 days. This is mainly due to a longer induction where it depends on the starting cell density. Cultures inoculated with a higher cell density or with more exponentially growing algae generally have a shorter induction period. Exponentially growing cells adapts more rapidly and are able to grow and divide rapidly.

For this *Chlorella vulgaris* culture, it reaches a stationary plateau of slightly more than 60 million cells per ml in a course of 60 days. Based on the growth dynamics curve (Figure 2.9), it will generally take about 2-3 weeks after inoculation to reach the exponential phase and it will take another 2 weeks to reach the declining growth phase. In this study, *Chlorella vulgaris* in the exponential phase were selected due to the possibility of having inferior algae after the 2nd phase (declining and stationary). In these stages, the algae growth slows down and may produce toxic metabolites which are not favorable and not required for this intended study[176]. Before proceeding forward to perform experiment, the cell density was determined and ensured that from the start to the end of the study, the cell density of the growing cultured algae are in the same growing dynamics phase which is in the exponential phase. This is done to ensure that there are no other possible factors affecting the different metabolites of the algae and the metabolites evolving is not due to the change in growth dynamics phase.

2.3.3 UV-VIS – assessment of the effect of PFCs on cell viability

With regards to the concentration ranges of PFOA and PFOS used in this study, it is well-noted that the concentrations are much higher than those found in the aquatic environment which is about at parts-per-billion (ppb) range. These concentrations are selected because PFCs are persistent in the environment and

bioaccumulation occurs resulting in a higher concentration detected. In addition, at the source of pollution or contamination, the concentration determined at the site may be close to what is being used in this study or even higher. The effect is then diluted due to the volume or size of the aquatic environment. Therefore the actual effect and fate of high concentration PFCs to this alga are still sought after. So it is necessary to study the effect of PFCs in this range of concentrations stated to better evaluate the effects and it can provide a starting point for further studies and applications.

For each PFOA and PFOS concentration-time study, all collected samples as indicated in Table 2.5. and 2.6 were analyzed for cell densities and viability using the optical density UV-VIS method illustrated in Section 2.3.1. The results were plotted and shown in Figures 2.11 and 2.12 (PFOA) and Figures 2.13 and 2.14 (PFOS).

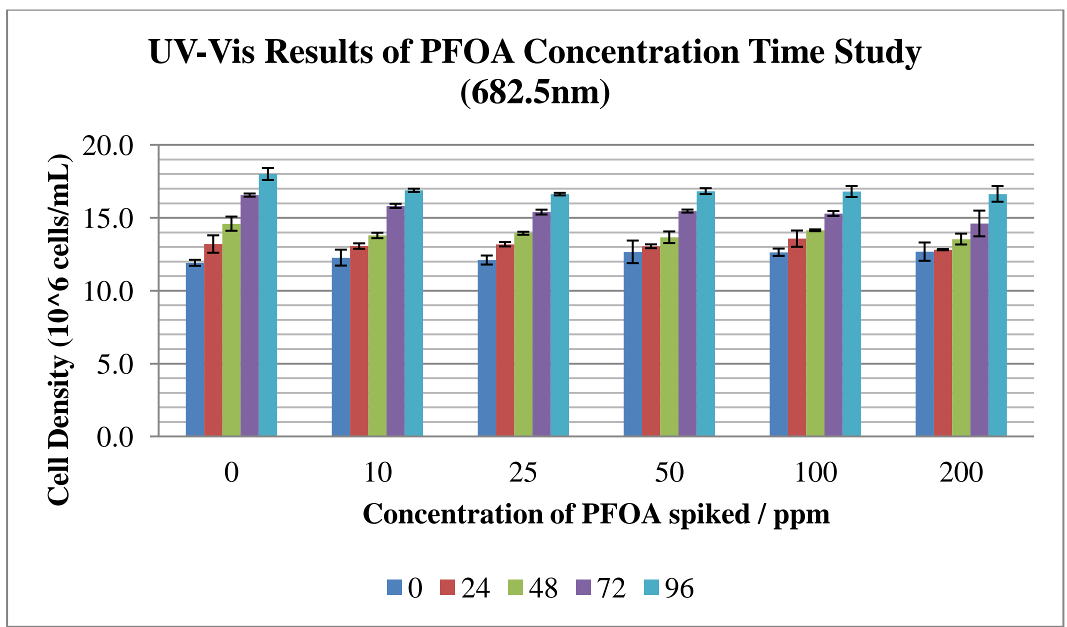


Figure 2.11 Cell densities of *Chlorella vulgaris* (UV-Vis at 682.5nm) in PFOA concentration-time study

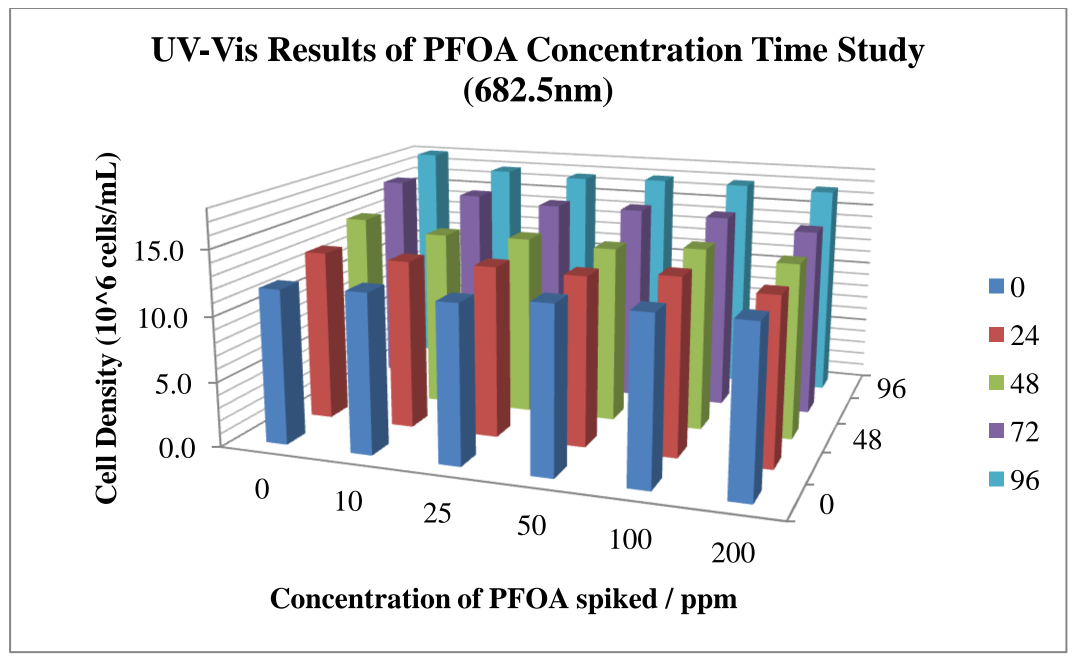


Figure 2.12 3D-graphical representation of cell densities of *Chlorella vulgaris* (UV-Vis at 682.5nm) in PFOA concentration-time study

A 3-dimensional graphical representation of the plots (Figures 2.12 and 2.14) are shown to better visualize and compare the cell densities between the different concentrations of PFCs and also at different time points.

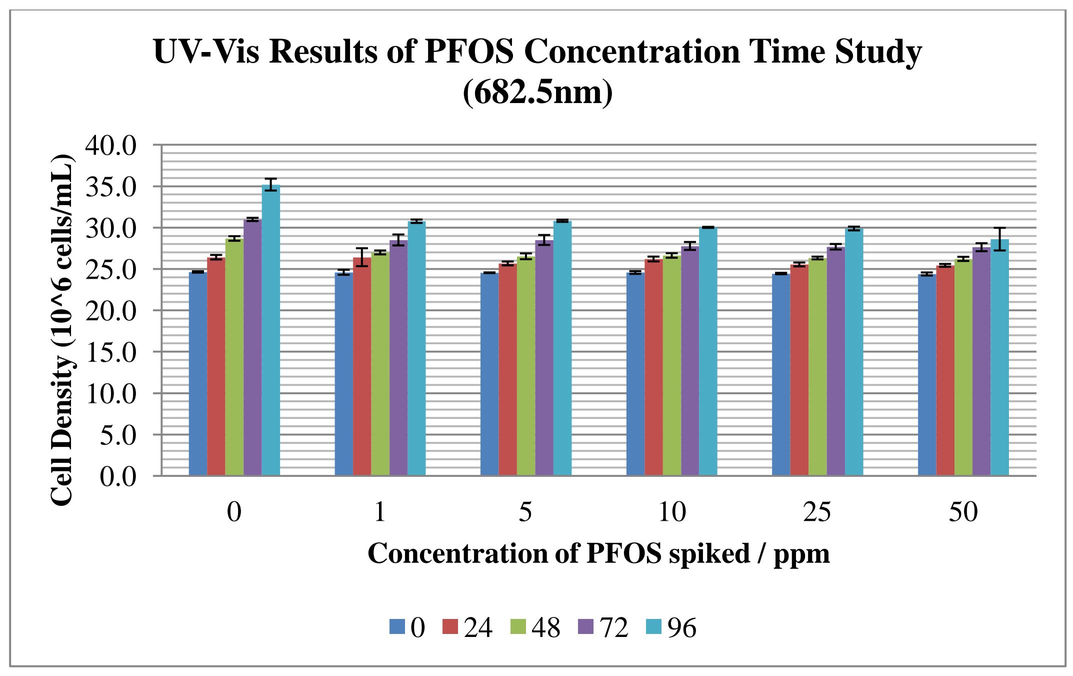


Figure 2.13 Cell densities of *Chlorella vulgaris* (UV-Vis at 682.5nm) in PFOS concentration-time study

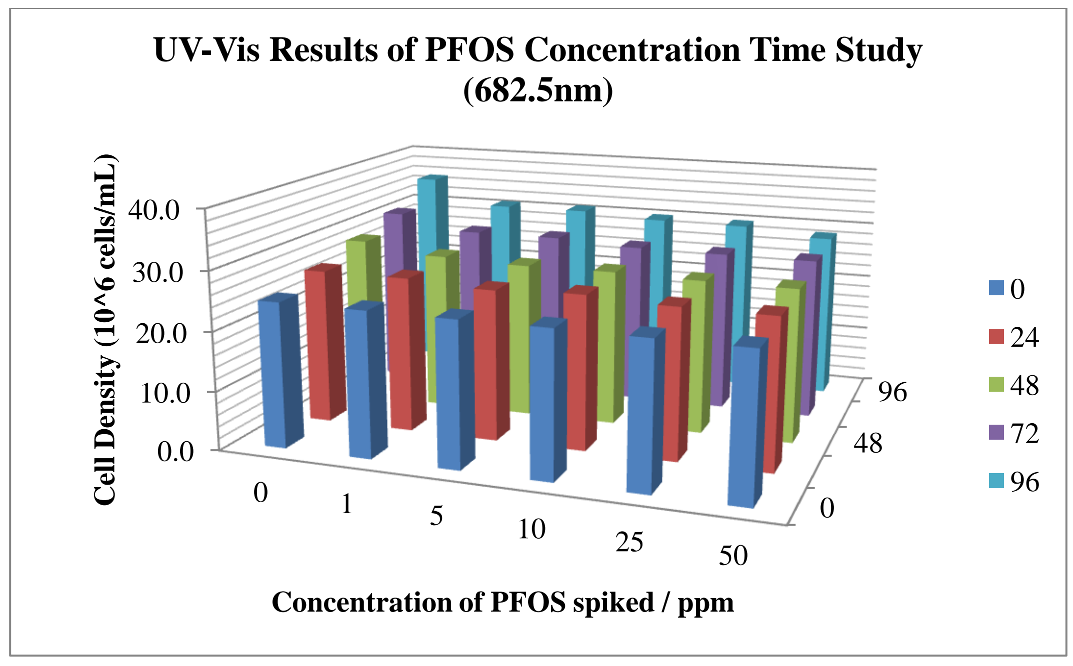


Figure 2.14 3D graphical representation of cell density of *Chlorella vulgaris* (UV-Vis at 682.5nm) in PFOS concentration-time study

From these observations, PFOA and PFOS, 2 of the most toxic PFCs are clearly affecting the *Chlorella vulgaris* cultures in these studies. It is observed in both concentration-time studies, the cell count and cell growth is delayed upon increasing concentration of PFCs and also upon increasing period of PFCs-exposure. However, in both studies, despite the acute exposure of high concentration of PFCs, the algae are not affected to a large extent and have at least 80% surviving alga cells after the 4-day study. PFOS shows a bigger impact on the green microalgae compared to PFOA based on the percentage of surviving cells of the PFCs- exposed compared to the control.

As mentioned in several literatures, PFOS tend to have a greater impact on organisms compared to PFOA[177-181]. Similarly, this is also observed here. PFOA and PFOS accumulation have been compared in organisms such as earthworms[182], rotifier[183] and human cells[184], where results indicating PFOS being more toxic. Wen et al have determined that PFOS have a higher degree of uptake compared to PFOA in earthworms. Likewise, Midgett et al determined that PFOS have shown to suppress

specific growth and production in human cell lines. Also, Ding et al investigated that PFOS is more toxic than PFOA in zebrafish and they went on to investigated the combined effects of PFOA and PFOS together, resulting in PFOS being the major contributor to toxicity[185]. Most importantly, a group of researchers have determined the effect of several PFCs on another freshwater alga species and observed that PFOA did not inhibit growth at all while PFOS suppressed algae growth and the extent increases as concentration increases.

In all, this UV-VIS optical density method of determining cell density presents a fast technique to evaluate the cell quality and growth. However, there are limitations to this method such as its specificity, sensitivity and the information it provides. With regards to these surviving cells that are observed, more insights and information needs to be obtained and it is covered in the next section – microscopic images of cultures.

2.3.4 Microscopic images of PFC-spiked Chlorella vulgaris

Images of *Chlorella vulgaris* cell cultures from day 0 to 4 were taken but only little differences were observed under the microscope. Following which, the cultures of both PFOA and PFOS-spiked *Chlorella vulgaris* were kept even further to 21 days for imaging. This further shows the acute and chronic visible effects of PFCs to the algae cells. Significant visible differences can be spotted when comparing these images as shown in Tables 2.7 (PFOA) and 2.8 (PFOS).

Chlorella vulgaris cells which are not spiked with PFCs are imaged as sample A of each section and also Figure 2.1. From these images, these algae cells can be observed to be spherical in shape, have sizes about 2-10μm and are evenly spaced or dispersed.

Table 2.7 Microscopic images of PFOA-spiked *Chlorella vulgaris* at Day 4 and 21

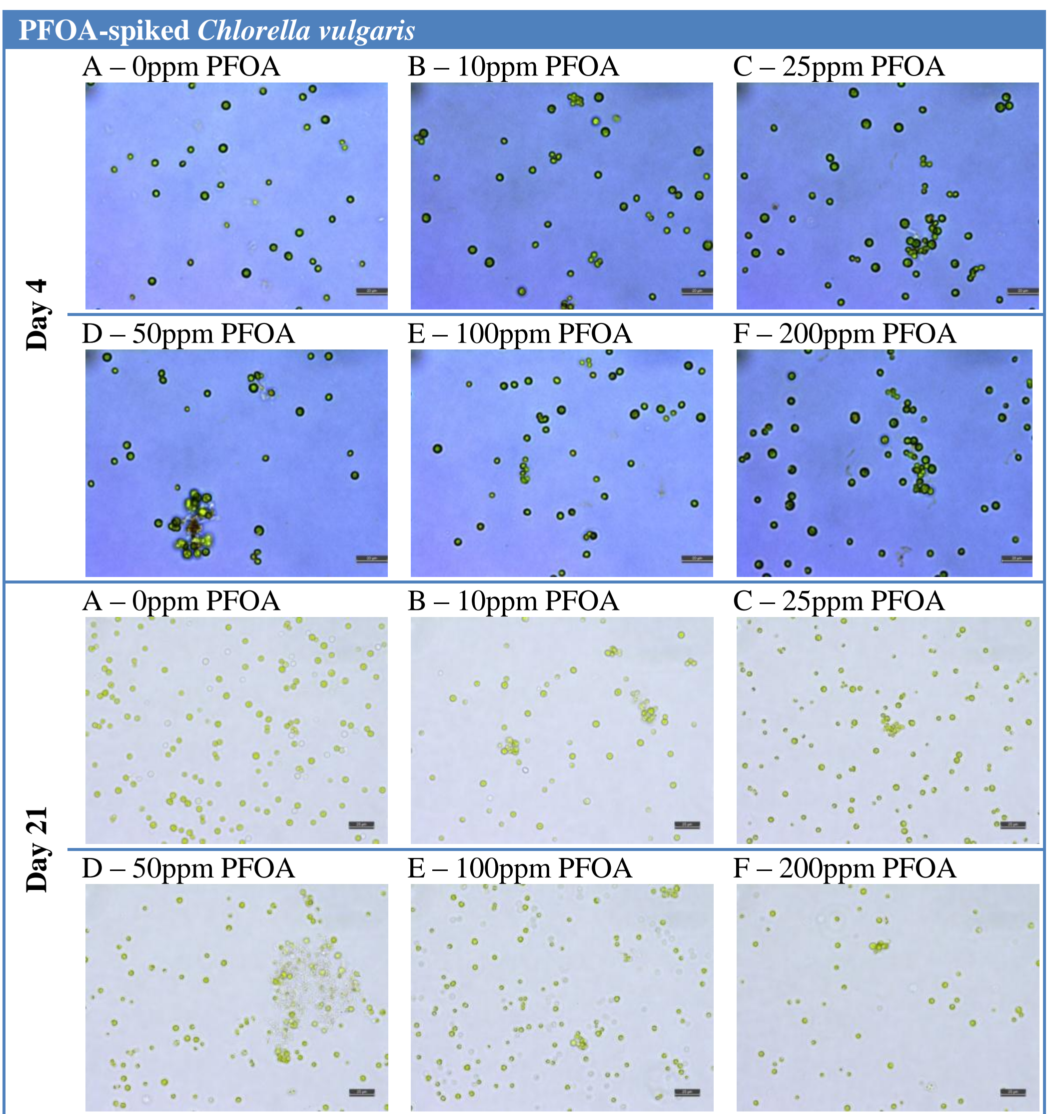
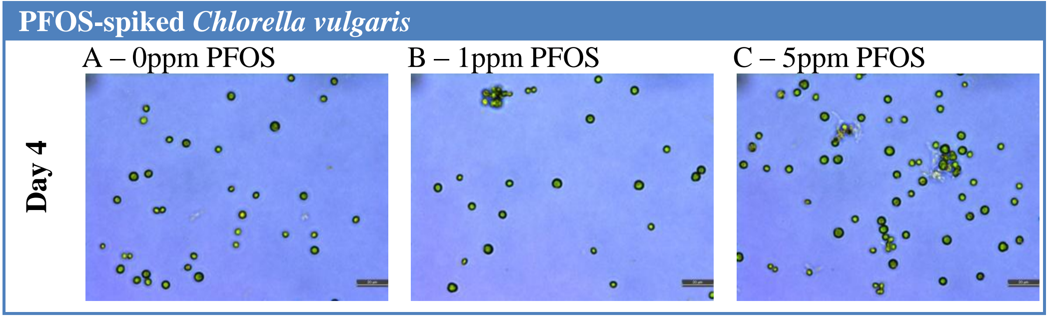
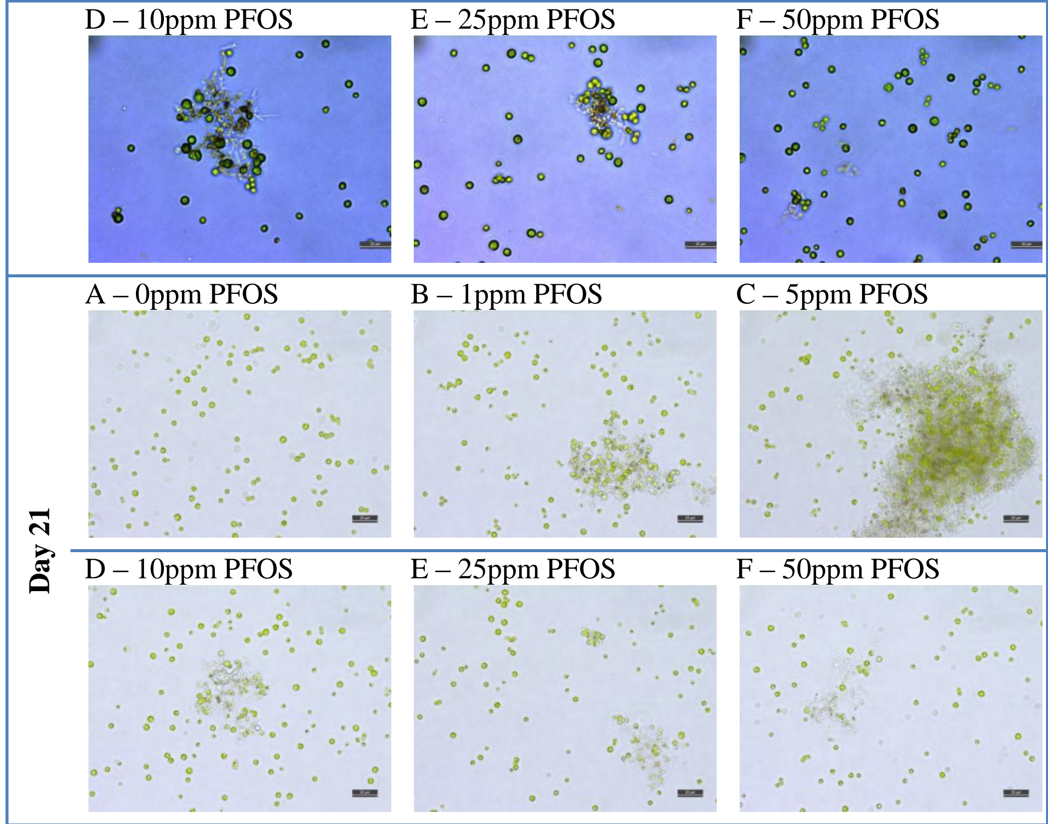


Table 2.8 Microscopic images of PFOS-spiked *Chlorella vulgaris* at Day 4 and 21





However, upon exposure to different concentrations of PFOA and PFOS for several periods of time, changes are observed such as the sizes and shapes of the cells, coagulation and the existence of other foreign masses. The cells tend to aggregate together and form clumps as shown predominantly in Day 21 – Sample C (5ppm PFOS) in Table 2.8. From these observations, these clumps form when PFCs concentrations are high and also when the period of exposure prolongs. Also, the spherical cells may change to elongated shapes upon exposure to PFCs. These formations are mainly suspected to be due to the cells having either reached the stationary phase or the declining phase resulting in this auto-flocculation phenomenon[146,152]. In the presence of these PFCs contaminants in the aquatic culture medium, these algae cells are in a very undesirable condition and have died or deteriorated in health and quality as compared to the control (not spiked with PFCs)[154].

In addition, it is suspected that PFCs adhere onto the algae cell wall upon contact and causes severe disruptions to the cell wall surfaces. This further proves that PFCs is the major reason of the clumps formation and shape change. Similarly as mentioned by Liu et al[186], PFCs does change and affect the algae's wall surface properties which can be observed by the coagulation of the algae cells and shape change. This occurs even at low concentration which will suppress algae growth (as shown in Section 2.3.3). Therefore it strongly presents the point that PFCs have an effect on the algae even before the occurrence of growth inhibition and its cell wall surface properties are being compromised and thus affected the optimal conditions of algae.

Moreover, *Chlorella vulgaris* tends to possess a stronger structure where an indigestible cellulose is the main constituent in the cell wall[154]. One such example will be to compare the cell strength of *Chlorella vulgaris* and the gram-negative Cyanobacteria[187]. Furthermore, Latala also concluded that different species of algae may have different responses to PFCs accumulation and toxicity. It is speculated to be mainly due to the differences in cell wall components, structures and cell sizes. With a thicker cell wall, it resulted in lower permeability to PFCs and less intrusion effect observed. Therefore, in an acute exposure, PFOA and PFOS are suspected only to be able to adsorb on the surface of the algal cell wall-water interface via both ionic and hydrophobic interactions. This eventually causes it to accumulate on the surface and effectively disrupts the integral algae cell wall. With the evidences from the surface properties, it is evident as to why the *Chlorella vulgaris* cells have such a high rate of 80% surviving even after a high dosage of PFCs in an acute study. After prolonged PFCs exposure and strong interferences, PFCs may cause the death of cells and also cause the breaking of cell walls.

The effect of PFOS on *Chlorella vulgaris* is more pronounced as compared to that of PFOA. The severity is more evident may be due to the structure of PFOS and

chemical interaction and bonding with the algae cell surface which resulted in more surface interaction and disruptions. Based on the images shown, these algae cells are still mainly green in color. They will be picked up by the UV-VIS optical density method due to the chlorophyll present and are not classified as dead (without chlorophyll). However, by referring to the shape and the coagulation, the quality and conditions of these cells are not optimal. These observations are observed in the previous UV-Vis optical density method. So with regards to the cell viability, there may be lesser surviving/optimal cells for the PFCs concentration-time study. Therefore in order to understand better the fate of PFCs upon acute exposure to green microalgae *Chlorella vulgaris*, both algae and culture medium samples are being isolated and analyzed for PFOA and PFOS individually and will be discussed in the next section.

2.3.5 PFCs extraction techniques for algae and medium

Several articles have reported extraction and determination of PFCs in different organisms and aqueous medium using methods such as liquid extraction followed by clean-up step and also other techniques such as solid phase extraction (SPE)[54,55,65,66,188-195]. In this study, the algae and culture medium samples were prepared and extracted using small volume solid-liquid or liquid-liquid extraction. For PFCs extraction in dried algae biomass, the method described in Section 2.2.6 were obtained from Yoo et al with some modifications to fit the sample size and mass in this study[196]. Optimizations for the extraction procedure were performed with regards to the variables such as the sample mass, solvent volume and the number of times of extractions.

For the PFCs extraction in aquatic medium, the method described in Section 2.2.6 were obtained from several similar samples sources[197,198] and also used with

slight modifications to fit the appropriate sample matrix, composition and size. Extraction efficiencies/recoveries were tested for both methods using PFOA and PFOS spiked samples and standards. Three different ranges of concentrations (which includes low, medium and high) for the algae biomass and culture medium were evaluated for the extraction recoveries. The recoveries of analytes were all determined to be within the acceptable range of 80% to 120% before proceeding forward with the actual samples. The samples were then analyzed using the LC-QTRAP-MS method (Section 2.2.7).

2.3.6 LC-QTRAP-MS standards preparation and method development

Analyses of sample extracts were performed using a HPLC-QTRAP-MS. MS optimization were first performed to obtained the essential MRM parameters such as the identity and mass/charge of the precursor and product ions, collision energy (CE), accelerator cell voltage and other parameters listed in Section 2.2.7. After obtaining these MS MRM parameters for each of the individual analytes, the LC gradient method were optimized further to obtain the best possible separation based on several literatures[62,65,66,189,199-203]. Since MRM LC methods were utilized, baseline separations for all the analytes were not required. Among the product ions obtained, 3 were chosen and 1 of which were classified as quantifier while the other 2 are qualifiers. Care was taken to ensure no tailing and fronting of the peaks. The respective values and calibration curves were obtained and tabulated for all PFCs as indicated in Table 2.9.

Table 2.9 Mass spectrometry parameters, regression equations and correlation coefficients for PFCs using HPLC-QTRAP-MS

Analyte	Chemical Formula	Molecular Weight	Precursor Ion	Product Ion#	Retention Time (minutes)	Calibration Curve	R^2
Perfluoropentanoic Acid	C_4F_9COOH	264.0	263.3	218.9	6.37	Y = 0.0212X + 0.0204	0.9998
				68.9			
				63.3			
Perfluorohexanoic Acid	$C_5F_{11}COOH$	314.1	313.6	270.1	6.84	Y = 0.0018X + 0.0044	0.9997
				118.9			
Perfluoroheptanoic Acid	$C_6F_{13}COOH$	364.0	363.4	318.9	7.16	Y = 0.0117X + 0.0204	0.9988
				168.9			
				118.9			
Perfluorooctanoic Acid	**$C_7F_{15}COOH$**	**414.1**	**413.3**	**368.9**	**7.46**	**Y = 0.0190X + 0.1466**	**0.9990**
				168.8			
				218.9			

Analyte	**Chemical Formula**	**Molecular Weight**	**Precursor Ion**	**Product Ion#**	**Retention Time (minutes)**	**Calibration Curve**	**R^2**
Perfluorononanoic Acid	$C_8F_{17}COOH$	464.1	463.3	418.8	7.78	Y = 0.0159X + 0.0575	0.9980
				218.9			
				168.8			
Perfluorodecanoic Acid	$C_9F_{19}COOH$	514.1	513.3	468.9	8.19	Y = 0.0139X + 0.0243	0.9990
				218.9			
				269			
Perfluoroundecanoic Acid	$C_{10}F_{21}COOH$	564.1	563.3	518.9	8.67	Y = 0.0121X + 0.0097	0.9994
				218.9			
				268.9			
Perfluorododecanoic Acid	$C_{11}F_{23}COOH$	614.1	613.3	568.8	9.24	Y = 0.0178X + 0.0093	0.9996
				319			
				168.9			

Analyte	Chemical Formula	Molecular Weight	Precursor Ion	Product Ion#	Retention Time (minutes)	Calibration Curve	R^2
Perfluorotridecanoic Acid	$C_{12}F_{25}COOH$	664.1	663.3	<u>619</u> 318.9 368.8	9.74	Y = 0.0171X + 0.0077	0.9995
Perfluorotetradecanoic Acid	$C_{13}F_{27}COOH$	714.1	713.3	<u>669</u> 218.9 319.1	10.30	Y = 0.0148X + 0.0037	0.9999
Perfluorooctane Sulfonate (Potassium Salt)	**$C_8F_{17}SO_3^-K^+$**	**538.2**	**499.6**	**<u>80.0</u>** **99.0** **129.9**	**7.77**	**Y = 0.0012X + 0.019**	**0.9997**

#Product ions used as quantifiers for each analyte are underlined; the other product ions are used as qualifiers.

The PFCs LC method was completed within 16 minutes with all the analytes being eluted and detected by 11 minutes. However, the elution of PFOS and PFOA in the C_{18} column is very similar due to its comparable interaction with the column. MRM mode in the MS is utilized for this specific reason as

there is another variable that can be used to differentiate these analytes. In addition with the use of the carbon-isotope-labeled internal standard (MPFOA), these three analytes are differentiated based its MRM transitions (precursor → product ions). These transitions are unique to this particular analyte and can be detected accurately by the LC-QTRAP-MS.

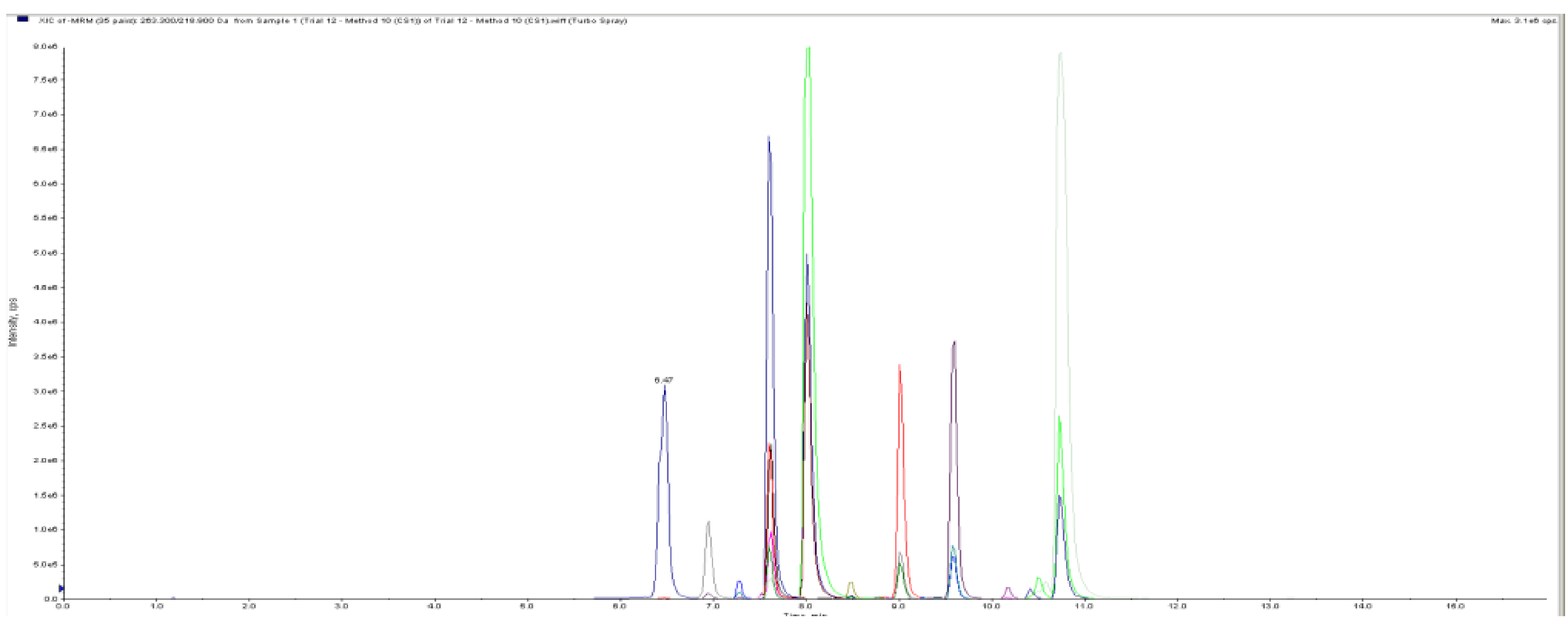


Figure 2.15 Multiple-reaction-monitoring (MRM) chromatogram of the PFCAs and PFOS (with quantifier and qualifier ions)

2.3.7 Bioaccumulation of PFCs in Chlorella vulgaris

With the chromatogram results obtained from HPLC-QTRAP-MS, the quantification data of PFOA and PFOS for the algae and culture medium at the different time periods were obtained and tabulated as shown in Figure 2.16 and 2.17. PFOA and PFOS were determined at the different time points (0, 24, 48, 72, 96 hours upon exposure to PFCs) for the algae biomasses and the liquid culture medium. The main objective of this study is to determine whether PFCs are indeed bio-accumulated in the green microalgae. The information obtained from the culture medium was used to further substantiate and validate this point. Care was taken to ensure that the peak was correlated to the right analyte (e.g. PFOA, PFOS and the IS) by checking that the MRM parameters and fragmentation ions were all input correctly.

Each experiment set was repeated thrice for both the PFOA and PFOS concentration-time study to ensure that the observation of the trend is similar. At least 5 biological replicates were also analyzed per concentration in each experiment set. It is important to note that it is not possible to combine all 3 data set results together to obtain the average as the algae cell density or cell count for each experiment set differs and it will greatly affect the accurate determination of PFCs. However the relationship and trend observed is similar for all 3 experiment sets and follows that of the one shown in the Figure 2.16 and 2.17. One of each PFOA and PFOS study are shown for easier illustration.

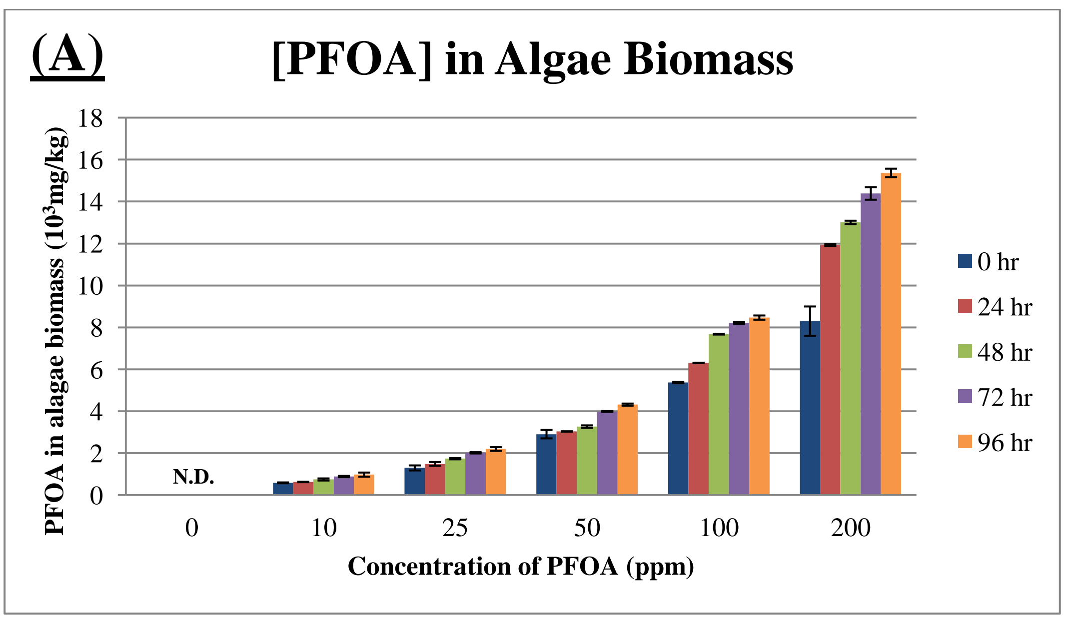
There are a few major observations from these PFCs bioaccumulation results. Firstly, when comparing between different concentrations of PFCs that the algae was initially exposed to, it can be observed that as the spiked PFCs concentrations increase, the quantity of bio-accumulated PFCs detected in the algae biomass also increases. This trend is true for all PFOA concentrations in Figure 2.16 and for all PFOS concentrations in Figure 2.17. The main reasoning behind this is that as

concentration increases, the availability of PFCs increases and there are more molecules available to attach and adsorb itself on the algae surface and eventually bioaccumulates due to poor elimination. These values are in a general upwards trend with a lower rate of increase as time increases. This is suspected to be because initially, PFOA and PFOS are able to adsorb onto the available algae surface in the first few period, resulting in a sharper increase rate and $[PFC]_{algae}$ increases too. However, as time increases, the amount of space available for adsorption decreases due to the limited surface area availability and also equilibrium of PFC between the algae and medium may be established. Secondly when comparing at different time points, the longer the algae is exposed to the PFCs, the higher the concentration of PFCs is detected in the algae biomass. This trend is observed across the x-axis in Figure 2.16 for PFOA and Figure 2.17 for PFOS. This is expected since having a longer time means a longer adsorption and interaction allowed between the algae cells and the PFCs molecules.

From both results obtained, it is evident that the UV-VIS results are consistent with that of the HPLC-QTRAP-MS results. The concentration effect seems to be more significant as compared to the time effect as there are more differences and changes observed. Both PFOA and PFOS have similar bioaccumulation trends and pattern but PFOS does have a larger bioaccumulation potential compared to PFOA. It is suspected that this occurs due to the difference in structures and the possible stronger interaction of PFOS with the algae cells.

These results indicate that PFCs does accumulate either in the algae itself or are adsorbed strongly on the microalgae surface. Therefore to build on the algae biomass results, the PFCs in the culture medium were also isolated, extracted and analyzed. This is performed to determine the remaining amount of PFCs in the medium and to prove that the PFCs are actually being uptake or adsorbed and in turn affecting the algae. The PFCs extracted from the culture medium were analyzed similarly as the

algae biomass. The results obtained are in good agreement with that of the algae biomass.



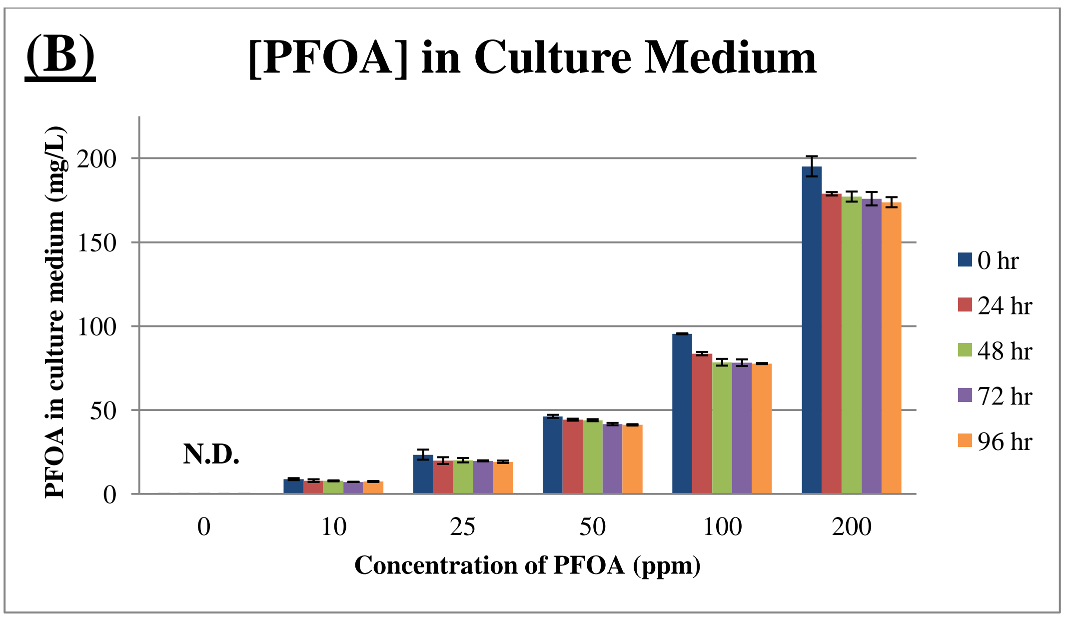
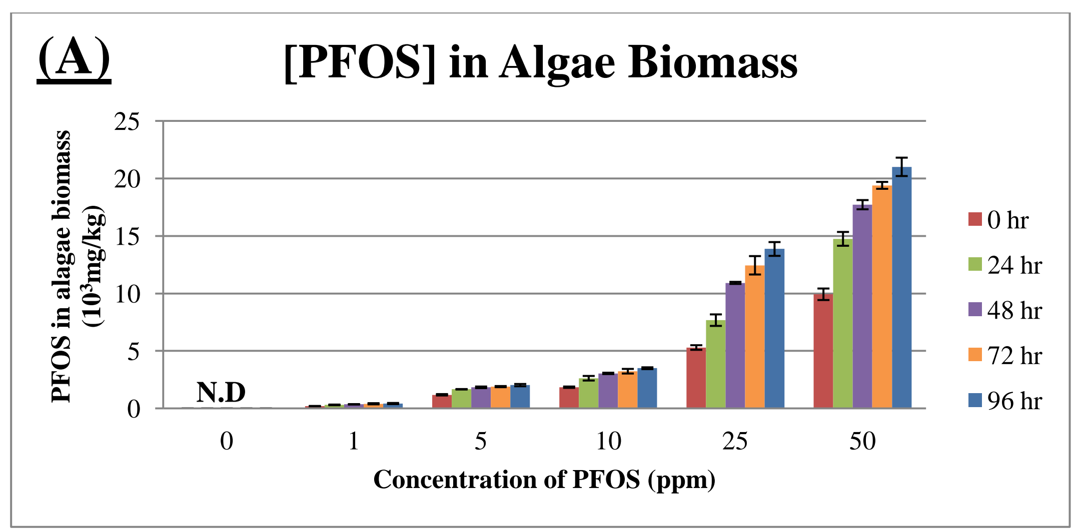
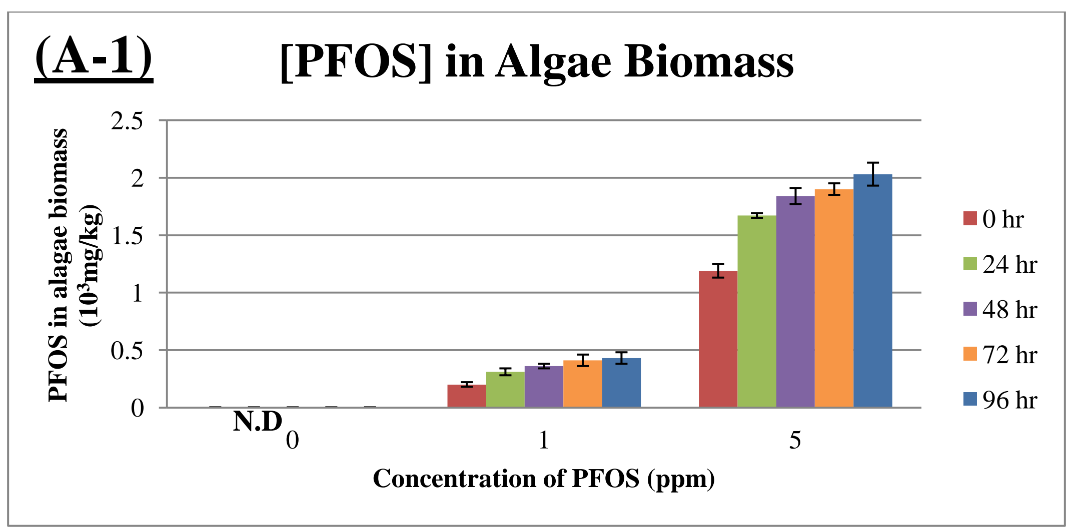
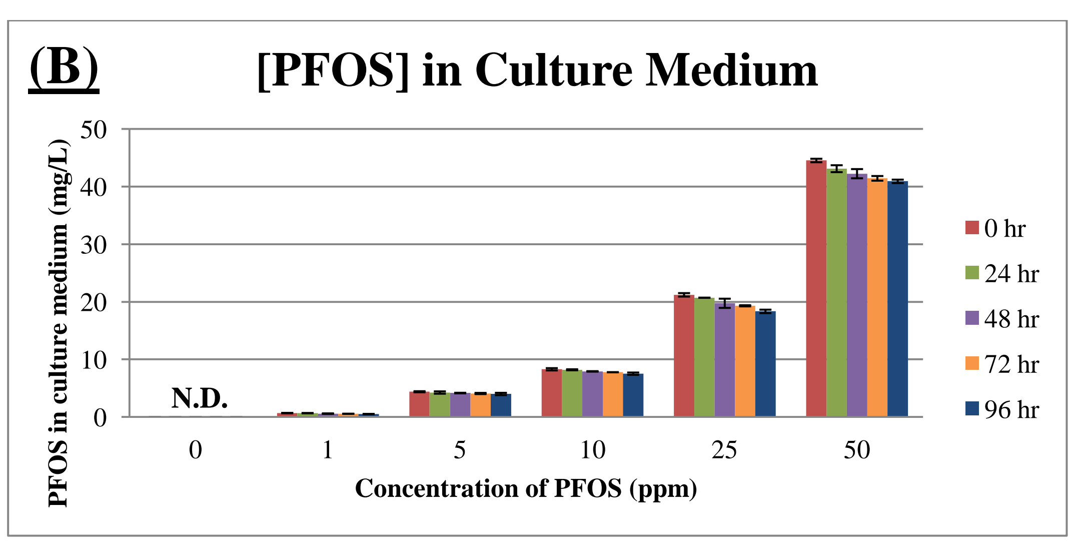


Figure 2.16 Concentration of PFOA detected in algae biomasses and culture medium for concentration-time study - (A) displays the PFOA concentration present in the algae biomass while (B) the PFOA concentrations present in the culture medium. N.D. means not detected.


(A)
[PFOS] in Algae Biomass
PFOS in alagae biomass (10³mg/kg)
25
20
15
10
5
0
N.D
0
1
5
10
25
50
Concentration of PFOS (ppm)
0 hr
24 hr
48 hr
72 hr
96 hr


(A-1)
[PFOS] in Algae Biomass
PFOS in alagae biomass (10³mg/kg)
2.5
2
1.5
1
0.5
0
N.D
0
1
5
Concentration of PFOS (ppm)
0 hr
24 hr
48 hr
72 hr
96 hr


(B)
[PFOS] in Culture Medium
PFOS in culture medium (mg/L)
50
40
30
20
10
0
N.D.
0
1
5
10
25
50
Concentration of PFOS (ppm)
0 hr
24 hr
48 hr
72 hr
96 hr

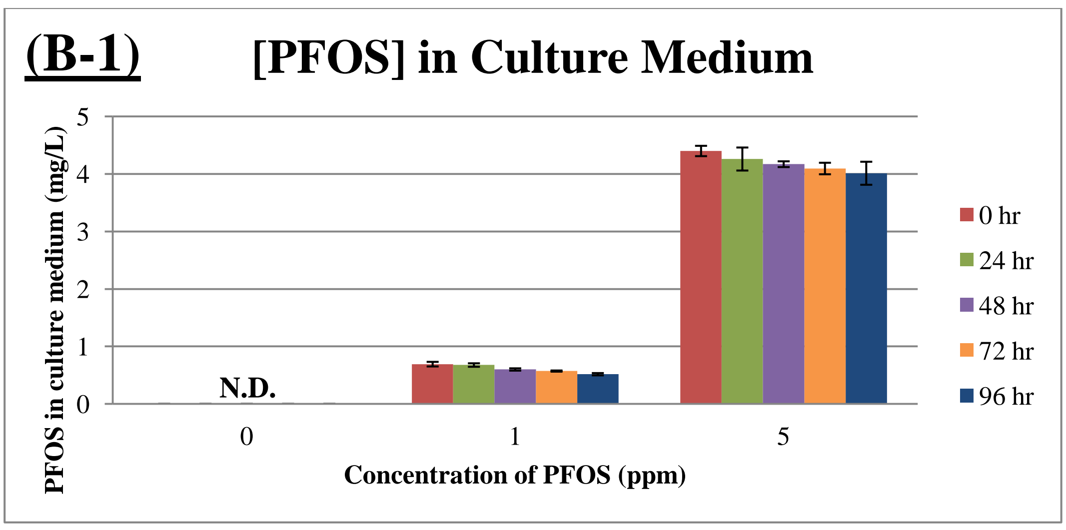


Figure 2.17 Concentration of PFOS detected in algae biomasses and culture medium for concentration-time study - (A) displays the PFOS concentration present in the algae biomass, (A-1) just shows the enlarged version for 0-5ppm PFOS treatment. while (B) shows the PFOS concentrations present in the culture medium. (B-1) displays the enlarged portion for the 0 – 5ppm PFOS treatment. N.D. means not detected.

EC_{50} is defined as the effective concentration for half of the population to have a response to the toxicant where in this context is to die. However, the comparison of EC_{50} values, for example the EC_{50} of PFOA for *Chlorella vulgaris*, may not be that reliable and applicable for comparison across different studies. This value is obtained based on a specific growing cell density, volume, environment and spiked PFOA concentration and these factors and the surrounding environment and apparatus can contribute to the differences in the reported EC_{50}.

Theoretically, the concentration of PFCs detected in the algae biomass and the culture medium should add up to 100%, assuming that there is no PFCs present initially. However, it is noted that these values for each experimental setup do not necessarily add up in actual fact[204]. These values are expected to be less than 100% due to the fact that there are other contributing factors and phases apart from just the algae and medium. As a matter of fact, PFCs have a tendency to adsorb on glass surface which will contribute to some PFCs adsorption. The washing of biomass in

the sample preparation step may also induce some washing off of PFCs that are adsorbed on the algae surface.

These two areas should not be overlooked when considering the possible fate of PFCs in this experiment. Due to the growth of algae and the requirement to place and expose it to light, plastics containers, which have a lower tendency for adsorption to occur, were not employed in the use of the study. Glassware and other glass apparatus were rinsed with ultrapure water and baked at high temperatures before use. Precautions of contamination due to PFCs from the glassware and the environment were taken. Apart from the growth setup where algae needs light, the rest of the apparatus and containers were plastics that do not contribute significantly to the experimental results and contamination.

There have been only a few studies on PFCs bioaccumulation in algae but many on PFCs and their bioaccumulation in aquatic organisms, birds and larger organisms[204]. In the early 2000s, Martin et al studied the bioaccumulation patterns of PFCs in several fishes and invertebrates in the Ontario's aquatic environment[7]. PFCs effect and accumulation was also studied in mussels by Liu et al. Similarly, the results were all concentration-dependent. Xu et al have studied and determined that the trophic magnification factors of several PFCs and also of PFOA and PFOS[205]. Although all these values and amount of PFCs detected in these aquatic organisms were currently not causing any immediate health effects to these organisms and humans who consume these aquatic organisms. However, this needs to be revised.

Importantly, there have been studies on PFCs and its toxicity response in several other algae such as the single-celled green algae *Chlorella pyrenoidosa* and *Selenastrum capricornutum* by Xu et al[206]. Similarly as shown in the results presented in this study, the growth was inhibited due to the presence of PFCs as shown by the chlorophyll wavelength in the UV-Vis results. It was also determined that PFOA does

affect the cell wall permeability (passive transport) of these algae. As a result, accumulation of PFOA on the algae surface does affect the metabolic activity of the algae.

There have been some studies that conclude that PFOA and PFOS show little acute or chronic toxicity to freshwater aquatic organisms such as *Chlorella vulgaris* at the respective environment conditions[207-209]. Although the bioaccumulation is quite low generally for both PFOA and PFOS in *chlorella vulgaris*, the overall eventually effect should not be underestimated. This is because PFCs are not easily eliminated from the algae bodies which results in significant accumulation as time progresses. It is a slow, but nevertheless an ongoing serious process.

It is important to take concern that such effects are accumulating and have a possibility of causing harm and complications following which[205]. This study serves as a good starting point for a PFCs bioaccumulation study for the aquatic food web as *Chlorella vulgaris* is a food source for most aquatic organisms. In addition, from these results, there is a possibility of using *Chlorella vulgaris* as a bioremediator in order to remove these pollutants and contaminants in aqueous environments.

These results strongly present that PFCs are being accumulated in the algae by adsorption on the algae surface and it have a detrimental effect on the algae. As such its growth is inhibited and its cell wall surface properties are being compromised and thus affected the optimal culture conditions of algae.

2.4 Conclusion

PFCs, even at low concentrations, are found to be highly toxic to the environment. These persistent compounds have the ability to cause adverse effects to human health, wildlife and the environment. With its persistent nature and its ability to bioaccumulate through the food web, it is important to study in detail and realize that the bioaccumulations of PFCs in the environment and wildlife are still ongoing and the consequences of it are still largely unclear and very concerning.

Since this green microalga, *chlorella vulgaris*, is a key source of food for aquatic organisms and is essential in the aquatic food web, it is a good starting point for determining the fate of PFCs upon acute and chronic exposure. In this study, with all the analyses and evidences from the UV-vis cell density counts, the microscopic images and the LC-MS/MS quantification results of PFCs in the algae and medium, it is determined that upon PFCs exposure, the algae growth is inhibited and the algae cell wall surface properties are being affected resulting in clumps and aggregations forming. A comprehensive concentration- and time- study of PFOA and PFOS interaction with *chlorella vulgaris* was established and it serves to better understand the fate of PFCs upon contact and exposure to these green microalgae. In conclusion, from this study, it is evident that PFCs does accumulate in the body or on the surface of the green microalgae *Chlorella vulgaris* and it is concentration and time dependent.

In order to further investigate how the PFCs are going to affect the green microalgae and in turn the aquatic environment and those higher up the food chains, the following chapter will investigate more on the metabolomics effect of PFCs on the microalgae.

3 Metabolomics approach for the investigation of the effect of PFCs on *Chlorella vulgaris*

3.1 Introduction

In chapter 2, the effects and fate of PFCs in the green microalgae *Chlorella vulgaris* were investigated. With the intention to explore further, chapter 3 focuses on the use of untargeted metabolomics approach to examine the acute (short-term) bioaccumulation effect of PFOA and PFOS on *Chlorella vulgaris*. The objectives of this study are (a) to determine any changes to the metabolic profile of the microalgae, (b) to identify any possible potential biomarkers and metabolites that can be utilized for early detection or exposure to PFCs in the aquatic environment and (c) to develop a deeper understanding of the possible algal stress and defense mechanism observed upon the acute 96-hour exposure to PFCs, specifically PFOA and PFOS. Altogether, chapters 2 and 3serve to provide a comprehensive study of how PFOA and PFOS affect the green microalgae *Chlorella vulgaris*.

Currently, there has been increasing interest in the metabolomics research using microalgae and also *Chlorella vulgaris* but no studies have been published specifically on PFCs-exposed *Chlorella vulgaris* as of now[165,208,210-215]. In chapter 2 of this thesis, it is revealed that PFOA and PFOS do accumulate in the algae biomass or on the surface of *Chlorella vulgaris* based on the UV-Vis optical density data and LC-QTRAP-MS quantification results. In addition, it also has a detrimental effect on the algae cells as observed by the growth inhibition, cells aggregation and clumps formation in the microscopic images and also cell counts.

For this chapter, untargeted metabolomics approach is thus adopted to identify any possible changes in the algae metabolites upon the acute exposure to PFCs. Both nuclear magnetic resonance spectroscopy (NMR) and reverse-phase liquid chromatography coupled with tandem mass spectrometry in liquid chromatography quadrupole time-of flight mass spectrometry (LC-QTOF-MS) techniques are utilized

to investigate this metabolic effect of PFCs-exposed *Chlorella vulgaris* in the concentration-time studies.

In order to design the experiment specifically for metabolomics studies, planning of the experiment, as discussed in Chapter 1, was conducted carefully and the concentration of PFCs used, duration of exposure and other critical factors were taken into account and executed with care. Specifically, the PFCs concentrations have to be high enough for a significant change to be observed in the metabolites but not too high to incur death to all or most of the cells population. Therefore the concentration used were trialed and carefully chosen to be way below the lethal concentration that causes 50% of the cells population to die (LC_{50}). The acute LC_{50} value of PFOA for similar green microalgae was larger than 400mg/l and the no-observable-effects-concentration (NOEC) was determined to be 12.5mg/l[207]. The metabolites observed upon cell death are not desirable for this study and therefore avoided.

In addition, to make this study applicable and relevant to the real world PFCs contamination situation, a comparison between the actual concentration of PFCs found in the aqueous environment and the concentration used in this algae study was made. PFCs concentration detected in the aqueous environment (oceans, river, drinking water, etc…) are found to be in the range of ng/l to sub-µg/l[2,32,62,67,69,148,149,192,216-219]. It is well noted that the PFCs concentration chosen for this study is much higher than the $[PFCs]_{aqueous\ environment}$. However, it is important to consider that at the source of PFCs pollution, these values may go up to very high concentrations and the overall effect is only reduced due to the mixing and the large volume of the aqueous environment. The resultant $[PFCs]_{aqueous\ environment}$ detected is thus lower due to the mixing and eventual sampling at a site that may be far from the pollution source.

Furthermore, it must be remembered that PFCs bioaccumulates have poor elimination and have a long half-life. With such properties, the concentrations chosen for this experiment are shown below:

$$[\text{PFCs}]_{\text{aqueous environment}} < [\text{PFC}]_{\text{algae in this study}} < LC_{50} \text{ for } \textit{Chlorella vulgaris} - \text{PFCs.}$$

Therefore this chapter aims to detect any possible significant metabolite changes and to identify any potential biomarkers upon PFCs exposure with the use of LC-QTOF-MS and NMR techniques.

3.2 *Materials and methods*

The groundwork for this study is similar to the one described in Chapter 2. However, to understand even more and investigate further the effect of PFCs on the green microalgae, metabolomics techniques were employed.

Figure 3.1 Overview of workflow to investigate the metabolomics effect of PFCs on *Chlorella vulgaris*

The experimental design, setup, sample collection and harvesting are similar to the study as described in Chapter 2 (Section 2.2.4 and 2.2.5). The green microalgae samples analyzed in both chapters are identical. They are from the same sample sets and are homogenized before proceeding with their respective sample preparation and analyses. This ensures that there exists a correlation between these studies and also to

obtain a clearer understanding about the concentration-time impacts of PFOA and PFOS individually on *Chlorella vulgaris*. The dried algae biomasses obtained were then utilized in this study to further investigate the effect of PFCs on algae using metabolomics techniques. For this study, algae metabolites were extracted and analyzed using HPLC-QTOF-MS and NMR.

3.2.1 Chemicals and reagents

The chemicals and reagents used are similar to those described in Chapter 2 (Section 2.2.1). Apart from those chemicals, chemicals which are specifically used for NMR sample preparation and analyses are 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS), deuterium oxide (D_2O, 99.9% D), sodium phosphate (NaH_2PO_4) and disodium phosphate (Na_2HPO_4). All were purchased from Sigma Aldrich (St. Louis, MO, USA). NaH_2PO_4 and Na_2HPO_4 were used to prepare the phosphate buffer while DSS is used as a chemical shift reference and also an internal standard for NMR analyses.

3.2.2 Sample preparation

A 96-hour (4-day) concentration-time study on the exposure of PFCs to *Chlorella vulgaris* was conducted. The study parameters are identical to that in chapter 2. The concentrations investigated are listed in Tables 2.3 and 2.4. 500ml of algae were collected at time points of 0 (immediately), 24, 48, 72 and 96 hours for all treatments/concentrations. In total, there are 30 different samples of *Chlorella vulgaris* exposed to each PFOA and PFOS as shown in Tables 2.5 and 2.6 respectively. The whole set of experiment was repeated thrice for both PFOA and PFOS.

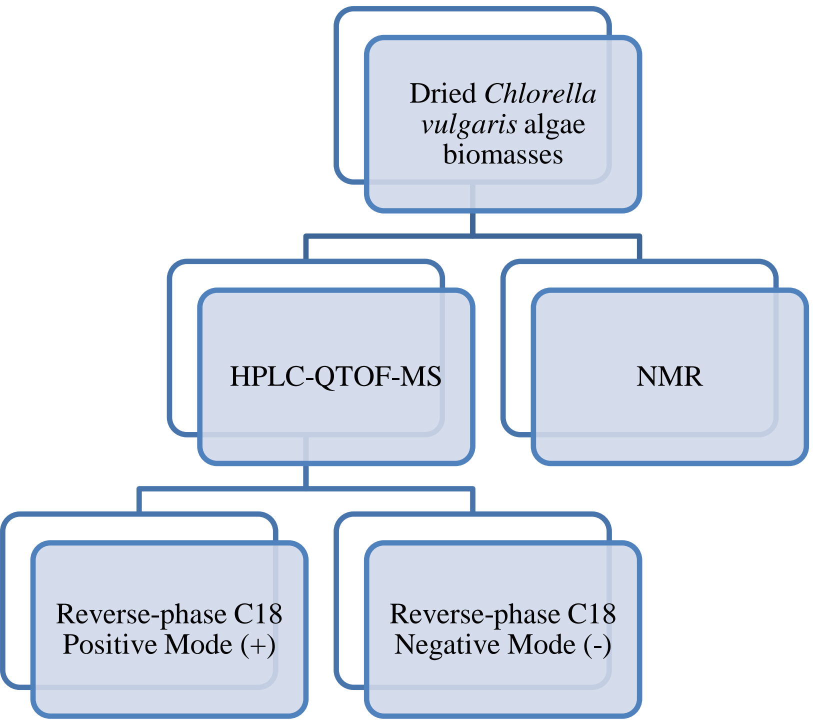


Figure 3.2 Sample preparation procedures for metabolomics study using HPLC-QTOF-MS and NMR

The following sections will describe the detailed sample preparation procedures for HPLC-QTOF-MS and NMR analysis.

3.2.2.1 Metabolites extraction procedure for LC-QTOF-MS

Dried homogenized algae biomasses were first mixed thoroughly and weighed. 600µL of cold methanol were added to 20mg of dried algae. The mixture was vortexed for 10 seconds. 200µL of cold ultrapure water was then added. The samples were then sonicated for 15 minutes in ice. The mixture was centrifuged at 19 000g for 10 minutes in 4°C and the supernatant was placed in a new vial. The extraction was repeated.

Combined supernatant undergoes 2 more rounds of centrifuging to remove any fine particles before removing all solvents using a speed vacuum concentrator. Dried metabolites extracts were then stored in -80°C freezer till analysis.

Quality control samples were prepared identically as the samples. Quality control samples were obtained by combining a fixed amount of algae (15mg) from samples of all the 30 treatment groups. The combined biomasses was then mixed and homogenized thoroughly and 20mg of dried algae mass were weighed into vials and prepared in the same procedure as the samples.

Before analyses, 600µL of 5% methanol was added, vortexed and pipette into amber LC vials. 3 biological replicates were prepared for each sample. This extraction procedure was optimized prior to the actual sample extraction where the extraction time and algae mass: solvent ratio was all optimized specifically for these algae metabolic studies.

3.2.2.2 Metabolites extraction procedure for NMR

2ml of 20% methanol was added to 20mg of dried *Chlorella vulgaris* biomass. This mixture was vortexed for 10 seconds followed by a 30 minutes sonication. The mixture was then centrifuged at 13 000rpm at 4°C for 15 minutes. 1.80ml of the supernatant was isolated into a new tube. The extraction method was repeated and the supernatant was combined and froze in a -80°C freezer and lyophilized overnight. The samples were stored in -30°C freezer before analysis.

0.10M phosphate buffer with 10% D_2O and 0.5mM DSS at pH 7.4 was used as the NMR solvent. 600µL of this NMR solvent was added in to the samples for NMR measurement and analysis. 3 biological replicates were prepared and analyzed for each sample.

3.2.3 LC-QTOF-MS analysis parameters

The liquid chromatography mobile phases used for positive mode were (A) 0.1% formic acid in ultrapure water and (B) 0.1% formic acid in acetonitrile. Gradient elution chromatography for positive mode started with 2% B. This was held constant for 4 minutes before increasing it to 70% B in 5 minutes. % B composition was then increased to 90% in 6 minutes, followed by 100% B in 7 minutes. This composition was kept for 3 minutes before allowing it to re-equilibrate to 2% B (initial conditions).

For the negative mode, the mobile phases used were (A) 2.5mM ammonium acetate in ultrapure water and (B) 2.5mM ammonium acetate in 95% acetonitrile: 5% ultrapure water. Similarly for negative mode, solvent B started with 2% and was kept at this value for 2.5 minutes. At 5 minutes of the run, 85% of solvent B was reached. %B composition was then increased to 100% in 5 minutes and kept constant for 10 minutes. The system was then allowed to return to its equilibrium initial conditions.

The detailed gradient elution chromatographies for both negative and positive mode are shown in Figure 3.3. For both positive and negative modes, the gradient elution chromatography setup had been optimized separately. All mobile phases were degassed for 15 minutes in an ultrasonic bath prior to use.

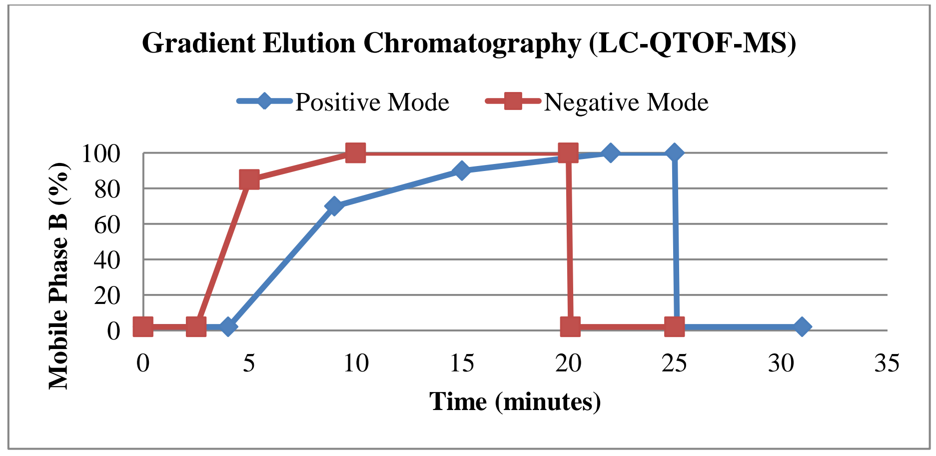


Figure 3.3 Gradient Elution Chromatography (LCQTOFMS) for untargeted metabolomics study

Extracted metabolites samples were reconstituted in 5% methanol. 5µL of these samples were injected in to a reverse-phase C_{18} Acclaim RSLC column with dimensions of 2.1mm x 100mm, 2.2µm and 120Å. The column oven and sampler tray temperatures were maintained at 30°C and 4°C respectively.

Mass spectrometry was performed in both the positive and negative modes. The parameters set during the analyses were as listed in Table 3.5. Samples were all randomized for analyses and quality control sample was inserted for every 10-15 samples depending on the mode of the runs. The LC column and MS were all equilibrated properly using matrix blanks and quality control samples before the actual injection of algae samples.

Table 3.1 Mass spectrometry parameters for LC-QTOF-MS metabolomics studies

Parameter	Value
Mass range	50 – 1000 Da
Nebulizer gas (GS1)	50psi
Heater gas (GS2)	50psi
Curtain gas (CUR)	25psi
Heater temperature (TEM)	400°C
Ion spray voltage (ISVF)	5400V (positive mode) -4500V (negative mode)
Declustering potential (DP)	100V
Collision energy (CE)	10V

3.2.3.1 LC-QTOF-MS data analysis

For this study, an untargeted metabolomics approach was applied. The data files (.wiff format) from the AB Sciex LC-QTOF-MS were first converted to a suitable format (mzXML format) and then uploaded and processed using the XCMS online interface[113]. Upon the pre-processing using XCMS Online, the data were corrected for retention time, and the features and peaks were extracted and detected. The pre-processed results which includes the mass/charge (m/z) values, retention time and corresponding peak areas were obtained and compiled in an excel sheet. Total

area normalization (total intensities) was performed for all samples using the excel functions. Following which, multivariate data analysis was performed using SIMCA as discussed in Section 1.6.2. In short, the compiled results obtained were compared control versus samples exposed to PFCs for the identification of possible metabolite changes and biomarkers. The tandem MS spectrums were also used for verification.

3.2.4 NMR analysis parameters and data analysis

Proton Nuclear Magnetic Resonance (^{1}H-NMR) analyses were conducted on the algae samples using the Bruker DRX500 NMR instrument at 500.2MHz. DSS was used as the NMR chemical shift standard. Upon obtaining all the NMR spectra, pre-processing of the data were required before processing it for metabolomics data analyses. NMR pre-processing of the spectra was performed using Chenomx NMR Suite 7.6. DSS was used as the NMR chemical shift standard at 0ppm. The NMR spectra were firstly phase and baseline corrected, and then the water peak was deleted without any deletion of essential peaks from the spectra. Lastly the Chenomx Profiler was used to identify and profile the small molecule metabolites.

3.3 *Results and discussion*

For the metabolites analyses of algae samples using LC-QTOF-MS, the extraction method is first optimized as discussed in Section 3.3.1. Two main factors that greatly affect the extraction efficiency are being considered: (1) ratio of algae biomass to solvent and (2) time for extraction (ultra-sonication in 4°C). Secondly, the metabolic effects of PFOA and PFOS in an acute 96-hour study using positive and negative mode LC-QTOFMS untargeted metabolomics approach are discussed.

3.3.1 *Optimization of extraction of algae metabolites*

The ratio of algae biomass to solvent and time for extraction (ultra-sonication in 4°C) are the two parameters selected critically for optimization. The list in Table 3.6 indicates the different parameters varied for each of the factors. The number of peaks and corresponding intensity or area in the LC-QTOF-MS are used for comparison and determination of the finalized optimized parameter. The parameter that is bold and underlined indicates the final optimized parameter.

Table 3.2 Parameters for optimizing of *Chlorella vulgaris* metabolites extraction

Parameter	Values
Mass of algae (mg) used for extraction in a fixed volume (800µl) of extracting solvent	5 10 **<u>20</u>** 30
Time (minutes) for ultra-sonication in 4°C	5 10 **<u>15</u>** 20

The full LC-QTOF-MS chromatography runs were used as comparison. Several significant peaks were identified and used for more detailed comparison as shown by the x-axis in Figure 3.4. As shown in Figure 3.4, 20mg algae biomass have the best extraction efficiencies compared to 5, 10 and 30mg. 5mg seems to be insufficient for metabolites extraction since there are missing critical peaks as observed by the missing points in Figure 3.4.

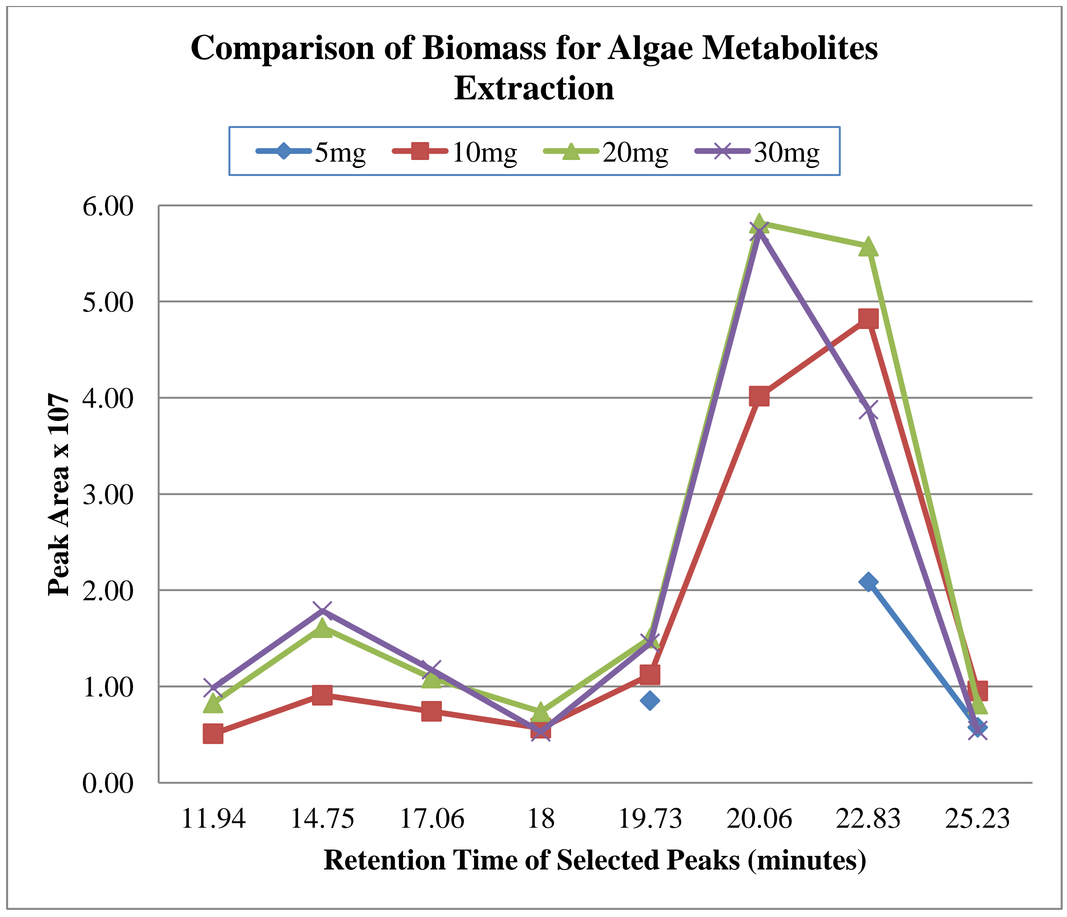


Figure 3.4 Comparisons of peak area of different algae biomass (5-30mg) for extraction method (analyzed by positive mode LC-QTOF-MS run)

Due to the limited algae mass available after sample collection and the most number of peaks and high intensities, 20mg is selected as the optimized mass of algae used for extraction in a fixed 800μl of extracting solvent. Similarly, by suing the full LC-QTOF-MS chromatography runs as comparison, 15 minutes is optimal for the ultra-sonication in ice for the metabolites extraction in algae.

3.3.2 Metabolomics study of algae when exposed to PFCs using HPLC-QTOF-MS

Algae cell cultures that are exposed to varied concentrations of PFOA and PFOS individually over a 96-hour period show some degree of growth inhibition. As concentration of PFOA and PFOS increases and the time of exposure increases, the growth inhibition was observed to be more significant. By comparing the UV-Vis

results and cell densities, it indicated that *Chlorella vulgaris* appears to be under more stress due to the higher percent of growth inhibition. The microscopic images of the PFOA- and PFOS- affected algae cells showed that irregularities in the cell shape and size it has undergo stress and are in the state of defense by the membrane disruption aggregating and forming clumps.

To have a deeper understanding of the defense mechanism and cell response of algae and also the exact disruptions caused by exposure to these toxic PFCs, the algae metabolome at different concentrations of PFCs and time of exposure (0 - 4 days) was analyzed using LC-QTOF-MS in both positive and negative modes. Metabolomics responses for the control and PFCs-exposed algae samples were compared and the variations between the samples were possibly identified and determined. The relationship between the different concentration and time points are also illustrated in the figures in the subsequent sections. Statistical tests were also performed to determine fit and also the observed variations in the metabolome were statistically different from the control groups. This is conducted to ensure that the metabolic responses of the PFCs-exposed groups that are different from the control group are also statistically significant.

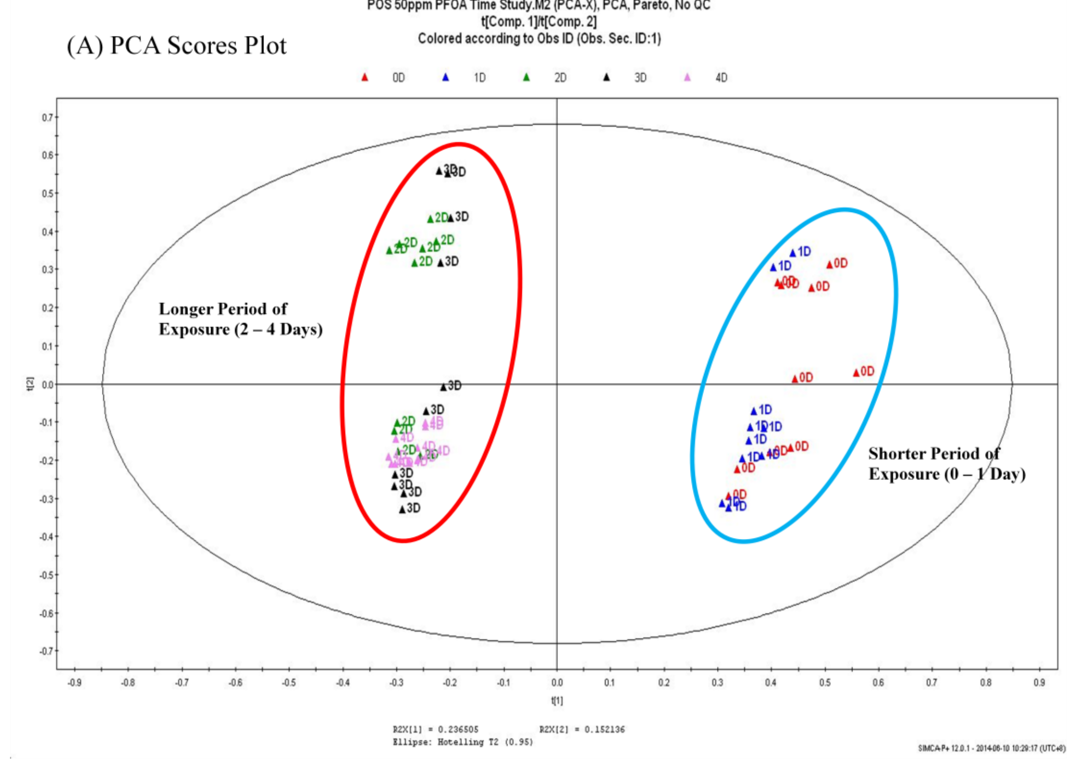
Due to the large number of samples, 2 different toxicants (PFOA and PFOS) and the positive and negative mode HPLC-QTOF-MS results, the effect of PFOA to *Chlorella vulgaris* are first discussed followed by PFOS. The sections itself includes and discusses the different factors and differences between the concentration and time study.

3.3.2.1 Effect of PFOA on Chlorella vulgaris: LC-QTOF-MS Results

Based on the liquid chromatograms of the algae samples, there were differences in peak intensities and area between the control and PFC-spiked samples but no entirely new metabolite peaks were identified. Since LC-QTOF-MS results

generally have a large quantity of data, multivariate analysis (MVA) which consist of principal components analysis (PCA) and orthogonal partial least square discriminant analysis (OPLS-DA) are employed to accurately process and generate differentiation easily.

The metabolomics study (positive mode, LC-QTOF-MS) of a 0 to 4 days exposure of 50ppm PFOA to *Chlorella vulgaris* is illustrated in the PCA plot in Figure 3.5, and OPLS-DA plot in Figure 3.6. Based on the time-based results shown, it was observed that as time progresses, especially from Day 1 to Day 2, the metabolic profiles of *Chlorella vulgaris* are different as shown in the PCA score plot. The profiles for Day 0 (immediately at time of exposure) and Day 1 are similar. This indicates that 50ppm PFOA spiked starts to have substantial effect on Day 2. Even in the longer period of exposure group (circled in red), there are still variations as observed by the close group of Day 4 samples and some of the Day 3 samples. This further indicates a change in the metabolic profiles in Day 3 due to the increased stress and exposure caused by the PFOA. The clustering of the different time exposure groups (Day 0, 1, 2, 3 and 4) was more evidently differentiated according to the number of days in the OPLS-DA plot compared to the PCA plot as shown in Figure 3.6. The time effects were also better observed in the OPLS-DA plots, indicated by the arrows showing the trend as time progresses. Therefore based on the results, as shown there are significant differences in the metabolic profiles of *Chlorella vulgaris* when it is exposed to 50ppm PFOA over a 4-day study. The corresponding metabolites that are significant to the specific groups were isolated and identified with the use of the mass spectrums , fragmentations and databases.



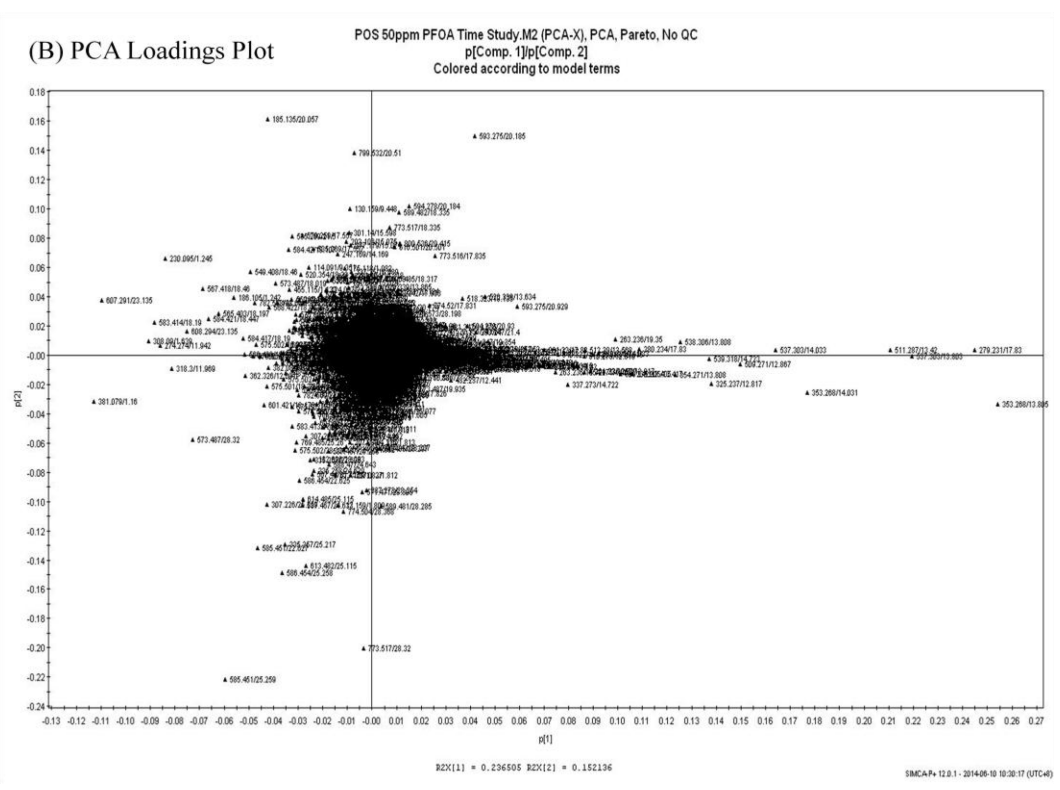


Figure 3.5 PCA plots for 50ppm PFOA spiked to *Chlorella vulgaris* (time-based study, positive mode LC-QTOF-MS) where the different colors represents the period of exposure: 0D, 1D, 2D, 3D and 4D. Figure A shows the score plots while B indicates the loadings plot.

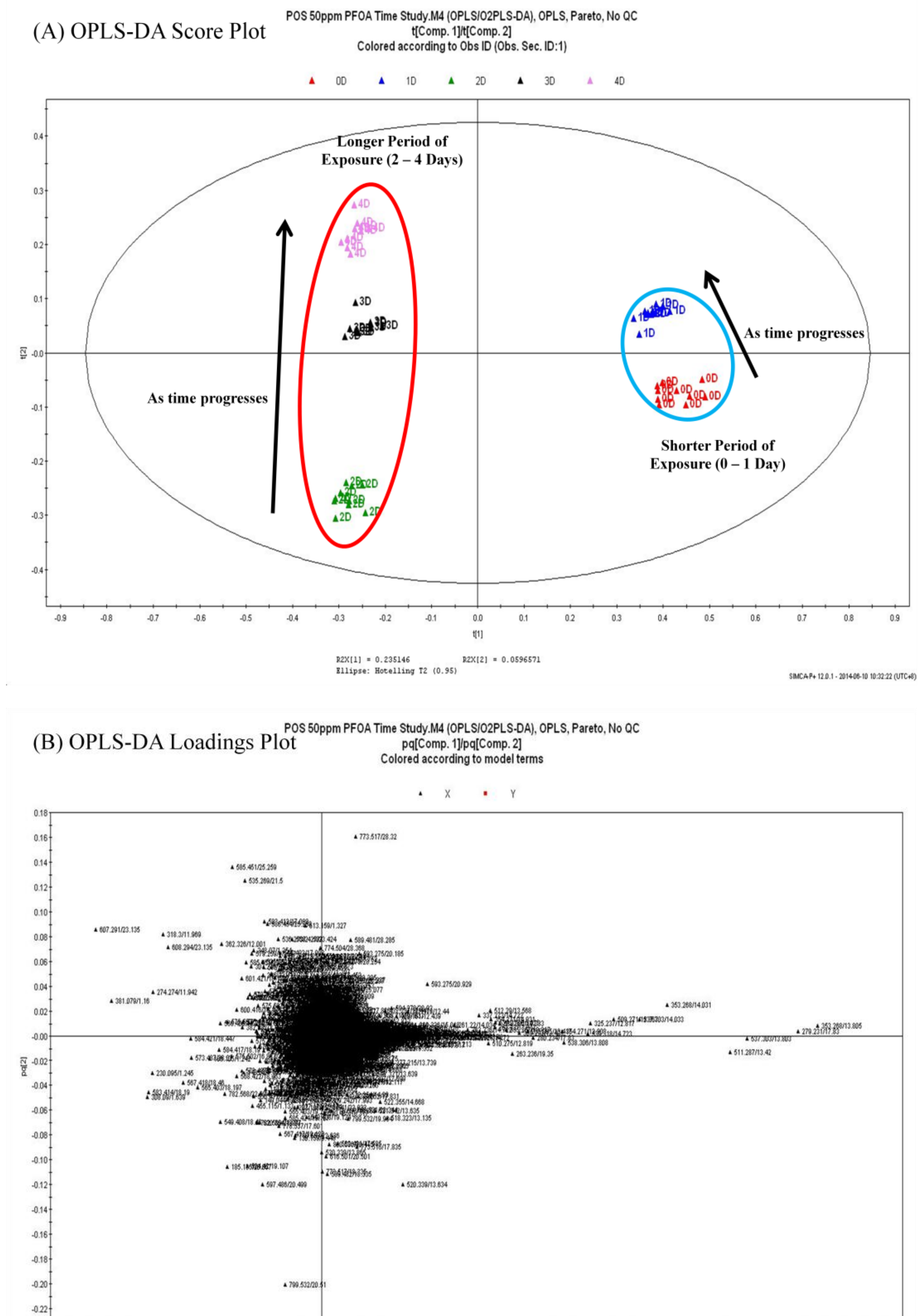


Figure 3.6 OPLS-DA plots for 50ppm PFOA spiked to *Chlorella vulgaris* ((time-based study, positive mode LC-QTOF-MS) where the different colors represents the period of exposure: 0D, 1D, 2D, 3D and 4D. Figure A shows the score plots while B indicates the loadings plot.

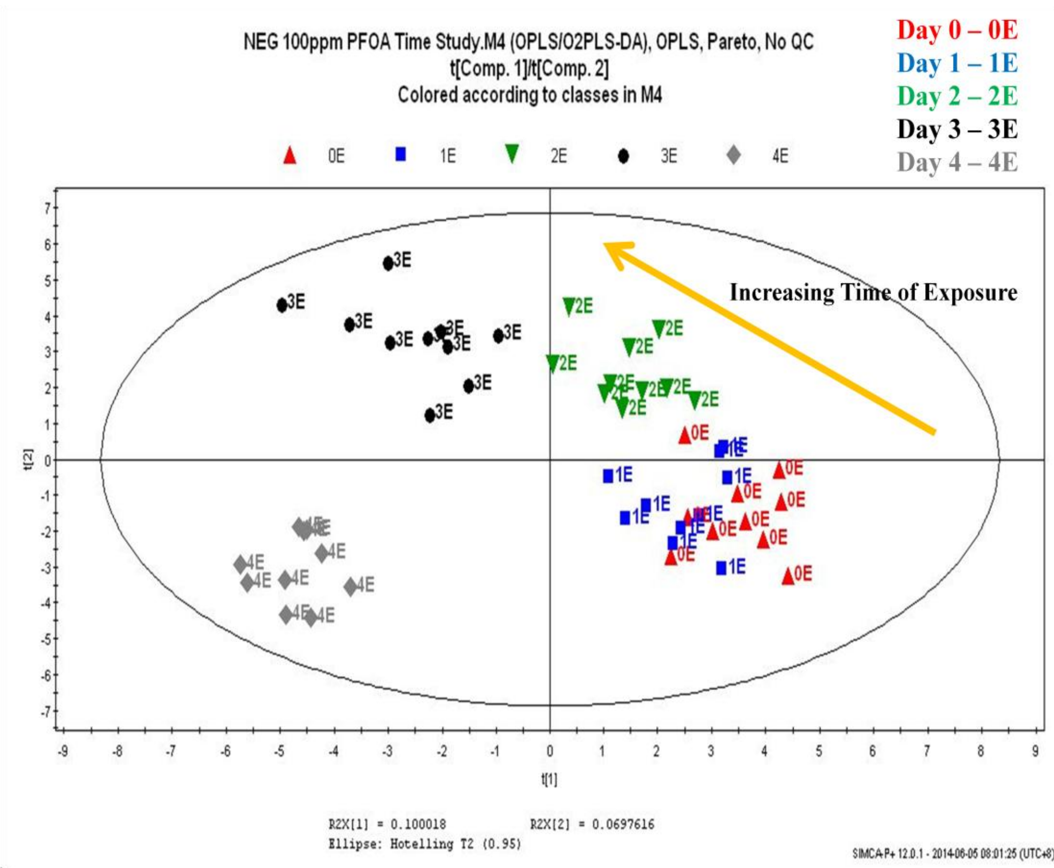
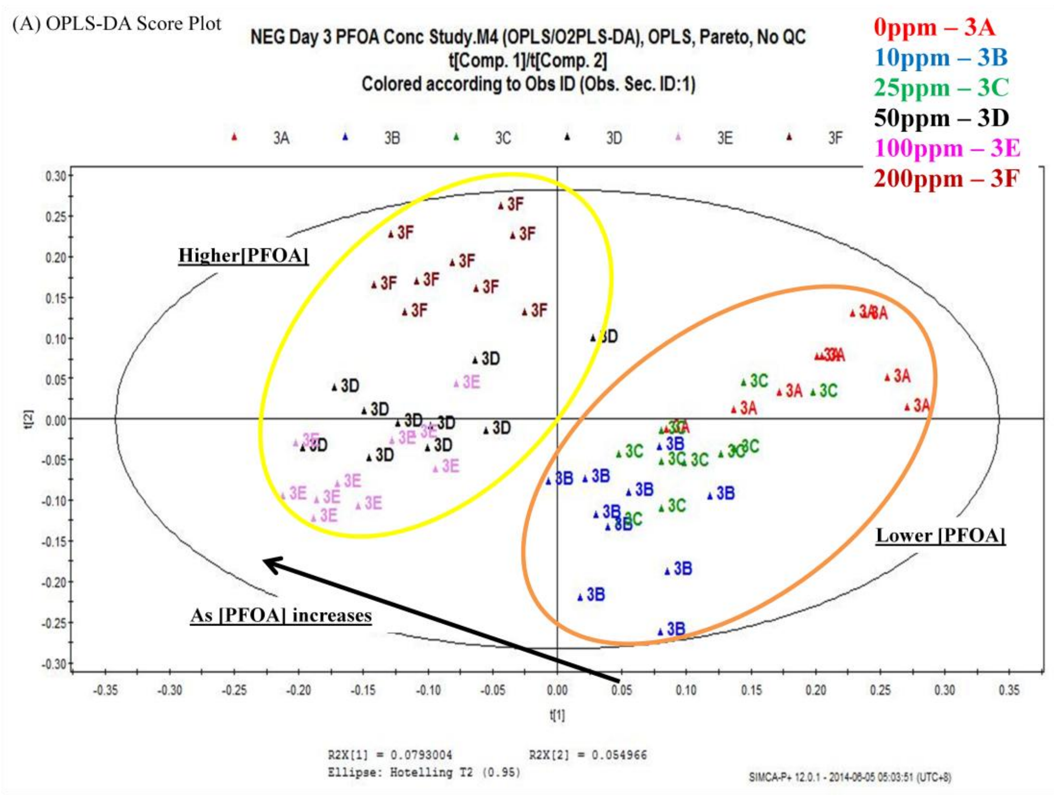
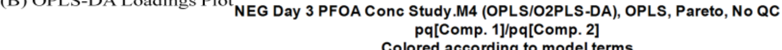


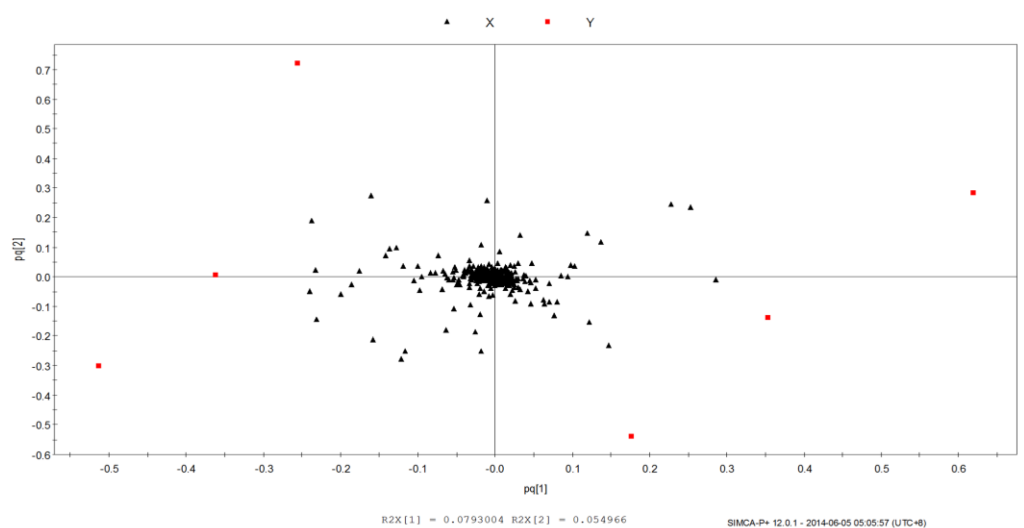
Figure 3.7 OPLS-DA score plot for 100ppm PFOA spiked to *Chlorella vulgaris* ((time-based study, negative mode LC-QTOF-MS) where the different colors represent the period of exposure: 0E, 1E, 2E, 3E and 4E.

This trend was also observed in the LC-QTOF-MS negative mode runs of the algae metabolites. Figure 3.7 shows the OPLS-DA score plots of the 100ppm PFOA-spiked *Chlorella vulgaris* over a 4 day exposure. The trend in Figure 3.6, and 3.7 is slightly different as the time factor where there is vast variation and when it starts to deviate away is distinct. For Figure 3.6, where 50ppm PFOA is spiked to Chlorella vulgaris, the time that the profile is different is on Day 2 while that for 100ppm PFOA as shown in Figure 3.7, it is on Day 4. After the time factor comparison for PFOA-spiked *Chlorella vulgaris*, the variation in the concentration when keeping the time constant will be illustrated. Day 3 of the acute PFOA exposure to green microalgae was analyzed based on the OPLS-DA plot in Figure 3.8. As observed in the LC-QTOF-MS negative mode runs and the corresponding score plot, a trend in

the shift my metabolic profiles are observed as concentration of PFOA increases. Similar trends were observed in Day 4 of the study.







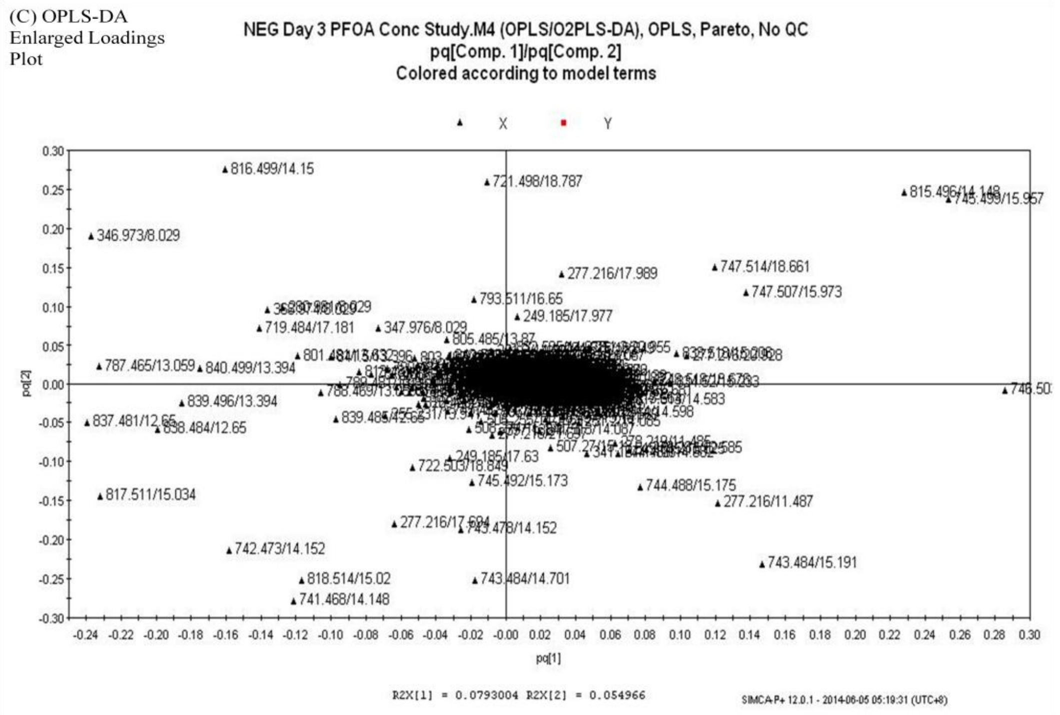
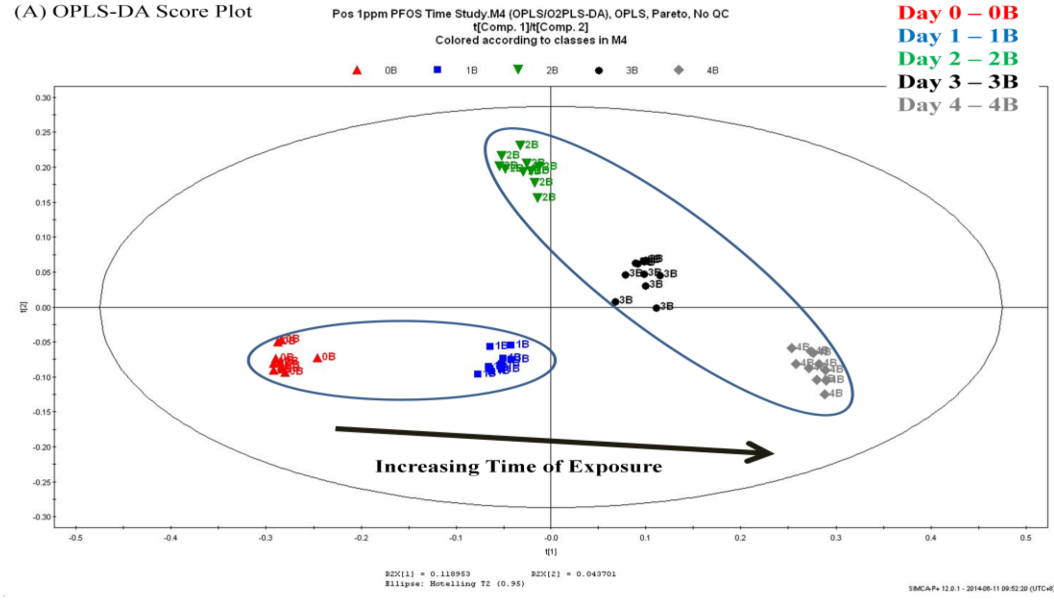


Figure 3.8 OPLS-DA plots for Day 3 of varying concentrations of PFOA spiked to *Chlorella vulgaris* ((concentration-based study, negative mode LC-QTOF-MS) where the different colors represents the [PFOA]: 0ppm, 10ppm, 25ppm, 50ppm, 100ppm and 200ppm. A: score plot, B: loadings plot, C; loading plot (enlarged).

3.3.2.2 Effect of PFOS on Chlorella vulgaris: LC-QTOF-MS Results

Similarly for the effect of PFOS on the green microalgae, the time and concentration factors are all evident in the study as illustrated in Figure 3.9 to 3.11.



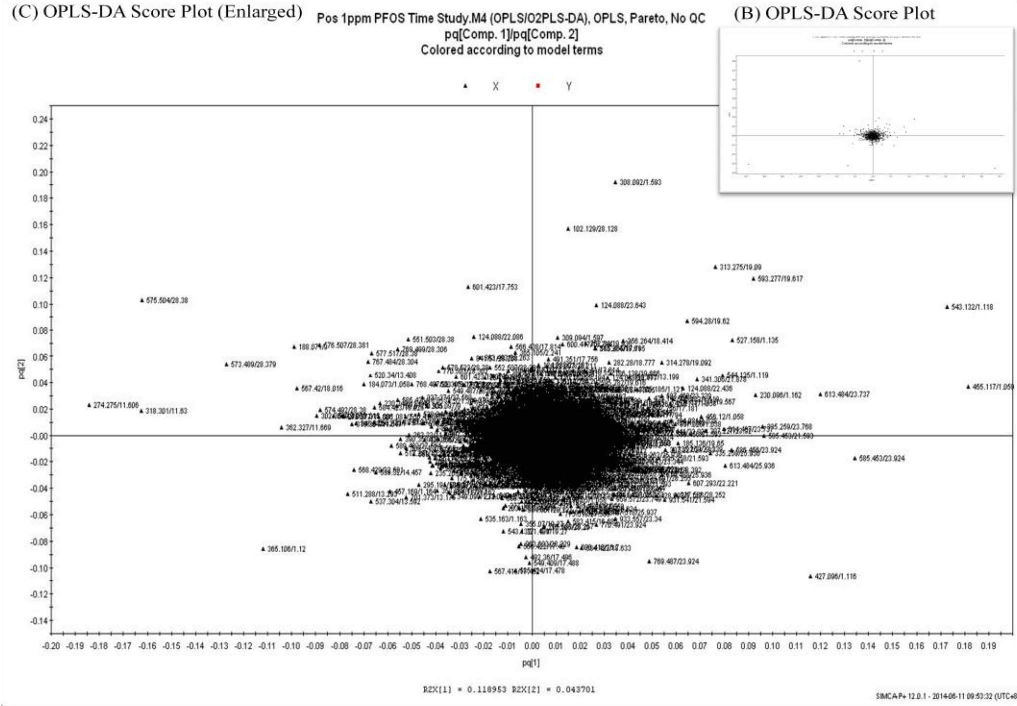


Figure 3.9 OPLS-DA plots for 1ppm PFOS spiked to *Chlorella vulgaris* ((time-based study, positive mode LC-QTOF-MS) where the different colors represents the period of exposure: 0B, 1B, 2B, 3B and 4B. A: score plot, B: loadings plot, C; loading plot (enlarged).

For the exposure of 1ppm PFOS, the metabolic profiles follow a trend to the right as time of exposure increases. This trend indicates that as time of exposure increases the metabolites identified on the loadings plot contributes significantly to that factor.

3.4 Conclusion

It has been revealed that upon PFCs exposures to green microalgae, these toxicants adsorbed on the surface of the algae and interfere with the algae's cell membrane permeability and therefore also the cell membrane function[209]. Under further stress from varying concentrations and periods of PFCs as shown in this study, these toxicants affect the composition of lipids (Chapter 3) in the cell membrane which eventually caused the aggregation and clumps formation of algae (observed in the microscopic images in Chapter 2). With such changes and loss in the algae cell membrane and transport activity, the growth inhibition occurs. In fact, it has been confirmed that even at low concentrations of PFOA (PFCs), long term exposure may actually lead to the generation of excess reactive oxygen species in algal cells which eventually result to membrane lipid peroxidation and cell oxidative damage[209].

Concentration and time plays an important factor in the metabolome profile of the *Chlorella vulgaris* as shown in the results in this study. Based on the possible metabolites identified using the reverse phase LC-QTOF-MS, there were signs of cell growth inhibition and occurrence of oxidative stress and damage found as concentration of PFCs increases. The occurrence of these compounds may be majorly due to the adsorption of PFCs resulting in the disruption of cell membrane transport activities. However, further validations and confirmations need to be performed in order with commercial standards. In conclusion, an integrated analysis and understanding of the microalgae systems and their response and exposure to toxicants would greatly enhance the PFCs toxicity determination.

4 Investigation of the use of *Daphnia magna* from different age groups for metabolomics study and its application in eco-toxicity determination

4.1 Introduction

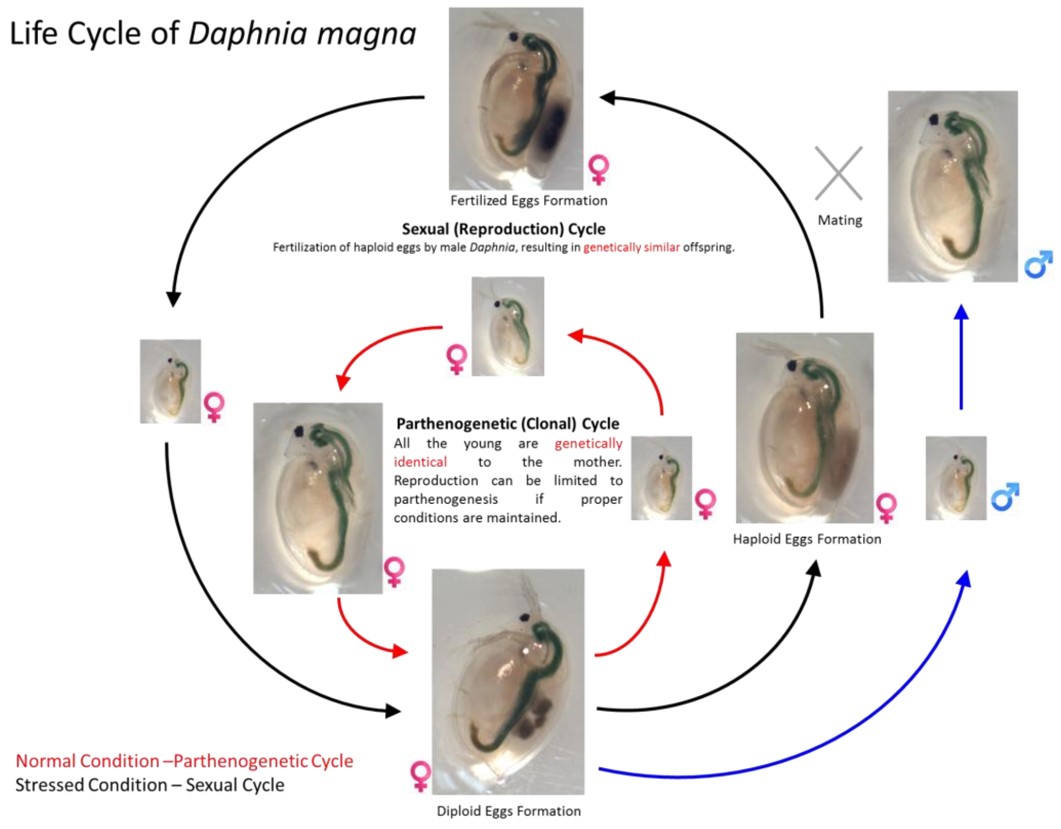


Figure 4.1 Microscopic images of *Daphnia magna* at different life stages

Freshwater water fleas, *Daphnia magna*, are small crustaceans which measure only about 3 to 5mm in length. There are several species of the genus *Daphnia* but for the purpose of this study, this larger-sized water flea, *Daphnia magna* species, is used. The anatomy and physical traits of *Daphnia magna* at different life stages are given in Figure 4.1. The young *Daphnia magna*, also known as neonates (aged between 0-2 days) and juveniles are generally smaller in size. At all stages, its two antenna, eye and gills are easily visible through the microscope as shown in Figure 4.1. In addition, since it is transparent, its gut can be clearly observed to be green due to the green microalga (*Chlorella vulgaris*) that is its food source. The different genders and age groups of the water flea (adult and adult with eggs) are all illustrated

in Figure 4.1. There are different types of eggs (diploid, haploid and fertilized). Reproduction of *Daphnia magna* via parthenogenesis, as observed in the red arrows and cycle in Figure 4.1, can only be achieved in optimal culture conditions. Upon stress and suboptimal conditions, reproduction can only proceed via the sexual reproduction cycle (blue and black arrows).

This small aquatic organism is very sensitive to their environment and growing conditions and therefore the culturing conditions (temperature, lighting, culture medium, food, etc…) are very critical to its growth, survival and reproduction. The most important factors in maintaining good cultures are mainly sufficient food and space (to avoid overcrowding). Due to its sensitivity to the environment and toxicants, these aquatic fleas are commonly used in aquatic toxicological studies and also ecological monitoring. Moreover, they are used to test water quality and to ensure water safety before releasing the test samples to the environment[220]. Its sensitivity is evident in several tests and studies where the 48-hour immobility observations and the no-observable-effect concentration (NOEC) values for *Daphnia magna* is considered a lot smaller than the other aquatic test species[172]. These characteristics are observed in both the acute and chronic tests toxicity tests.

As early as 1978, Adema introduced the use of *Daphnia magna* as a test organism in acute and chronic toxicity test[221]. Since then, it was used for prescreening of chemical toxicity and other environmental purposes. Guilhermino et al have also determined that this water flea is more sensitive and specific than a rat in acute toxicity testing[222]. In addition, there are a few research groups investigating the toxicity of PFCs to these water fleas[172,178,207,208,223-229]. In particular, Pablos et al observed the effect of PFCs on *Daphnia magna* by focusing on the reproduction and immobility of the water fleas after a fixed time period[208]. However, experiments were not further conducted to investigate why and how the PFCs affect these aquatic organisms.

In fact, the use of *Daphnia magna* is not widely explored in environmental metabolomics studies but it is starting to gain interest. There are only a few studies of using *Daphnia magna* as subjects for metabolomics studies[92,230-234]. In particular, Nagato et al uses it for NMR metabolomics to investigate the stress of arsenic, copper and lithium to aquatic organisms. This study provides evidence that indeed the metals are toxic, affected the water fleas and disrupted the metabolism in them.

Daphnia magna is used in toxicological studies because of the advantage discussed above. Similarly, most of these factors make *Daphnia magna* a very suitable choice for metabolomics studies too. Apart from it being sensitive, it is small, and easy to culture in a laboratory environment. It can also be reared in freshwater lakes easily and have high population densities. There are no ethical issues involved too. *Daphnia magna* and its full anatomy and organs can be easily observed (Figure 4.1) and deformations and growth can be monitored easily. Furthermore, it can multiply quickly and has a relatively short lifecycle of about 60 days, dependent on temperature and culture conditions. To add on, the short lifecycles enable toxicological and metabolomics studies to fully consider and monitor various effects during its whole life span (e.g. mortality, reproduction, offspring, growth development etc). Most importantly, it is a key trophic to the aquatic food chain where many aquatic organisms and fishes feed on *Daphnia magna*.

The aims of this chapter are firstly to demonstrate and culture *Daphnia magna* successfully for the use in toxicological and metabolomics studies, secondly determine which life stages of *Daphnia magna* is the most suitable for toxicological and metabolomics studies, thirdly conduct eco-toxicological studies of *Daphnia magna* being exposed to PFCs and leachates in an acute 48-hours study and lastly applies metabolomics techniques in leachates eco-toxicological determination.

One of the factors that are crucial in metabolomics studies is actually the age group of the test organisms. It is because different age group or life stages do have corresponding effects on the metabolites and also the sensitivity and effect of it upon exposure to toxciants[235-238]. Many test species of the earlier life stages were commonly used as they are assumed to be more sensitive and responsive to environmental stressors. However, this may not be entirely true for these water fleas and needs to be studied in greater detail. Arzate-Cardenas et al have also acknowledged and identified this situation and have conducted experiments for chromium using *Daphnia magna* of different age groups[235]. It was concluded that age is one of the key factors that can contribute to the differences in response and sensitivity and it has to be considered carefully for toxicity bioassays and metabolic studies using *Daphnia magna*.

Another important factor to consider during the toxicity and metabolomics studies is the gender of the water fleas. *Daphnia magna* are cyclical parthenogens where in favorable conditions, it will produce an all-female clonal offspring. According to several articles[239,240], it is observed that in organisms and also humans, different metabolic profiles for different genders resulted in having gender-specific metabolites and biomarkers. In particular, Thévenot et al analyzed urines from 183 adults and determined clusters and concentration variations with regards to age or gender. In conclusion, age and gender of *Daphnia magna* test organisms need to be considered critically in the planning, design and execution of toxicity and metabolomics studies. This chapter carefully examines the selection and planning of using Daphnia magna in toxicological and metabolic studies and applies it to PFCs and leachates examples.

4.2 Materials and methods

4.2.1 Chemicals and reagents

Growth medium used for culturing *daphnia magna* was prepared as described by Hilham et al[241]. All chemicals used to prepare the COMBO medium for these water fleas were obtained from Sigma Aldrich (St. Louis, MO, USA). It was prepared using ultrapure water (Smart2Pure, Thermo Scientific, TKA) and was filtered through a 0.22µm large volume filter (Corning ®) before use. The medium is stored in autoclaved glass bottles after filtering.

Ammonium acetate (≥ 98%) and ammonium formate (≥ 97%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Ultrapure water (Smart2Pure, Thermo Scientific, TKA) was used to prepare the mobile phases used in liquid chromatography-mass spectrometry. Methanol, hexane, acetone and acetonitrile, both of LC-MS grades, were purchased from Sigma Aldrich (St. Louis, MO, USA). The PFCs chemicals and reagents used are similar to those described in Chapter 2 (Section 2.2.1) and will not be elaborated further.

4.2.2 Daphnia magna cultivation

COMBO medium was chosen as the culture medium used for *Daphnia magna*. As described by Hilman et al, this medium was prepared using 7 major elemental solutions, 2 trace elemental solutions, 1 vitamin solution and ultrapure water[241]. The pH, conductivity, dissolved oxygen and water hardness of the COMBO medium were measured and recorded upon preparation. If it falls below the expected range of values, the medium will be discarded and re-prepared. Water hardness was determined using the EDTA titrimetric method while conductivity and dissolved oxygen content was measured using Hach HQ40d multi-parameter meter. The medium was filtered through a 0.22µm large volume filter (Corning ®) before use.

Daphnia magna (Carolina Biological Supply Company, Burlington, North Carolina) were cultured in 2.5 litres plastic fish tanks as shown in the figure. The culture is kept in a controlled condition, at 20 ± 2°C and is given a 16 hours light: 8 hours dark photoperiod and 24 hours aeration. *Daphnia magna* were fed daily with green microalgae *Chlorella vulgaris*[208]. The algae growth conditions are similar and as described in Chapters 2 and 3. *Daphnia magna* cultures were monitored daily and were maintained by renewing 75% of the medium twice each week. Stresses such as overcrowding and overfeeding were avoided and constant clean, well-fed and good conditions were ensured to prevent suboptimal conditions and the induction of male water fleas.

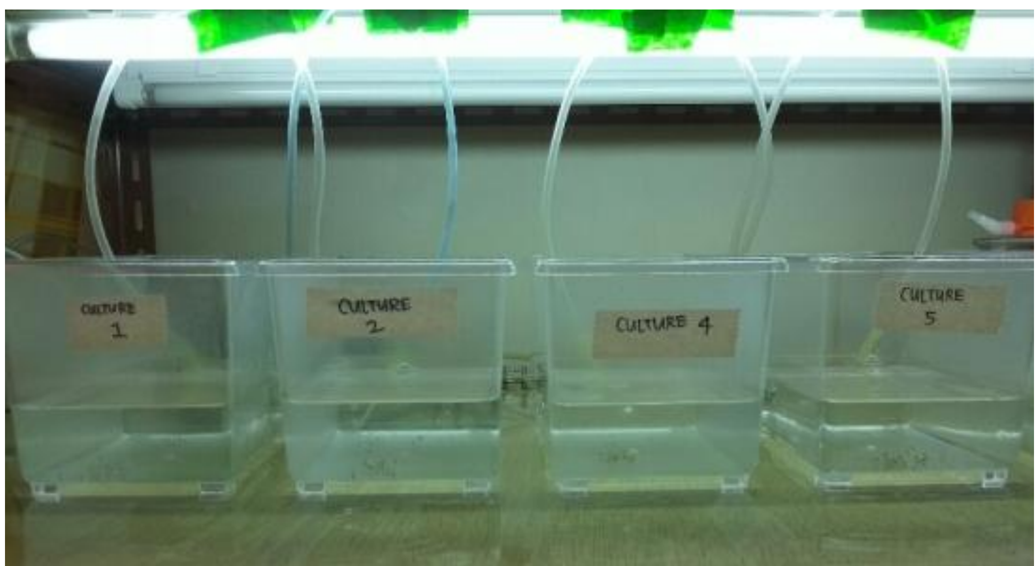


Figure 4.2 ***Daphnia magna*** **cultures in plastic tanks with aeration and lightings**

4.2.3 Experimental design and workflow

The entire workflow for this study is as shown in Figure 4.3. Planning is crucial before the start of the study so as to understand and fulfill the objectives and work towards the right sample collection and method for the study. Each section will be discussed in further details in the following sections.

Figure 4.3 Experimental design and workflow for *Daphnia magna* studies

4.2.4 Daphnia magna sample collection and preparation

4.2.4.1 Sample collection

For this study, *Daphnia magna* of different age groups were collected. In order to minimize any discrepancies in the ages and the gender (female) of the water fleas collected and to prevent any huge differences in the growing conditions, *Daphnia magna* with fertilized eggs were first isolated out in a separate tank or beaker. Once the neonates were born, the date of birth and their corresponding ages (days old) were recorded. The culture conditions and the food for these water fleas were as described in Section 4.2.2.

Once it reaches the required ages, the water flea will be removed from the tank into a 2ml vial. It was then carefully immersed in ultrapure water thrice to remove any medium or algae on its surface. After rinsing, all liquid were removed with the use of a pipette and *Daphnia magna* were flash-frozen using liquid nitrogen or

placing it in -80°C freezer. The *Daphnia magna* samples were then lyophilized overnight and stored in -80°C freezer till analysis.

The water flea samples collected for the different age groups are illustrated in Table 4.1. Due to the lighter mass and smaller size of the neonates and juveniles, 5 daphnia of these 2 groups were used per sample instead of the usual 1 daphnia required per sample for the other age groups. The numbers of daphnia required have been optimized prior to selecting the required quantity.

Table 4.1 *Daphnia magna* samples of different age groups

Daphnia description	Abbreviation	Ages	Number of daphnia required / sample
Neonates	NB	0 – 2 days	5
Juveniles	Y	2 – 9 days	5
Adult	A	10 – 40 days	1

Daphnia description	Abbreviation	Ages	Number of daphnia required / sample
Adult with fertilized eggs	AE	10 – 40 days	1
Adult with winter eggs	AW	10 – 40 days	1
Adult with unfertilized eggs	UFE	10 – 40 days	1
Old	O	> 40 days	1

4.2.4.2 Extraction of metabolites

For the extraction of metabolites, the biomasses have to be accurate and kept the same. However the mass of each water flea used cannot be measured accurately by the electronic mass balance. So in order to keep everything accurate for each experiment set, the quantity of *Daphnia magna* is used instead of the mass. For this study, the selections of daphnia magna were all picked at random so no bias is allowed.

The optimized *Daphnia* extraction for C_{18} HPLC-QTOF-MS are as described: 1ml of cold 70% methanol was added to the *Daphnia magna* samples. It was homogenized using three 2.8mm ceramic (zirconium oxide) beads in 2ml standard tubes (CK28 beads) for 25 seconds at 5000 rpm. The mixture was then cooled in ice for 10 seconds before homogenizing it at 5000 rpm for another 25 seconds (Minilys Homogenizer, Bertin Technologies, France).

The mixture was centrifuged at 14 000rpm at 4°C. The supernatant was then pipette out into a new vial and stored in ice. The process was repeated to get another extract from the sample. Both supernatants were then combined and centrifuged at 14 000rpm at 4°C for 10 minutes. The combined supernatant was then centrifuged again to remove any remaining debris or particles.

850µL each of every sample were pipette out into 2 different vials for positive and negative mode. It was then subjected to drying using a speed-vac machine (Labconco, CentriVap Cold Trap and Concentrator, Thermo Fisher Scientific Inc.). The dried samples were stored in -80°C freezer prior to analysis.

The sample was reconstituted using 42.5µL of 5% methanol before HPLC-QTOF-MS analysis. Quality control (QC) samples were prepared in the similar method as the samples. QC samples consist of *Daphnia magna* of all age groups being pooled together.

4.2.4.3 Optimization of parameters in Daphnia magna extraction procedure

The described method shown in section 4.2.4.2 is the finalized optimized method for the extraction of metabolites from *Daphnia magna* samples. In order to obtain this method and to have good signal intensities, the following parameters in Table 4.2 were being varied and optimized individually beforehand. A blank control was included during the optimization.

Table 4.2 Parameters for optimization in *Daphnia magna* metabolites extraction

No.	Parameters	Descriptions
1	Extraction technique	(A) Ultra-sonication in ice for 10 minutes (B) Homogenize for 25 secs and repeat once (C) Homogenize for 10 secs and repeat once
2	Percentage of extraction solvent (methanol)	60% 70% 80%
3	Type of homogenizing beads (size and number)	Size of beads: CK14, CK28 and CKmix Number of beads: 3 and 6 beads
4	Number of *Daphnia magna* required	Young (neonates and juveniles): 1, 3, 5 and 8 Adult: 1 and 3

The lysing kits (Precellys Bertin Technologies, France) consist of a specific size of ceramic (zirconium oxide) beads in standard 2mL tubes. CK14 (1.4mm diameter), CK28 (2.8mm diameter) and CKmix lysing beads are generally used for hard and soft tissue homogenization. CKmix consists of a mixture of CK14 and CK28 beads.

An adult *Daphnia magna* was first tested to ensure that there is good reproducibility and signal of the metabolites in the HPLC-QTOF-MS. The optimization then continues as numbered from 1 – 4 in Table 4.2.

4.2.5 LC-QTOF-MS analysis and processing of data

4.2.5.1 Metabolomics LC-QTOF-MS analysis

For both positive and negative modes, the gradient elution chromatography setup had been optimized separately. All mobile phases were degassed for 15 minutes in an ultrasonic bath prior to use. The liquid chromatography mobile phases used for positive mode were (A) 0.1% formic acid in ultrapure water and (B) 0.1% formic acid in acetonitrile. Gradient elution chromatography for positive mode started with 2% B. This was held constant for 4 minutes before increasing it to 70% B in 5 minutes. % B composition was then increased to 90% in 6 minutes, followed by 100% B in 7 minutes. This composition was kept for 3 minutes before allowing it to re-equilibrate to 2% B (initial conditions).

For the negative mode, mobile phases (A) 2.5mM ammonium acetate in ultrapure water and (B) 2.5mM ammonium acetate in 95% acetonitrile: 5% ultrapure water was used. Similarly for negative mode, solvent B started with 2% and was kept at this value for 2.5 minutes. At 5 minutes of the run, 85% of solvent B was reached. %B composition was then increased to 100% in 5 minutes and kept constant for 10 minutes. The system was then allowed to return to its equilibrium initial conditions. The mobile phases and the gradient elution were similar to the one described in Section 3.2.3 (Figure 3.3).

Extracted metabolites samples were reconstituted in 5% methanol. 5µL of these samples were injected in to a C_{18} Acclaim RSLC column with dimensions of 2.1mm x 100mm, 2.2µm and 120Å. The column oven and sampler tray temperatures were maintained at 30°C and 4°C respectively.

The mass spectrometry parameters are similar to that stated in Chapter 3. Mass spectrometry was performed in both the positive and negative modes. The parameters set during the analyses were identical to the parameters listed in Table 3.5. Samples

were all randomized and quality control sample was inserted for every 10 samples. The LC column and MS were all equilibrated properly using matrix blanks and quality control samples before the actual injection of *Daphnia magna* samples.

4.2.5.2 Data processing and interpretation

For this *Daphnia magna* study, an untargeted metabolomics approach was applied and the data processing and interpretation procedure are similar to that in Section 3.2.3.1. In short, the compiled results obtained were compared between the different age groups. The tandem MS spectrums were also used for verification of high intensity metabolites.

4.2.6 Acute Daphnia magna toxicity tests with PFCs and leachates

This acute *Daphnia magna* toxicity tests were conducted for each of the different toxicants: PFOA, PFOS and the various leachates that are discussed in the sections below. The ISO6341:2012 method that is commonly used for water quality determination is conducted with only slight modifications. All tests were conducted with *Daphnia magna* neonates who are less than 48 hours old (2 days old). The ISO test medium, prepared similarly as in ISO5667-16:1998 and ISO6341:2012[242], were used as a control solution and used as a dilution medium for the rest of the test treatments.

The total volume in each of the 6-well plates is 5ml. 5 *Daphnia magna* neonates were utilized per treatment with 6 concentrations designed per group in a 6-well plate. Each set of experiments were conducted three times. An example of an acute toxicity test setup using leachate toxicant is as shown in Figure 4.4.

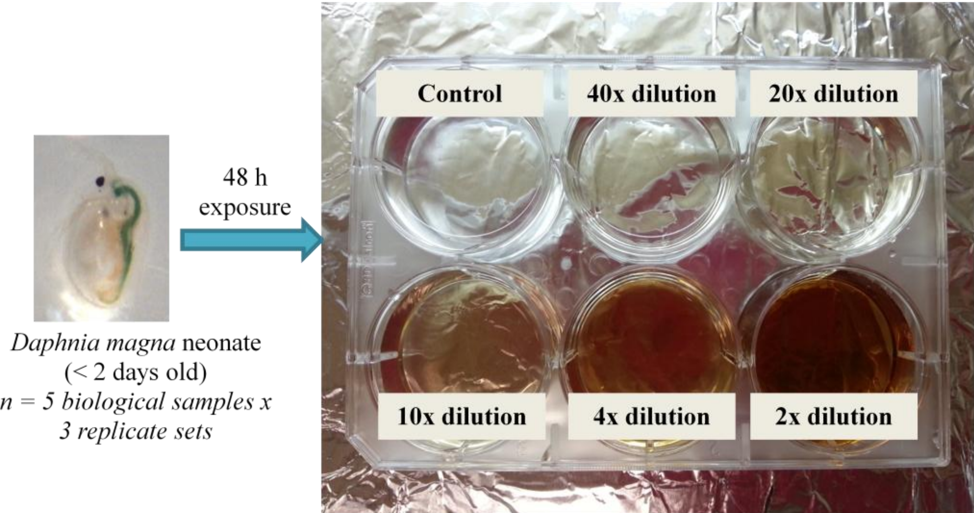


Figure 4.4 Illustration of the *Daphnia magna* toxicity test using 6-well-plates

The parameters and culture conditions such as the temperature and lighting cycle conditions, were similar to their growing conditions. The test species were not fed throughout the experiment to avoid any interference from the algae (food) being introduced. The oxygen concentrations and pH levels were also determined at the

start and end of the experiment. Immobilization and immortality was observed every 24 hours from the start of the experiment and were recorded.

The following sections 4.2.6.1 and 4.2.6.2 describes the following test treatments procedures for PFCs and leachates respectively. Some of the test *Daphnia magna* from the PFCs and leachates studies were also extracted and used for metabolomics analysis.

4.2.6.1 PFOA (PFCs)

The concentrations tested for PFOA in the *Daphnia magna* acute toxicity test are indicated in Table 4.3. The test treatment mediums were all prepared using serial dilutions of PFCs standards by using the ISO test medium.

Table 4.3 Concentrations of PFOA spiked in the respective treatments

Treatment	A	B	C	D	E
Concentration of PFOA (ppm)	0	20	50	100	200

4.2.6.2 Leachates

The descriptions of the leachates used in the study are illustrated in Table 4.4. The leachates were obtained from waste sludge or ashes and were all filtered though a 0.2μm filter before further testing. The pHs of all leachates obtained were all either measured to be in the neutral range or being acidified to the required pH by adding 0.3M nitric acid.

Table 4.4 Description of leachates obtained from different sources

Number	Leachates obtained from various samples
1	Singapore Public Utilities Board (PUB) Sludge
2	Fly ashes from construction wood gasification (CW FA)
3	Bottom ash from construction wood gasification (CW BA)
4	Bottom ash of pure woodchip gasification (PWG BA)
5	Bottom ash of 20% weight sewage sludge and 80% weight woodchip co-gasification (20SWCG)
6	Petroleum chemical process waste ash carbon soot (Carbon Soot)

For leachate acute toxicity test, the different test concentrations are all indicated in the Table 4.5. The leachate concentration (C/C_o) prepared is defined as C being the final diluted concentration and C_o as the initial untouched leachate concentration. The total volume of the each test treatment is 5ml.

Table 4.5 Concentrations (C/C_o) of various leachate samples in the *Daphnia magna* acute toxicity tests

Leachate Sample	Concentrations Tested (C/C_o)							
	0 Control	0.025	0.05	0.1	0.25	0.5	0.01	0.005
PUB **CW FA** **CW BA** **PWG BA** **20SWCG**	✓	✓	✓	✓	✓	✓		
Carbon Soot	✓	✓	✓	✓	✓	✓	✓	✓

4.2.7 Screening of toxic substances in environmental samples by LC-QTOF-MS and metabolomics

The objectives of this study are to illustrate that LC-QTOF-MS untargeted screening can be used as a technique for differentiating the different leachate composition and identify toxicants that are specific to a certain type of leachate sample. This is a follow up from Section 4.2.6 to determine what exactly is affecting the *Daphnia magna* based on the leachates' properties. Non-target screening of organic substances in leachate samples are then performed where all substances in the cleaned-up leachate extracts are detected by LC-QTOF-MS. By doing so, it is possible to identify certain groups of compounds which correlate to the toxicity of the leachate on *Daphnia magna*. The typical workflow consists of samples preparation, sample analysis, data processing and interpretation (Figure 4.5). Sample clean-up was performed using SPE using resin-based sorbent Isolute ENV+ cartridges at pH 3 and 7. A sample blank and SPE blank which constituted of ultrapure water and methanol respectively were also conducted.

(1) Sample Preparation	(2) Sample Analysis	(3) Data Processing	(4) Data Interpretation
•Leachate sample generation •Solid phase extraction (clean-up step) using Isolute ENV+ SPE cartridges	•Untargeted LC-QTOF-MS analysis •Dionex µHPLC and AB SCIEX Triple TOF 5600 •C_{18} Column •Positive and Negative Modes	•Analyses were conducted using MarkerView software and databases such as METLIN, Toxin and Toxin Target database and Mass Bank	•Compound verification, identification and toxicity evaluation

Figure 4.5 Workflow for untargeted analysis of toxicants in environmental samples

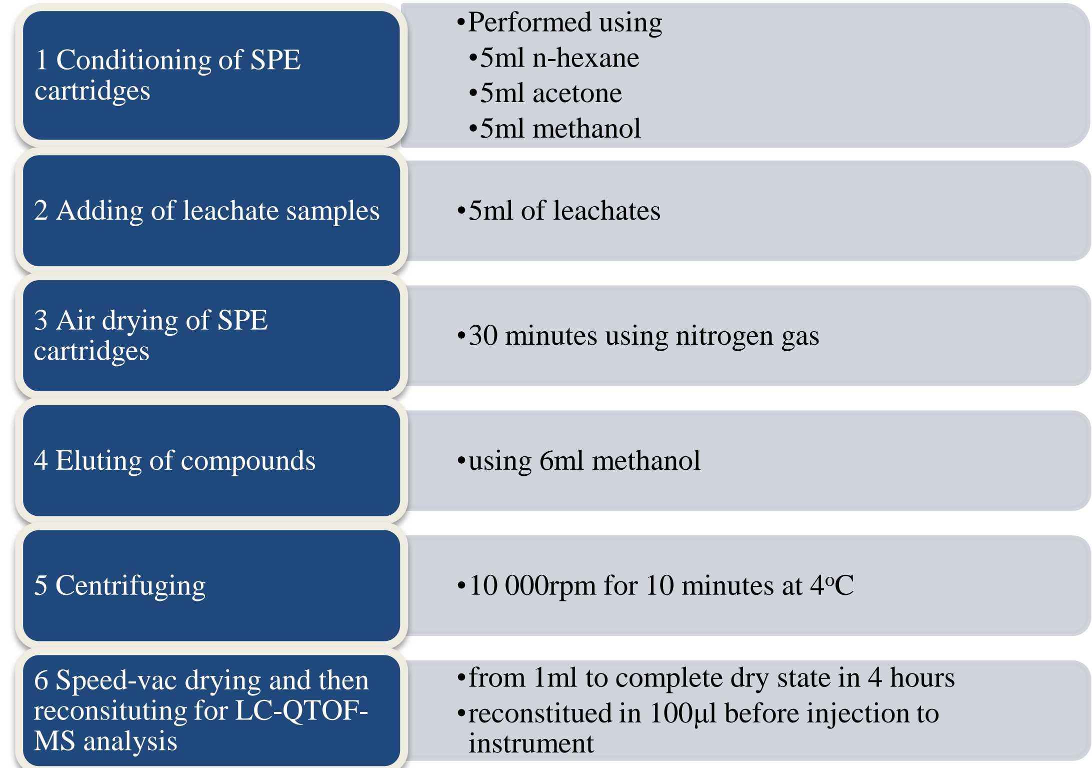


Figure 4.6 SPE sample preparation procedure

The extracts were then analyzed using HLPC-QTOF-MS. The LC column, mobile phases, LC and MS parameters are as programmed similarly in Section 4.2.5.1, with the exception of having injection volume of 10µL. The data processing is similar to the metabolomics section and with the inclusions of other relevant databases for toxins[243,244].

4.3 Results and discussion

4.3.1 Optimization of extraction of Daphnia magna in metabolomics studies

Each dried water flea was weighed using the electronic mass balance. However, due to the small mass and size of each *daphnia,* and taking into consideration the wind and laboratory conditions, difficulties were encountered and fluctuations to the mass of each water flea were observed. As a result, measurements of a pool of 20, 30 and 40 *daphnia magna* were performed instead. A plot of total mass against the number of *daphnia magna* gave a linear line indicating a constant average biomass measurement. Thus, the number of organism was used instead of the accurate biomass measurement of the organism to simplify the procedure. To ensure unbiased condition, the water fleas were also selected at random.

Several parameters were identified as critical in the optimizing of extraction procedure and efficiency of the extraction protocol of *Daphnia magna* metabolites (Table 4.6). There are being optimized in the said sequence too. The parameters in bold and underlined are the finalized parameters used in the optimized method.

Table 4.6 Parameters for optimizing of *Daphnia magna* extraction

No.	Parameters	Descriptions
1	Extraction technique	(A) Ultra-sonication in ice for 10 minutes **<u>(B) Homogenize for 25 secs and repeat once</u>** (C) Homogenize for 10 secs and repeat once
2	Percentage of extraction solvent (methanol)	60% **<u>70%</u>** 80%
3	Type of homogenizing beads (size and number)	Size of beads: CK14, **<u>CK28</u>** and CKmix Number of beads: **<u>3</u>** and 6 beads
4	Number of *Daphnia magna* required	Young (neonates and juveniles): 1, 3, **<u>5</u>** and 8 Adult: **<u>1</u>** and 3

In order to determine which parameter is the best, the liquid chromatograms in both the positive and negative modes were being examined to find the most number of peaks and the overall high intensity of the peaks. In general, for the *Daphnia magna* metabolites, there were more metabolites being detected in the positive mode compared to the negative mode. Each parameter was performed twice to confirm the reliability of the results. For the extraction technique, the procedure 'homogenization for 25 seconds and repeat' is chosen due to the higher intensity of peaks and also the number of the peaks detected as observed in Figure 4.7 and 4.8. In addition, the ease of the method surpasses that of the ultra-sonicator.

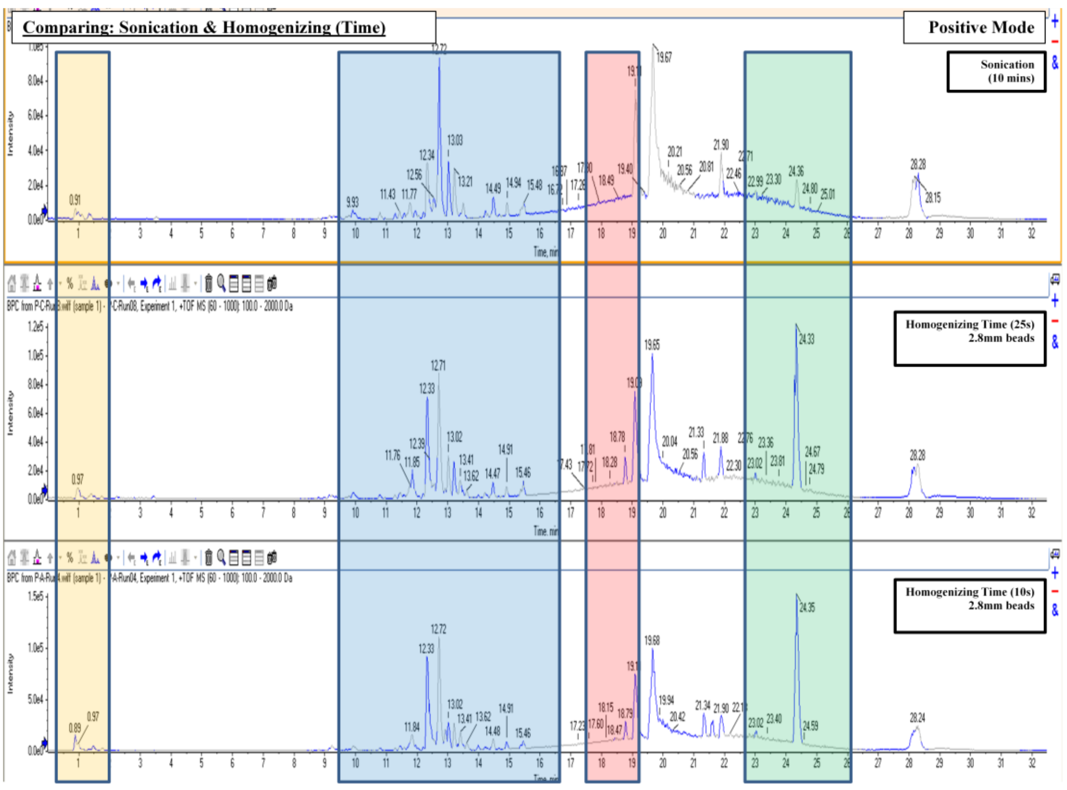


Figure 4.7 LC (positive mode) of *Daphnia magna* metabolites of different extraction parameters (top: sonication 10 minutes, middle: homogenizing for 25 secs, bottom: homogenizing for 10 secs)

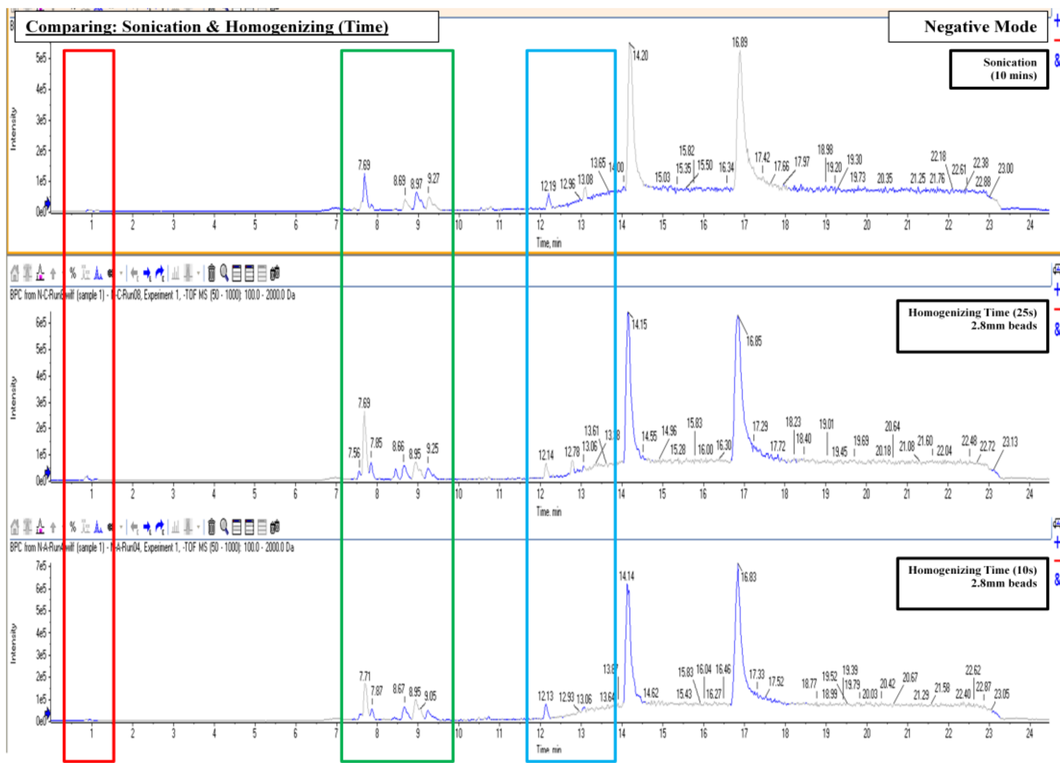


Figure 4.8 LC (negative mode) of *Daphnia magna* metabolites of different extraction parameters (top: sonication 10 minutes, middle: homogenizing for 25 secs, bottom: homogenizing for 10 secs)

For the percentage of methanol in the extracting solvent, the liquid chromatograms obtained (Figure 4.9 and 4.10) indicates that 60% methanol has generally lesser peaks while the extraction efficiency of 70% and 80% are comparable. Therefore 70% methanol was chosen in the optimal extraction protocol. For the homogenizing beads (type and number), three CK28 beads per homogenizing tube is sufficient to give the best extraction compared with the rest of the different type and number.

For the number of *Daphnia magna* to be used for analysis, one adult water flea was first extracted. With the biomass of just one *daphnia,* the intensities reproducibility and separation of the corresponding liquid chromatogram was sufficient for analysis. Therefore for analyses using adult *Daphnia magna*, just one is sufficient for a good analysis. This greatly reduces the number of samples and biological replicates required for a single set of experiment. The corresponding liquid

chromatograms for the different number of young *Daphnia magna* compared to an adult are shown in Figure 4.11. The chromatograms were all expanded to the same intensities (y-axis) to better observe the smaller peaks.

It is taken into consideration that the young neonates and the adult *Daphnia magna* may have different liquid chromatograms profile due to the presence of metabolites occurring at different growth stages. This may result in the occurrence of different peaks when comparing between the young and adult. Referring to Figure 4.11, 3 neonates will not be sufficient to give a good enough analysis due to the absence of some important peaks and the low intensities. 5 and 8 neonates are then considered in comparison to 1 adult water flea. Based on the chromatograms, it is concluded that 5 neonates give a sufficiently good intensity and peak number and is an optimum number of choice. Similarly, by having a lower number of water fleas required and with acceptable intensities greatly reduces the sample number and replicates required.

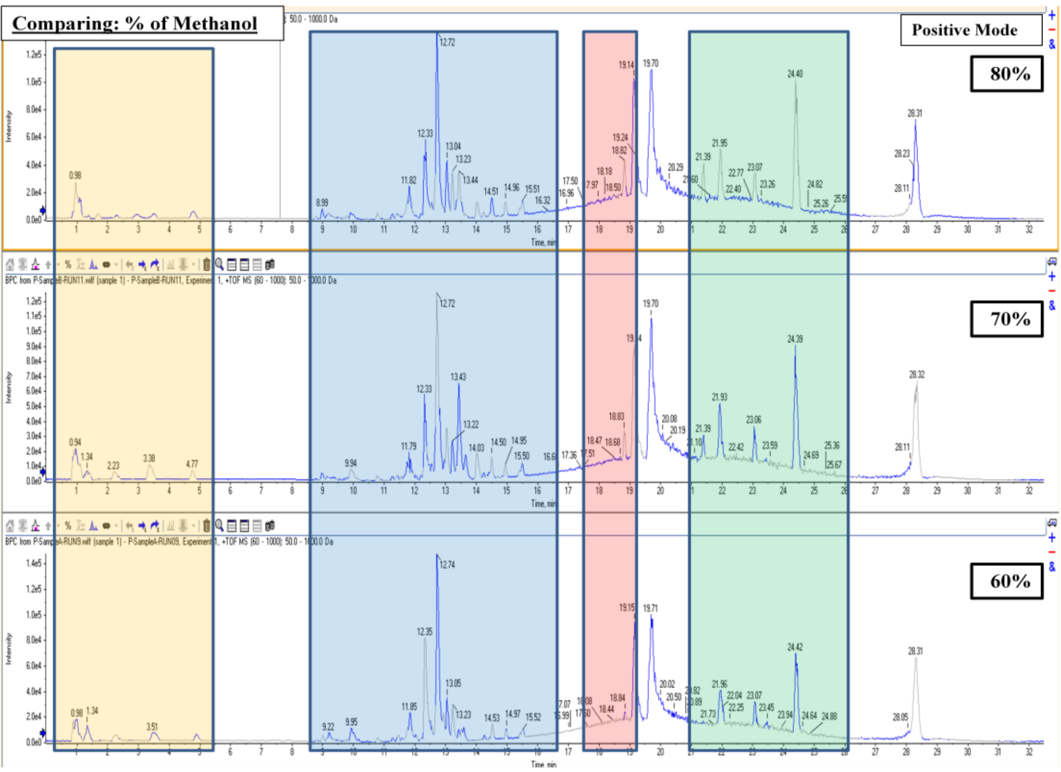


Figure 4.9 LC (positive mode) of *Daphnia magna* metabolites of different extraction parameters (top: 80% methanol, middle: 70% methanol, bottom: 60% methanol)

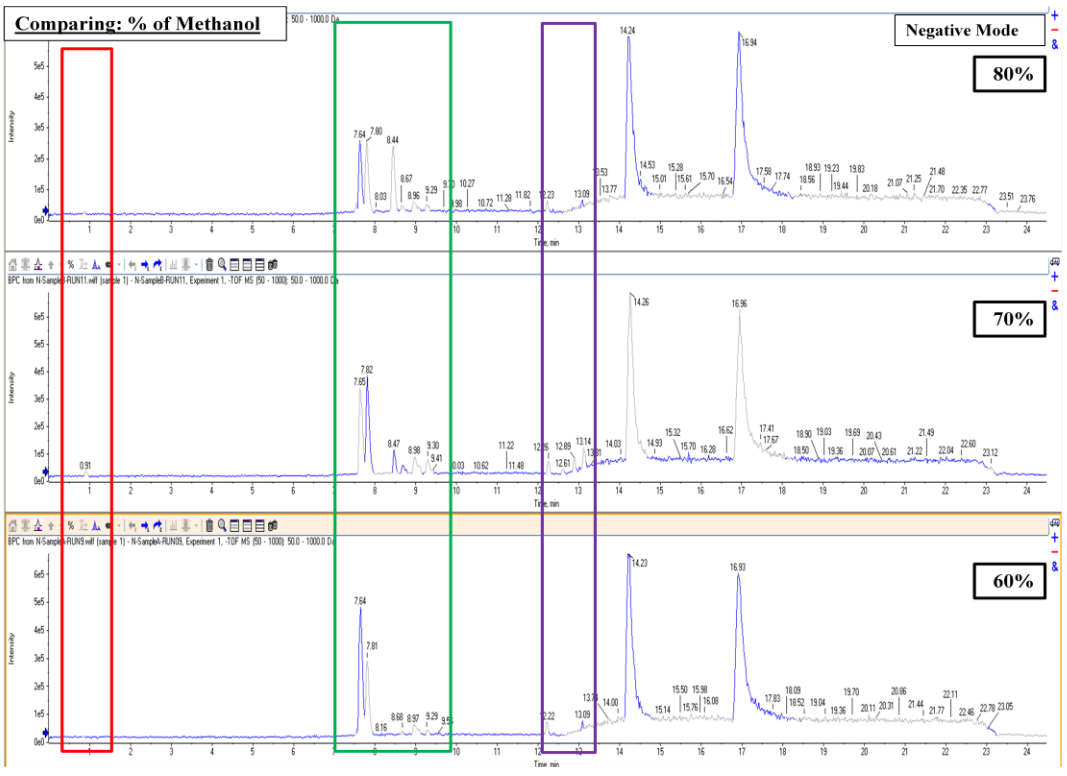


Figure 4.10 LC (negative mode) of *Daphnia magna* metabolites of different extraction parameters (top: 80% methanol, middle: 70% methanol, bottom: 60% methanol)

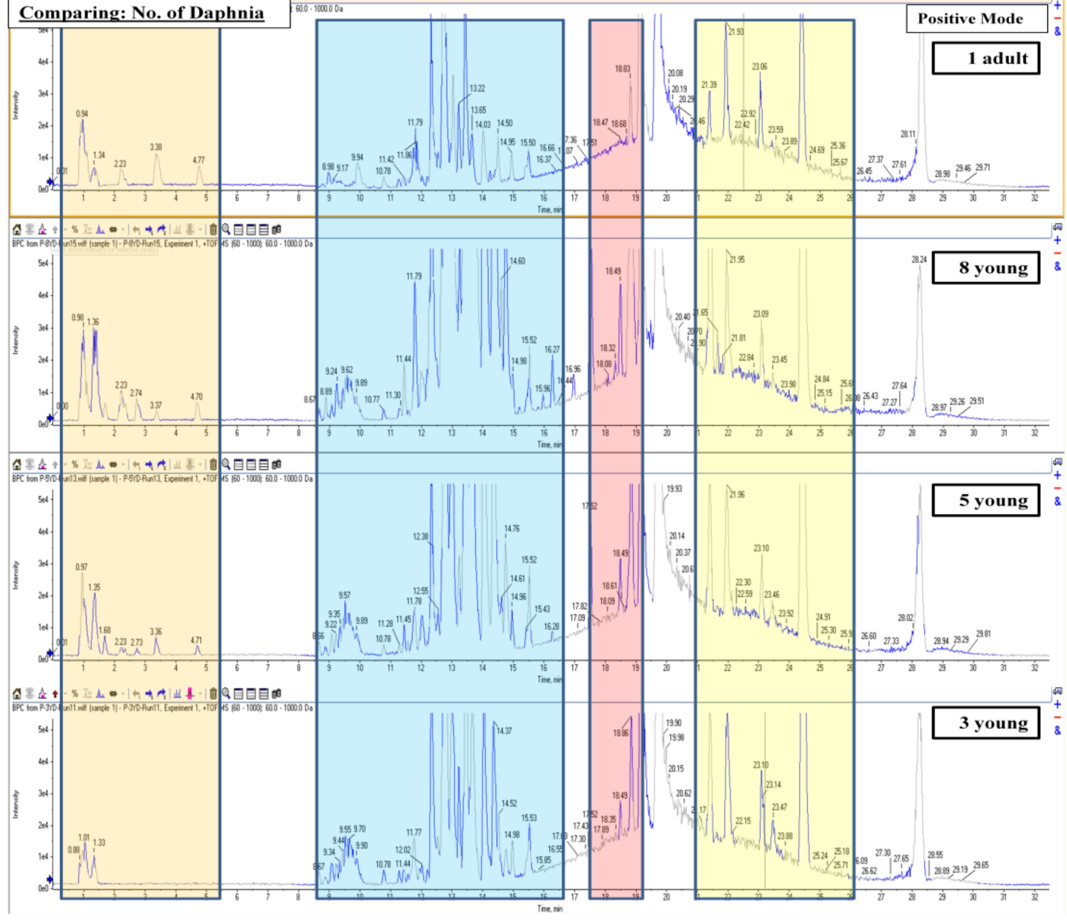


Figure 4.11 LC (positive mode) of *Daphnia magna* metabolites of different number of water fleas

Apart from the extraction optimization, sample degradation analysis was also performed to ensure that the nature of the sample is preserved and that the metabolites profiles are still accurate and indicates a good representative of the sample. It was concluded that the samples in reconstituting solvent should not be kept for more than 7 hours in $4^{o}C$ before injected into the LC-QTOF-MS. This is critical in running LC-QTOF-MS analyses in batches. In conclusion, the regions that consist of metabolites of interest are in the 0.5 to 5.5 mins, 8.5 to 16.5 mins, 17.5 to 19.5 mins and 21 to 25 mins for positive mode and 0.5 to 1.5 mins, 6.5 to 10 mins and 11.5 to 14 mins for negative mode. The optimized extraction method was then used for subsequent *Daphnia magna* metabolites extraction.

4.3.2 Metabolomics studies of Daphnia magna age group studies

Based on the few available *Daphnia magna* literatures[235], the metabolic profiles of it at different life stages do differ such as the lipids and fatty acid contents. It varies significantly in various developmental stages and this may in turn affect the sensitivity of it to certain pollutants and environmental factors. The aim of this study is to determine the most suitable *Daphnia magna* age groups for toxicity and metabolomics study.

For this study, the sample collection requires a large number of *Daphnia magna* at one time in order to get a good representation with identical growing conditions and no bias. Due to it being a micro-organism, it is difficult to synchronize all the reproduction timing, therefore the ages as stated in the samples may differ by up to a day in difference. This can cause slight metabolic differences and variations, especially to the neonates and juveniles age group *Daphnia magna*, as shown in the score plots in this section.

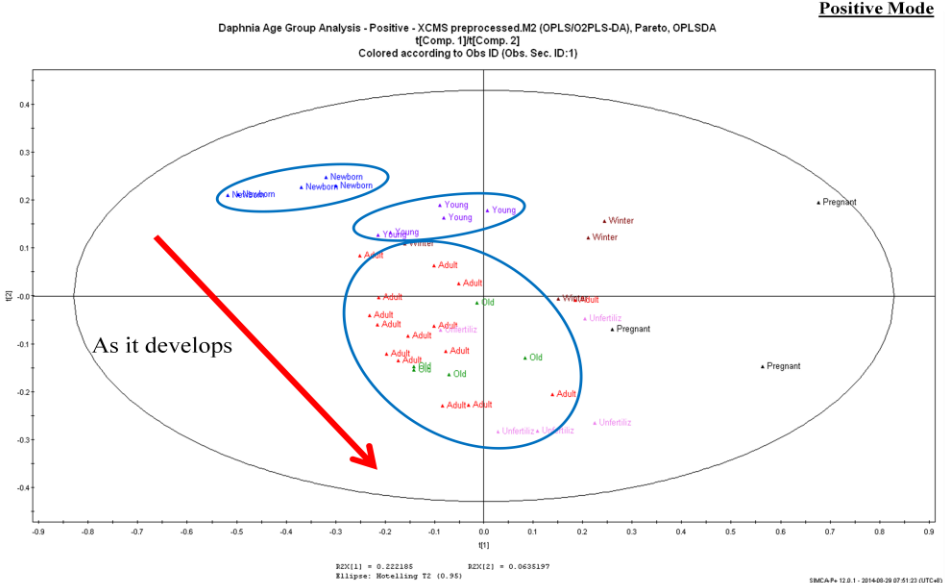


Figure 4.12 Score plot showing the relationship of different life stages of *Daphnia magna*. The LC-QTOF-MS data (positive mode) were normalized and pareto-scaled.

The water fleas from different age group were extracted and analyzed using LC-QTOF-MS. Result sets from both modes were processed using chemometrics and the obtained score plots are shown in Figure 4.12 and 4.13. From the results, there were large variations between the same samples. However, it is still possible to find segregation of certain groups especially the newborn (neonates) and juveniles (young). The metabolites in the newborn and young are quite different despite having only less than 2 days difference in ages. These young neonates are very susceptible to changes. However, it is difficult to really differentiate profiles for the rest of the age group, e.g. Adult, Old, and Pregnant with fertilized/winter/unfertilized eggs. There is a trend of changing LC profile (as shown in the red arrows) as the *Daphnia magna* matures.

Large variation found in “Pregnant/with eggs” category may be due to the size and number of eggs present in the *Daphnia*. According to these results, the effect of age is a very prominent factor in the metabolites in the water fleas, especially in the growing phase (first few days). As a result, the change in metabolites due to the

toxicant needs to be more significant and indicative than the change in metabolites due to the development phases in order to ensure that the effect is not masked out.

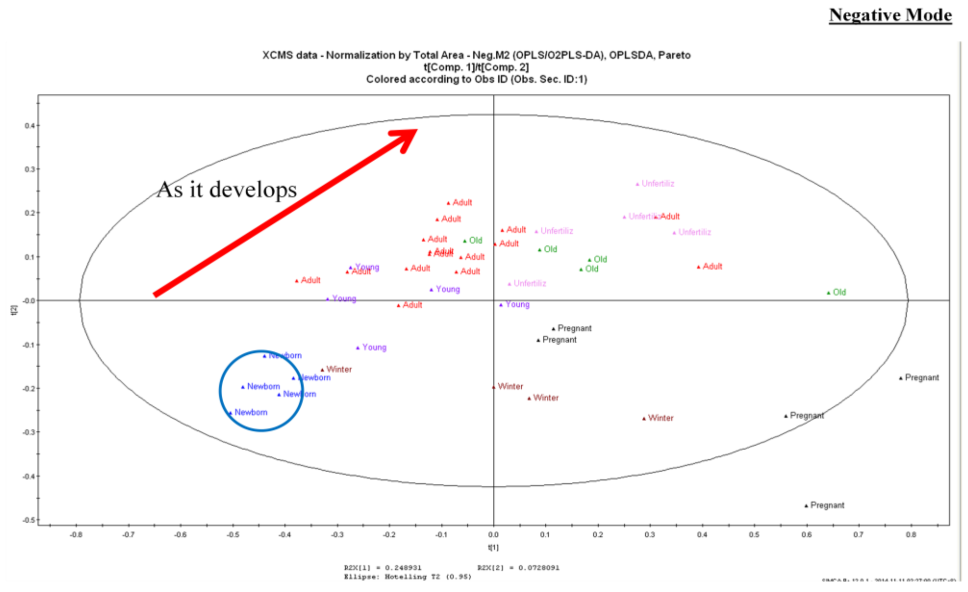


Figure 4.13 Score plot showing the relationship of different life stages of *Daphnia magna*. The LC-QTOF-MS (Negative mode) data were normalized and pareto-scaled.

Apart from the metabolomics study, there are several parameters that need to be considered carefully when using the various age groups for toxicity and metabolomics studies. Other than the age group, gender is also another issue that needs to be considered. It is ensured that favorable conditions in the culture are maintained to give an all-female clonal offspring. The type of reproduction and eggs are evidences of a good well-maintained favored culture conditions. In addition, throughout the toxicity and metabolomics experiment discussed in the next few sections, microscopic images of randomly selected *Daphnia magna* were performed and it was found that no significant male or inter-sex (gynandromorphism) characteristics such as an asymmetrical bi-valve-like carapace, were observed[245].

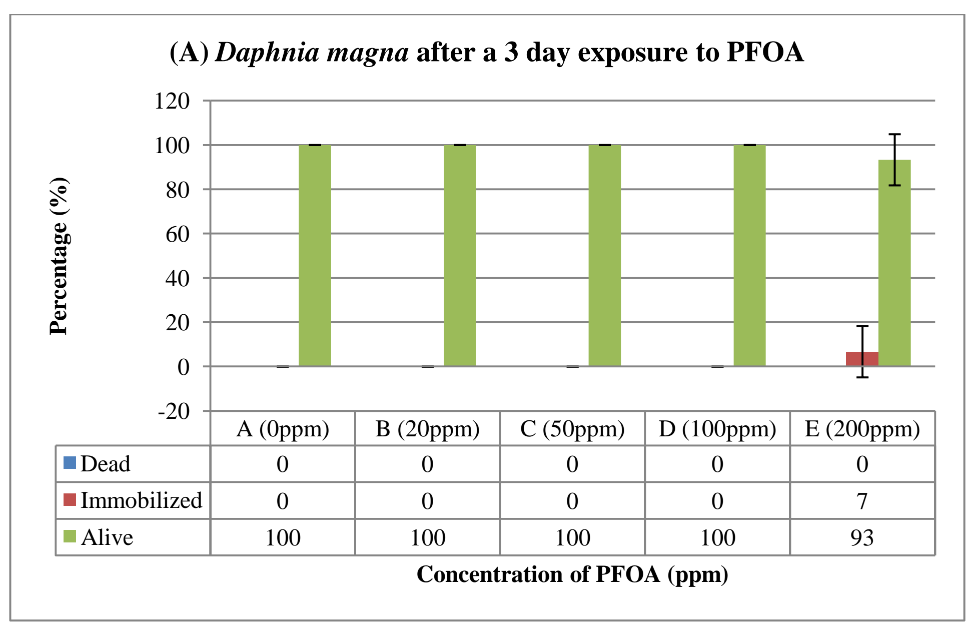
Table 4.7 List of considerations when using the *Daphnia magna* for toxicity and metabolomics analyses

Pros and Cons / Considerations when using the specific age group for toxicity and metabolomics analyses	
Young / Neonates	**Adult**
Food - does not need to be fed (no interference) - still surviving on maternal yolk	Number required for analyses - just require one per biological replicate for metabolomics
Very distinct growing phase - the development are obvious - only stays about 2-3 days in that life stage - sensitive	Very minute changes in developments during adult phase - hard to differentiate physically any differences between 10 and 24 days old
Number required for analyses - small in size and mass - require more samples to have sufficient biomass (As discussed in Section 4.3.1)	Sexually mature after 10 days - able to reproduce and have eggs to give new neonates - may mate during the experiment and give rise to unexpected variations

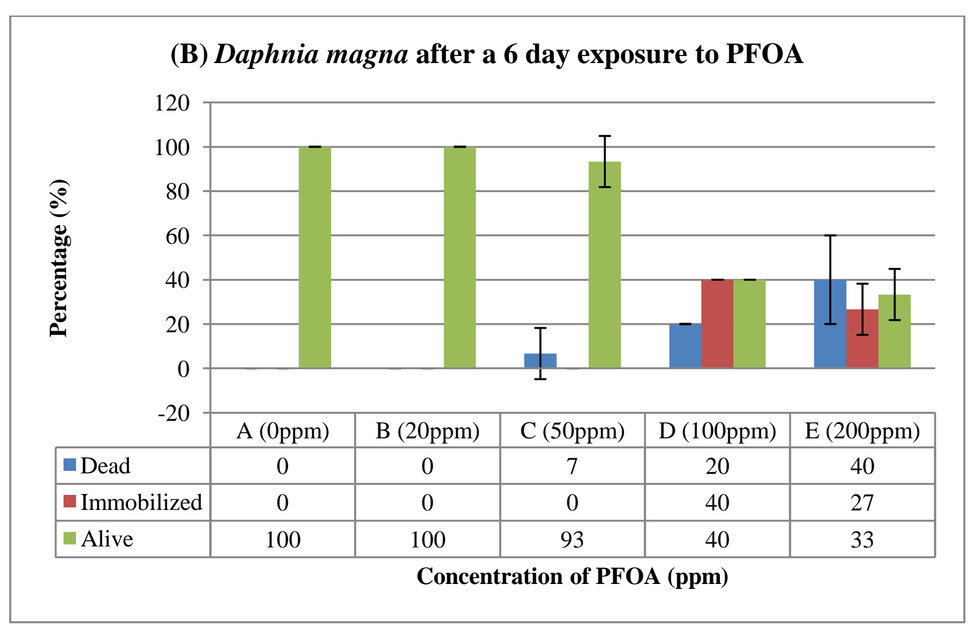
In conclusion, for studies lasting for last than 2 days (48 hours), it is possible to use the *Daphnia magna* neonates. For longer studies, the change in metabolites may be due to the change in the developmental stages instead of the actual toxicant. Apart from that, adult *Daphnia* are favored due to the ease of planning and preparation. However careful planning and execution needs to be performed for all *Daphnia magna* studies in order to obtain reliable results and achieve the aim.

4.3.3 Eco-toxicological studies of PFCs-exposed Daphnia magna

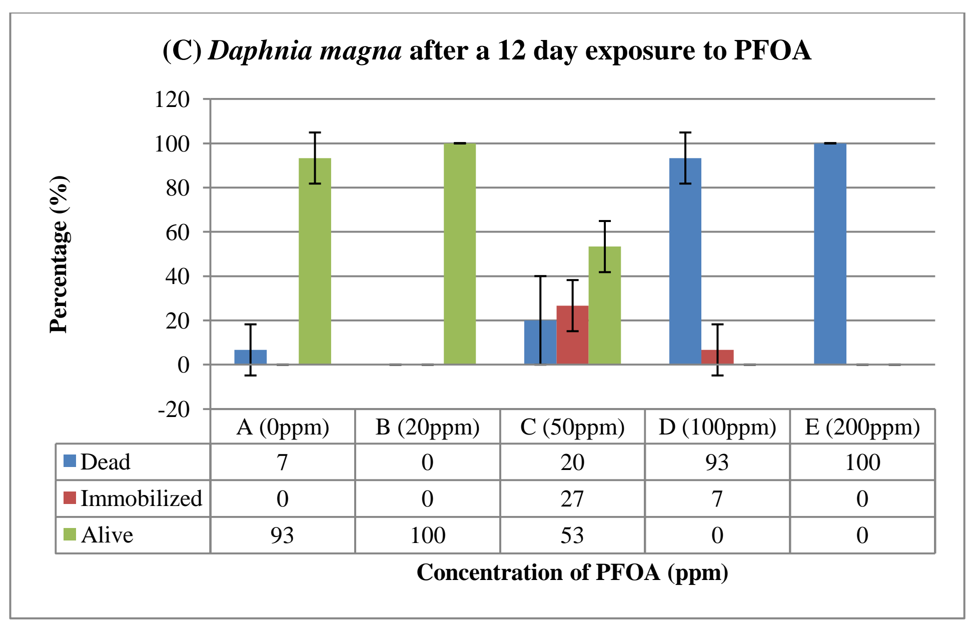
The concentrations explored for the PFCs-exposed *Daphnia magna* toxicity study was similar to the algae study in Chapters 2 and 3. This is to simulate the same toxic environment and to obtain any possible correlation if possible. The number of dead and immobilized water fleas were determined every 24-hour and the results for the 3, 6, 12 and 24 days after PFOA exposure is as shown in Figure 4.14 (A) – (D).



	A (0ppm)	B (20ppm)	C (50ppm)	D (100ppm)	E (200ppm)
Dead	0	0	0	0	0
Immobilized	0	0	0	0	7
Alive	100	100	100	100	93



	A (0ppm)	B (20ppm)	C (50ppm)	D (100ppm)	E (200ppm)
Dead	0	0	7	20	40
Immobilized	0	0	0	40	27
Alive	100	100	93	40	33



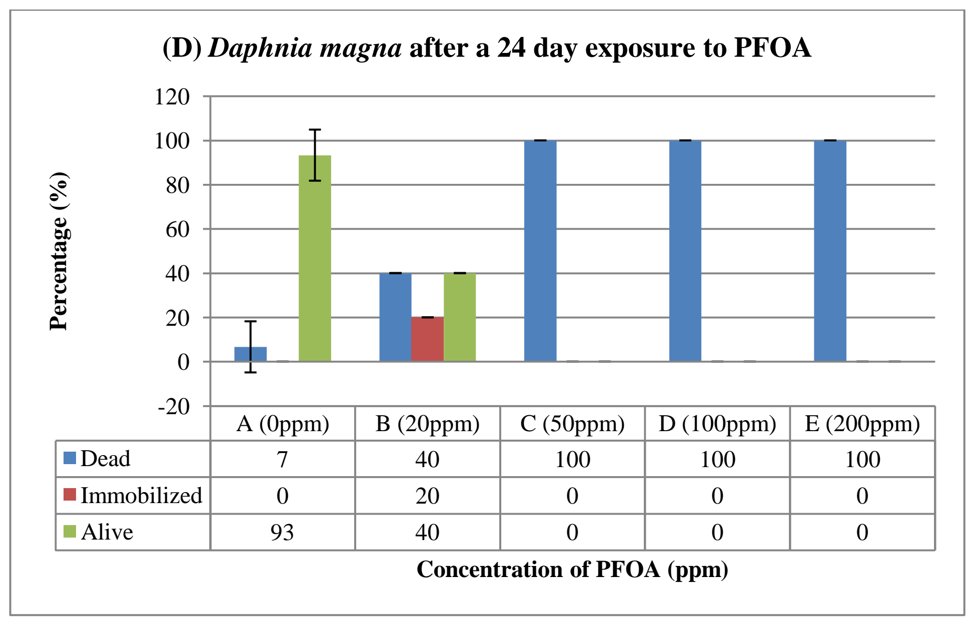


Figure 4.14 Outcome (dead, immobilized or alive) of *Daphnia magna* after a (A) 3, (B) 6, (C) 12, (D) 24 days after PFOA exposure.

Comparisons of the number of mortality or immobilization in the PFCs-spiked treatments were made with the control. For the 1st two days upon the exposure of PFCs, the control (with no PFOA spiked) and the 4 PFOA-spiked treatments, have no observations of having any immobilization or mortality effects. As the experiment proceeds, the effect of PFOA on the mortality and immobilization of *Daphnia magna*

is more significant. There are 2 different observations that can be obtained from these experiments, which are the higher the concentration of PFOA and the longer the exposure is, the larger the impact which causes the mortality and immobilization of the water fleas. With this study, it is observed that PFCs start to have an effect on *Daphnia magna* after 3 days at the highest PFOA concentration. PFCs does have an impact on regardless of the concentration they are exposed to, the effect will just take a longer period of time to surface.

The ages of *Daphnia magna* were less than 48 hours during the start of the toxicity experiment. As the experiment proceeds, within the concentration indicated initially, the water fleas have proceeded to grow out of the neonates phase and have also developed into the adult stage. With the comparison with the control, it is confirmed that the water fleas did not die due to suboptimal culture conditions but are fully due to the PFOA exposure. It is not fully understood that whether the PFOA have a greater effect on the neonates or on the *Daphnia magna* adults or whether the toxicant PFOA just require a longer time to fully impact and kill the water fleas. However the process of aging is not possible to stop and will always be a factor in toxicity and metabolomics study. This growing and aging process have to be well noted and be taken into consideration for any future studies.

Based on the literature, there have been reports of bioaccumulation of perfluoroalkyl substances in *Daphnia magna* in water[28,227]. It is evident that PFCs do accumulate in these water fleas via a body surface absorption. In addition, Dai et al have also observed that for shorter-chains PFCs, the concentration absorbed by the water fleas have reached a maximum and then equilibrated in 3 days. This coincides with the results obtained. In conclusion, the use of *Daphnia magna* for toxicity testing and water quality is demonstrated through this example.

4.3.4 Application of Daphnia magna in leachate toxicity and metabolomics analysis

The PFOA toxicity test described previously utilizes *Daphnia magna* where the bioaccumulation of PFOA can be determined and any acute and chronic effects caused by the toxicant can be monitored. On the other hand, this leachate toxicity test presented in this section is used for the toxicity evaluation for reusing and also safe disposal in the environment. Leachates from waste materials such as incineration ashes, sludge from waste water treatments and also domestic and food waste may pose critical consequences such as environmental pollution and contamination when disposed incorrectly. As reported by Clarke et al, landfill leachate is one of the sources of trace organic pollutants in the environment and PFCs are considered to be classified as one of the categories[246]. Apart from PFCs, there may be other unknown and new pollutants and toxicants that are present in the leachates[246,247].

In this section, it introduces a quick and reliable method to determine (a) the eco-toxicity of the leachates and (b) the biological and toxicological effect of the ashes by using *Daphnia magna*. The use of this test can be another form of control, where it is not available in current chemical analyses, to determine the synergistic effect of various contaminants present and other unknown compounds which are not monitored. This is due to the fact that the waste and or toxicant do not exist alone and everything is in a mixture, resulting that the environmental impact of a certain pollutant is not being classified only by its concentration but also by its interaction with various pollutants and organisms[248,249]. Therefore by having this test, it can ensure the safe disposal of waste and also bridge the gap of current chemical characterization tests and to achieve a more complete analysis.

The eco-toxicity tests of leachates from 6 different waste materials were determined using the *Daphnia magna* eco-toxicity test as described in the methods. The percent of immobilization and mortality observed are as shown in Figure 4.15. It

is noted that the immobilization and mortality refer to different degrees of toxicity where mortality of the water flea indicates a higher toxicity level in the leachate. However for the purpose of this rapid eco-toxicity test to determine the leachate toxicity level, immobilization and mortality of the *daphnia magna* are grouped together as a single parameter as it indicates an observed toxicity effect to the water flea as shown in the y-axis in Figure 4.15.

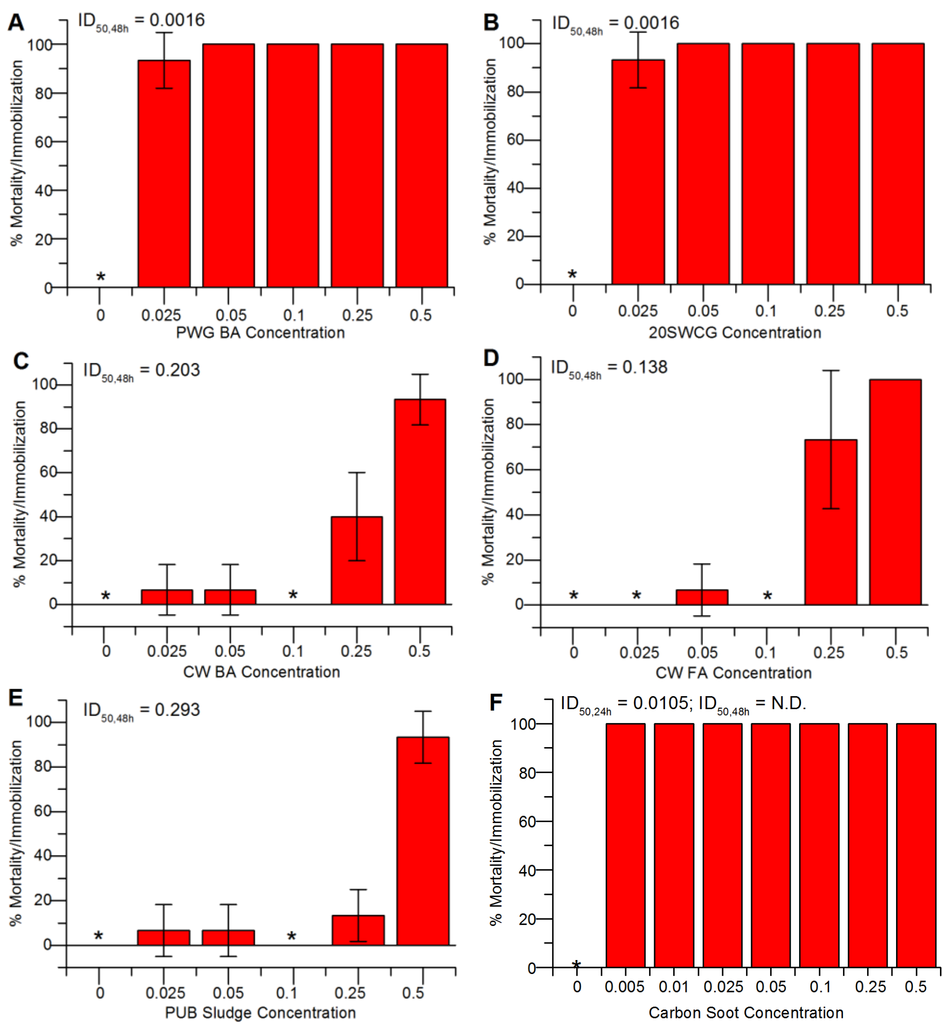


Figure 4.15 Mortality/Immobilization rate of *Daphnia magna* after 48h exposure to different leachates, ID_{50}, 48h represents the dose required to immobilize 50% of the test population at 48h. Test populations with no immobilized or dead organism are labeled with asterisk (*)

By referring to Figure 4.15, at least 5 different concentrations or dilution factors of the leachate samples and the effect to the water fleas were evaluated. All controls show no mortality/immobilization of *Daphnia magma* after 48 hours. Likewise, for 0.1 CW BA, 0.1 CW FA and 0.1 PUB Sludge, no mortality/immobilization was observed. However some toxic effects were noted for concentrations lower than 0.1 of CW BA, CW FA and PUB sludge. In these cases, 1 out of a total of 15 *Daphnia magna* neonates was immobilized/dead after a 48 hour exposure per treatment. Furthermore, based on the ID_{50} calculations and also the mortality and immobilization percentage, the toxicity of the leachates were found to be PUB sludge < CW BA < CW FA < 20SWCG & PWG BA < Carbon Soot, in increasing toxicity to *Daphnia magna*.

Surprisingly, the toxicity of PUB sludge is not as high as what was expected and has minimal effect on the water fleas. As a result, further dilutions of PUB sludge leachate can safely reduce the toxicity. This is also quite similar in CW BA and CW FA leachate samples. On the other hand, the leachates from carbon soot, PWGBA, 20SWCG is highly toxic to *Daphnia magna*, even with further dilutions. It is suspected that heavy metals present in the carbon soot, PWGBA and 20SWCG leachates are the main cause of the mortality/immobilization of the water fleas[234,250-256]. Based on these 48 hour acute toxicity tests, the results obtained can be used as a preliminary result for toxicity test and determine if any pre-treatment is necessary for waste treatment before disposal.

In addition, when comparing between the results for PFOA and for the leachates analysis, it is observed that these *Daphnia* are more responsive and sensitive to the toxicity found in the leachate analysis. This may have been due to the increased in toxicity in the leachate samples compared to the PFOA solutions. It has been determined that the quantity of PFCs found in all 6 of the leachate samples were only in the sub-ppb range and nowhere near the ppm range.

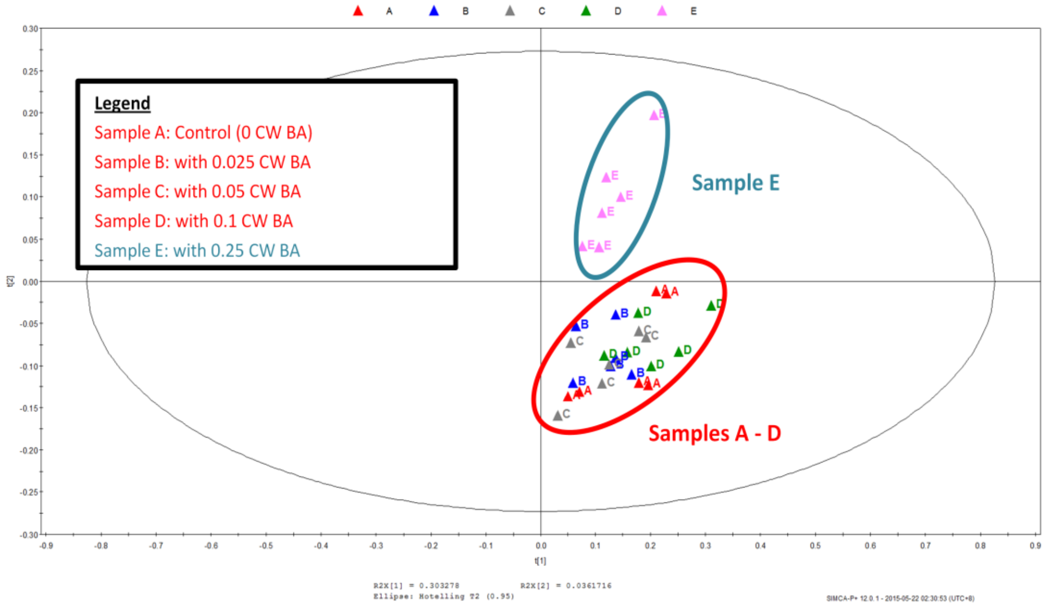


Figure 4.16 Score plot showing the relationship of *Daphnia magna* being exposed to different concentrations of CW BA sample. The LC-QTOF-MS data were normalized and pareto-scaled.

Apart from this, a preliminary metabolic analysis was performed using the *Daphnia magna* which was exposed to CWBA. The *Daphnia magna* exposed to (A) 0, (B) 0.025, (C) 0.05, (D) 0.1 and (E) 0.25 CW BA were extracted and the metabolites were analyzed using LC-QTOF-MS. *Daphnia magna* which is exposed to 0.5 CW BA was not included in this analysis, as the bodies of the water fleas are starting to disintegrate and the biomass obtained are not accurate and reliable. The results are illustrated in the score plot (Figure 4.16). The aim is to show a possibility of using *Daphnia magna* as a bio-monitoring species in aqueous environments and to identify any biomarkers specific to the toxicant.

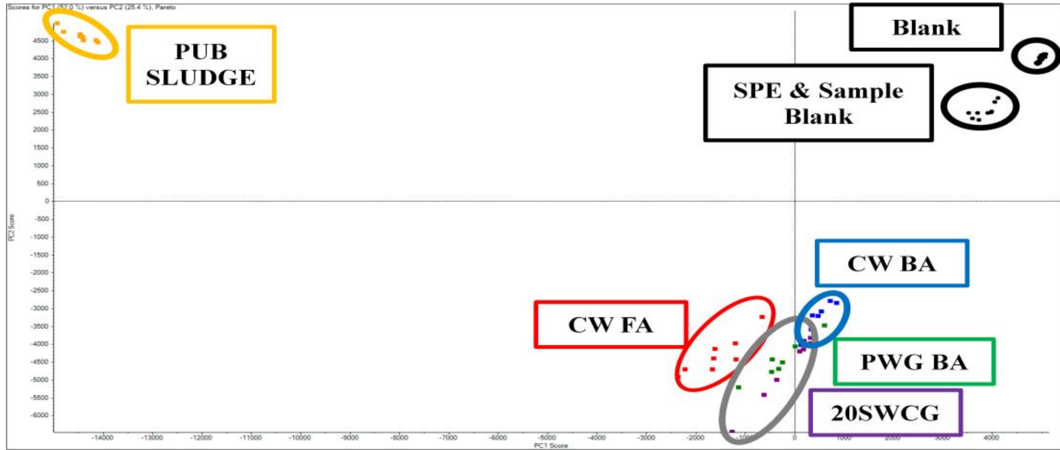
As observed, there is clear segregation of the metabolic profile between sample E and the rest of the samples A – D, including the control. When referring to Figure 4.15 Graph C, the ID_{50} is 0.203 and the leachate concentration in Sample E, 0.25 CW BA, is very close to this value. The reason postulated for this segregation and deviation from the control and the rest of the sample is due to the exposed concentration of 0.25 CW BA. The metabolites and the metabolic processes in the

test organisms in sample E have significant changes and is obviously different from the rest of the samples. This change also resulted in the immobilization and subsequent death of the water fleas. Given these points, the use of *Daphnia magna* as a test organism in toxicity and metabolomics analyses has been demonstrated and it can be further applied as a bio-monitoring species and biomarkers can be determined for environmental toxicity analyses.

4.3.4.1 Untargeted profiling of contaminants in leachates analyses

For the second part, in order to complement the previous analyses, a more comprehensive untargeted analysis of the leachates is performed by chemical characterization using LC-QTOF-MS. Currently, chemical characterization tests for toxicity waste determination are only searching for targeted groups of emerging contaminants, for example, personal care products and pesticides[257-259]. However this current method is relevant and important but it may not be sufficient. This technique that is introduced in this section can help to determine if there are any deviations from the various environmental samples and also detect and identify any newly found contaminants[260,261]. By using these complement methods; it is possible to monitor harmful pollutants as it may still pose a detrimental threat to the environment and humans.

(A) Principal Component Analysis (PCA) using MarkerView: LC-QTOF-MS (+) Mode



(B) Principal Component Analysis (PCA) using MarkerView: LC-QTOF-MS (-) Mode

Figure 4.17 PCA score plot (A) Positive mode and (B) Negative mode, showing the relationship between the different leachate samples. The LC-QTOF-MS data in both modes were normalized and pareto-scaled prior to PCA.

For this analysis, the leachates (PUB, CW FA, CW BA, PWG BA, 20SWCG) are subjected to the untargeted analysis using LC-QTOF-MS and undergo data processing using PCA. The results are as shown in Figure 4.17. Each LC-QTOF-MS runs represent a point on the score plot. The closer the points are, the higher in similarity in the LC chromatogram profiles. It is evident that the incinerator ash

leachate samples were chemically different in composition from the PUB sludge sample. Even the different type of ashes (bottom and fly ashes) can be differentiated using this method.

20SWCG consists of 80% weight of PWG BA ashes 20% weight of PUB sludge. However in the score plot (positive mode) shown in Figure 4.15A, there do not seem to have a clear distinct segregation for leachate samples PWG BA and 20SWCG. The chemical profiles appear to be similar. In contrast, in the negative mode, differentiation is observed. Therefore it is possible to even differentiate the different sources and composition of ashes by using this method in positive and negative modes.

Just focusing on Bottom and Fly Ash Leachates (LC-QTOF-MS Negative Mode)

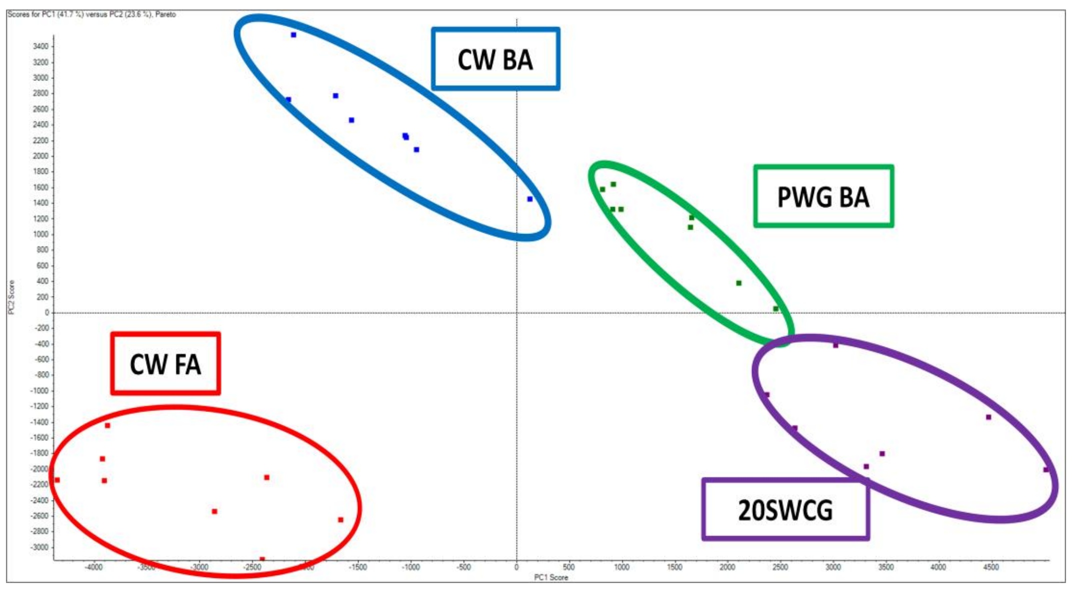
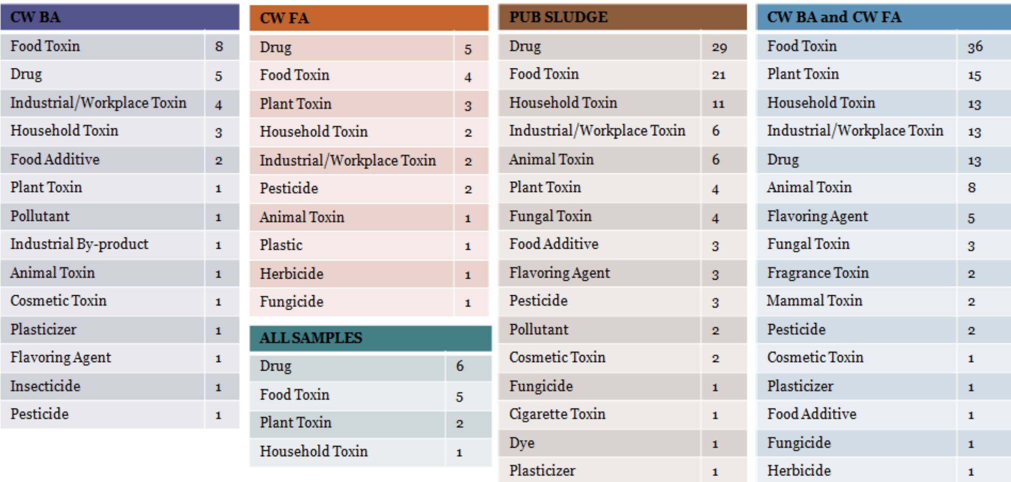


Figure 4.18 PCA score plot showing the relationship between the bottom and fly ash leachates. The LC-QTOF-MS data in positive mode were normalized and pareto-scaled prior to PCA.

Based on the loadings plot obtained, possible identities of contaminants present in these leachate samples were isolated using the mass/charge and fragmentation

values from the MS and listed. From the samples CW FA, CW BA PWG BA and 20SWCG, several classes of toxins found in the leachates are obtained. It is deduced based on the sample analysis obtained from the sources and also from the leachate. Further evaluations and identification needs to be performed using the commercial purchased toxins standards and more leachate samples.

Table 4.8 List of classes of toxins that are expected to be found in the respective leachates (based on LC-QTOF-MS positive mode analyses)



CW BA	
Food Toxin	8
Drug	5
Industrial/Workplace Toxin	4
Household Toxin	3
Food Additive	2
Plant Toxin	1
Pollutant	1
Industrial By-product	1
Animal Toxin	1
Cosmetic Toxin	1
Plasticizer	1
Flavoring Agent	1
Insecticide	1
Pesticide	1

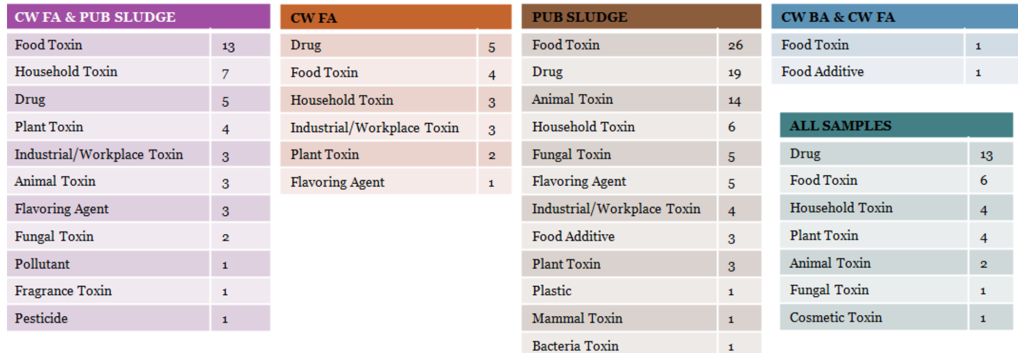
CW FA	
Drug	5
Food Toxin	4
Plant Toxin	3
Household Toxin	2
Industrial/Workplace Toxin	2
Pesticide	2
Animal Toxin	1
Plastic	1
Herbicide	1
Fungicide	1

ALL SAMPLES	
Drug	6
Food Toxin	5
Plant Toxin	2
Household Toxin	1

PUB SLUDGE	
Drug	29
Food Toxin	21
Household Toxin	11
Industrial/Workplace Toxin	6
Animal Toxin	6
Plant Toxin	4
Fungal Toxin	4
Food Additive	3
Flavoring Agent	3
Pesticide	3
Pollutant	2
Cosmetic Toxin	2
Fungicide	1
Cigarette Toxin	1
Dye	1
Plasticizer	1

CW BA and CW FA	
Food Toxin	36
Plant Toxin	15
Household Toxin	13
Industrial/Workplace Toxin	13
Drug	13
Animal Toxin	8
Flavoring Agent	5
Fungal Toxin	3
Fragrance Toxin	2
Mammal Toxin	2
Pesticide	2
Cosmetic Toxin	1
Plasticizer	1
Food Additive	1
Fungicide	1
Herbicide	1

Table 4.9 List of classes of toxins that are expected to be found in the respective leachates (based on LC-QTOF-MS negative mode analyses)



CW FA & PUB SLUDGE	
Food Toxin	13
Household Toxin	7
Drug	5
Plant Toxin	4
Industrial/Workplace Toxin	3
Animal Toxin	3
Flavoring Agent	3
Fungal Toxin	2
Pollutant	1
Fragrance Toxin	1
Pesticide	1

CW FA	
Drug	5
Food Toxin	4
Household Toxin	3
Industrial/Workplace Toxin	3
Plant Toxin	2
Flavoring Agent	1

PUB SLUDGE	
Food Toxin	26
Drug	19
Animal Toxin	14
Household Toxin	6
Fungal Toxin	5
Flavoring Agent	5
Industrial/Workplace Toxin	4
Food Additive	3
Plant Toxin	3
Plastic	1
Mammal Toxin	1
Bacteria Toxin	1

CW BA & CW FA	
Food Toxin	1
Food Additive	1

ALL SAMPLES	
Drug	13
Food Toxin	6
Household Toxin	4
Plant Toxin	4
Animal Toxin	2
Fungal Toxin	1
Cosmetic Toxin	1

4.4 Conclusion

In this chapter, the use of *Daphnia magna* as a test organism in toxicity and metabolomics research is being evaluated and discussed. Several applications involving persistent organic pollutants such as perfluorooctanoic acid and leachates are studied and shown. For the experiments using PFOA, the higher the concentration and the longer the exposure is, the greater the impact to the water flea. PFCs began to cause an effect on *Daphnia magna* after 3 days at the highest PFOA concentration.

Careful experimental planning with regards to the use of *Daphnia magna* in toxicity and metabolomics analyses need to be executed. As such, the procedure to obtain the optimized extraction protocol for *Daphnia magna* is obtained with several factors such as the extraction time, composition of extraction solvent, the number of biomass required. Also, the age group and life stages of *Daphnia* need to be taken in consideration carefully as it will greatly affect the results.

In all, it is demonstrated that the use of both 48-hour toxicity test using *Daphnia magna* and also the untargeted analysis using LC-QTOF-MS is critical in determining the toxicity and safety of any waste materials such as PFOA and leachates. These analyses presented a fast and also comprehensive method for determination of toxicities. Therefore, with the use of both chemical characterization and eco-toxicological characterization by *Daphnia magna*, it further complements and serves to be a better method in fully determining the eco-toxicity of the substance-of-interest.

5 Overall conclusion and future perspectives

PFCs are a class of POPs that have a long half life and they do not break down easily. Due to this unique PFCs' persistent nature, poor elimination and ability to bioaccumulate in the environment, it has been detected worldwide in air, water, soil and even in wildlife and humans. These compounds, even at low concentrations are extremely toxic to the environment. Cancer, reproduction and developmental deficiencies in animals and humans have been associated with the exposure of PFCs. In order to fully evaluate the toxicity and impacts in the environment, it is important to note that analytical quantifications of PFCs alone may not be a sufficiently good gauge to do so.

The main route of PFCs exposure is through ingestions. Being key trophic organisms in the aquatic food chain, *Chlorella vulgaris* and *Daphnia magna,* these aquatic organisms have been introduced in this thesis to aid and provide a more complete analysis of PFCs, its environmental effects, toxicity and bioaccumulation situations in the aquatic ecosystem. Different techniques of analytical determinations, toxicity studies and environmental metabolomics together were demonstrated to better understand the bioaccumulation of PFCs and its impacts to the aquatic environment and other organisms.

Chapter 2 investigated the fate of highly toxic PFCs, PFOA and PFOS upon acute 96-hour exposure to green microalgae *Chlorella vulgaris.* Bioaccumulations of PFOA and PFOS have been observed through the tandem LC-MS/MS algae biomass and culture medium quantification results. In addition, growth inhibition of Chlorella vulgaris upon exposure to PFCs has been determined by the UV-Vis cell optical density results. PFCs are adsorbed on the algae cell membrane upon initial exposure. Observation of algae cells through microscopic images have shown strong aggregations and clumps formation which represents evidences of severe cell membrane disruptions caused by PFOA and PFOS. Chapter 3 investigates further on

the metabolic changes occurring in *Chlorella vulgaris* upon acute PFCs exposure through environmental metabolomics approaches. Both nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography tandem mass spectrometry (LC-MS/MS) are used in untargeted metabolomics mode to investigate the metabolome. Optimizations of both techniques have been demonstrated. Based on the concentration-time study conducted, the LC-MS/MS profile of the metabolites shows significant differences. Several lipid molecules have been identified to show the significant cell membrane disruptions and activation of a defense mechanism.

In Chapter 4, *Daphnia magna*, a common aquatic toxicity test organisms, was being used for metabolomics studies. The various considerations, advantages and disadvantages of using *Daphnia magna* of different age groups and have been discussed and explained using metabolomics. The *Daphnia magna* extraction protocol for metabolomics studies have been optimized and have then been applied for eco-toxicity determination of PFCs and waste ash leachates. According to the eco-toxicity test described, the immobility and mortality of the water fleas observed in an acute or chronic period are used to determine and differentiate the level of toxicity and quality of the water sample. MS-based metabolomics approach was also applied to determine the possible contaminants present in these ash leachates. With this, these techniques of using Daphnia magna indicates the level of water quality quickly and adequately and also determine possible acute and chronic environmental effects.

Overall, these aquatic microorganisms *Chlorella vulgaris* and *Daphnia magna* serve as a good starting point for bioaccumulation, metabolomics and eco-toxicity studies. With the results obtained in this thesis, it can be a foundation for understanding the mechanism of PFCs bioaccumulation in the aquatic food chain. Since, *Daphnia magna* feeds on green microalgae, further investigations can be made by focusing on metabolic effects of *Daphnia magna* when consuming PFCs-adsorbed

Chlorella vulgaris. It can determine the biomagnification and environmental impacts of PFCs and provide more insights.

In reality, the environment consists of mixtures of toxicants and not just an individual pollutant. The introduction of these aquatic organisms in eco-toxicity determination can also take into consideration the synergistic effects of pollutant that may occur and give rise to a decrease or more critically, an increase to the toxic effects. The adsorption of PFCs and disruptions with the algae cell membrane or water flea may cause the other more toxic pollutants to interact effectively in a way to cause a more severe effect.

Furthermore, *Chlorella vulgaris* and *Daphnia magna* are also available in fresh water lakes in the environment. As the bioaccumulations of PFCs in the environment and wildlife organisms are still ongoing and the consequences of this bioaccumulation and biomagnification are still largely unclear and very concerning, the practical application of these organisms for biomonitoring and bioremediation purposes can be possible and more indicative in the future. Currently, with no strict regulations and restrictions imposed on these PFCs in all countries, the ongoing battle with these persistent compounds, its precursors and even similar substitutes will continue. Therefore the method proposed can assist in monitoring and understand the underlying theory, ultimate effects and impacts of PFCs to the environment.

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LIST OF PRESENTATIONS AND MANUSCRIPTS

List of Presentations and Manuscripts in Preparation

1. Lee, S.N., Chong, W.J.B. and Li, S.F.Y. *Detection of perfluorinated compounds in the aqueous environment and its metabolomics effects on microalgae, Chlorella vulgaris.* Poster presentation at Euroanalysis XVII 2013 (European Conference on Analytical Chemistry), Warsaw University of Technology, Warsaw, Poland, August 25-29, 2013.

2. Lee, S.N., Zhang, W.L., Rong, L., Maneerung, T., Wang, C.H., Neoh, K.G. and Li, S.F.Y. *Eco-toxicity determination of bottom ash leachates using Daphnia magna and MS-based metabolomics approach.* Presentation at All Material Fluxes on River Ecosystem 2015 (AMFR 2015), Peking University, Beijing, China, January 15-18, 2015.

3. Lee, S.N., Zhang, W.L. and Li, S.F.Y. *Investigation of the fate and metabolomics effect of perfluorinated compounds (PFCs) upon acute and chronic exposure to aquatic organisms, Chlorella vulgaris and Daphnia magna.* Manuscript in preparation.

4. Lee, S.N., Zhang, W.L. and Li, S.F.Y. *Eco-toxicity determination of bottom ash leachates using Daphnia magna and MS-based metabolomics approach.* Manuscript in preparation.