Toxicity and bioconcentration properties of Perfluorosulfonic acids (PFSAs) on the microalgae *Scenedesmus obliquus* UTEX 393

by

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Abstract

Recently, it has been found that Perfluorinated Alkyl Acids (PFAAs) are potentially harmful to humans and the environment. Used in some industrial applications for many years, these compounds are very persistent and do not easily degrade in the environment. Furthermore, they are suspected to be carcinogenic and endocrine disruptors in higher animals. Further evidence has shown the potential for bioaccumulation or transmission through food webs in aquatic systems. The purpose of this study is to test the toxicity and bioconcentration of a mixture of three Perfluorosulfonic acids (PFSAs) substances, a subclass of PFAAs, on the green algae *Scenedesmus obliquus* using a set of standard approaches. Bioconcentration of the PFSA mixture was investigated by exposing a growing culture of *S. obliquus* to two different starting concentrations of PFSAs. Sampling over time of the aquatic concentrations, and measurements of adsorbed and absorbed concentrations of PFSAs of algae biomass samples from the end of the growth trial, was performed to allow a mass balance on PFSA fractions.

The *Scenedesmus obliquus* Half Maximal Effect Concentration (EC_{50}) for mixed PFOS, PFBS, and PFHxS is 120.35 mg/L. The concentration of Chlorophyll a, Chlorophyll b, and total Chlorophyll gauge the cellular health, and the results show the PFSAs mixture does stress algae cells after seven days of exposure, as seen through declining chlorophyll concentration compared with the control group. In PFSAs mixture bioconcentration test, 0.733 μg/g PFOS, 0.653 μg/g PFHxS, and 1.33 μg/g PFBS were found on the algae cell, and 0.0248 μg/g PFOS, 0.00199 μg/g PFHxS, and 0 μg/g PFBS were detected in the algae cell from 0.01 mg/L PFSAs mixture concentration. At higher exposure concentrations of 0.1 mg/L PFSAs mixture, 13.8 μg/g

PFOS and 16.54 μg/g PFHxS were found on the algae cell, and 0.184 μg/g PFOS and 0.127 μg /g PFHxS were detected in the algae. Bioconcentration factors calculated to be 2.79 mL/g, 0 mL/g, and 0.23 mL/g for PFOS, PFBS and PFHxS, respectively, at the 0.01 mg/L PFSAs exposure concentration; and 2.63 mL/g and 1.83 mL/g PFOS and PFHxS, respectively, at the 0.1 mg/L PFSAs exposure concentration. All of the results from this experiment provided some basic knowledge about the PFSAs and inform further studies on ecological transfer in food webs of these and other PFAAs compounds.

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List of Abbreviations

AFFF	Aqueous Film Forming Foam
ANOVA	Analysis of Variance
BAF	Bioaccumulation Factor
BBM	Bold Basal Medium
BCF	Bioconcentration Factor
BMFs	Biomagnification Factors
CAT	Catalase
EC_{50}	Half Maximal Effect Concentration
EPs	Emerging Pollutants
HDPE	High-Density Polyethylene
MDA	Malondialdehyde
OECD	Organization of Economic Cooperation and Development
PCBs	Polychlorinated Biphenyls
POPs	Persistent Organic Pollutions
PFAA	Perfluorinated Alkyl Acids
PFAS	Perfluoroalkyl substances
PFBS	Perfluorobutanesulfonic Acid
PFCs	Poly- and Perfluorinated Compounds
PFHxS	Perfluorohexanessulfonic acid
PFOS	Perfluorooctanesulfonic Acid
PFSA	Perfluorosulfonic Acid

ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
SPE	Solid-phase Extraction
STPs	Sewage Treatment Plants
TLs	Trophic Levels
TMF	Trophic Magnification Factor
VOCs	Volatile Organic Compounds

1. Introduction

1.1. What are the PFSAs compounds

Widely used in industry applications, Perfluorinated Alkyl Acids (PFAA) are as desirable in industry as they are a risk to the environment. PFAAs is a class of compounds with hydrophobic (water resistant), fluorine-saturated carbon chain and a hydrophilic (water attractive) polar function group[1]. Chemical characteristics, which include thermal inertness, chemical stability, light transparency, and amphiphilic nature, make those compounds not only globally distributed, but also environmentally persistent and potentially harmful. Perfluorosulfonic acid (PFSAs) is one sub-class of Perfluorinated Alkyl Acids (PFAA) that have these same features. Through the manufacturing process by industrial factories, consumer applications, and post-use disposals, Perfluorosulfonic acid (PFSA) compounds such as Perfluorooctanesulfonic acid (PFOS), Perfluorobutanesulfonic acid (PFBS) and Perfluorohexanessulfonic acid (PFHxS) are discharged into the environment. Ongoing background studies and problems show increasing interest in the effects of PFAAs and PFSAs in the environment.

Perfluorosulfonic acids (PFSAs) are synthetic organic acids consisting of a fluorinated carbon chain as the main structure capped with sulfonate functional groups. The main elements of PFSAs are carbon and fluorine [2]. The chemical structure of PFSAs is C_nF_{2n+1}-SO_3H, similar to other perfluorocarbons but with a different functional group. Many of these compounds, such as Perfluorooctanesulfonic acid (PFOS), have been in production and use since the 1950s in many stain-resistant consumer products, waterproof materials, and non-flammable and non-sticky surfaces. These compounds form the functional component of many products such as aqueous film-forming foam (AFFF), used in fire-fighting, leather [3], waxes, polishes, and detergents for metal, fabric protectors [4], and carpet surfaces.

Environmental traces of PFSA compounds are found worldwide, especially in industrialized countries such as in Europe (UK, Poland, Spain, Germany et al.), North America (USA, Canada) and Asia (China, Japan) [5]. There are a variety of pathways for PFSA compounds to get into the environment. However, there are two potential major exposure pathways of PFSAs to humans, shown in Figure 1. For example, people can be exposed to the PFASs directly through a contaminated environment and from consumer products. PFAS compounds have been used in industry to form the materials of non-stick surfaces, such as AFFF and consumer products. Wastes from these industrial processes still contain PFSAs and enter into the waste infrastructure, often with direct release into the aquatic environment and drinking water sources [6]. Currently, PFSA compounds have been found in in the environment of many countries all around the world, including Australia [7], Germany [8], Norway [9], Sweden [10], United Kingdom [11], United States [12], Japan [6], China [13-15], India [16], Thailand [17], France [18], Netherlands [19], Spain, and Korea [20, 21]. Humans can consume PFSAs by ingesting contaminated drinking water and seafood [10, 22]. In addition, infants can be exposed through breastfeeding and cord blood [23]. In the United States, PFASs have been found in nearly every state [24]. In some states, such as California, North Carolina and New York, many people were exposed to PFASs from drinking water and on military sites [25]. The broad level of contamination and exposure globally, and the persistence of PFSAs in environmental systems, demands a level of concern over the effect of PFAS contamination on human health.

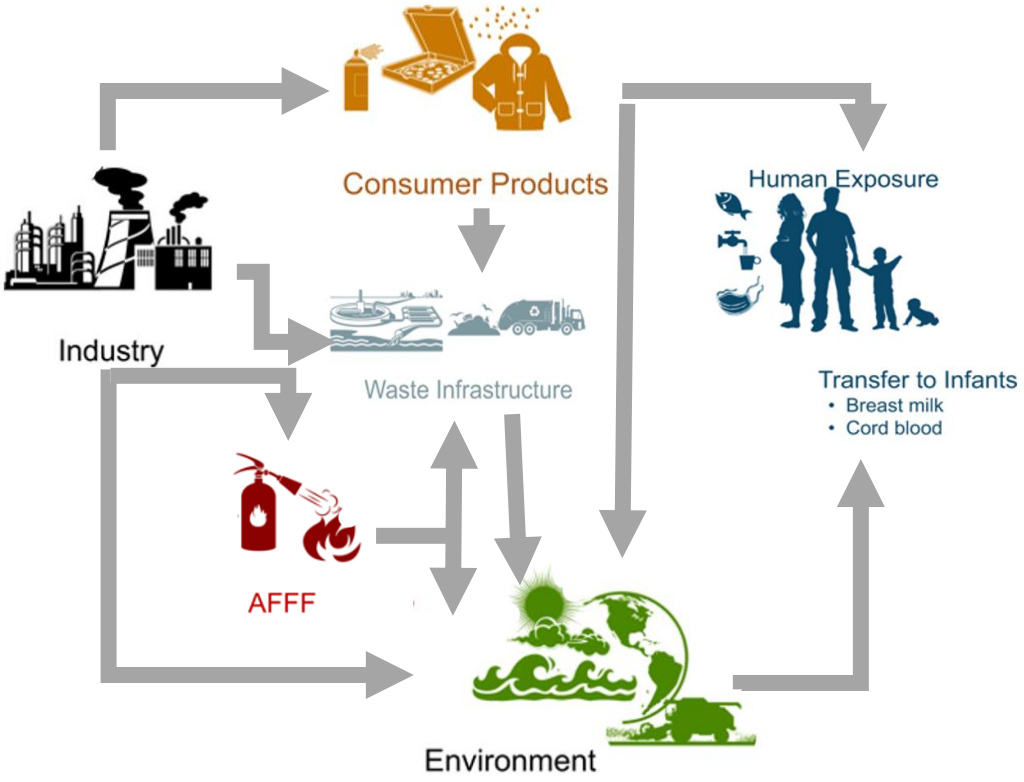


Figure 1. Potential major exposure pathways of PFSAs to humans and the environment (adapted from Smith et al. 2011)

The PFSA compounds are subclass of an overall class of manufactured industrial compounds called perfluoroalkyl acid (PFAA). A recent report indicates 2060 perfluoroalkyl substances (PFAS) compounds are known for use on the global market, and it is estimated that at least 3000 PFASs have been manufactured and are in use globally [26]. PFOS-based compounds were invented and produced through electrochemical fluorination to obtain the synthetic precursor perfluorooctane sulfonyl fluoride by the 3M Company in 1949 [27], and have been in use globally since that time, creating a global-scale problem of concern.

The family tree of PFASs can indicate the relationship between different compounds (Figure 2.). All PFASs are classified according to their molecular size, determined by the number of carbons in the carbon chain, and the different number and arrangement of functional groups.

These molecular characteristics set the reactivity, persistence, and solubility properties of the molecule, determining their transport and presence in environmental and biological systems. Perfluoroalkyl acids (PFAAs) and PFAA precursors are two main classes of the perfluorooctanoic acids (PFASs). PFAA precursors are the large (eight or greater carbons) fluorochemical compounds. They can be converted into PFAAs by biological and abiotic degradation in the environment [28]. Currently, many PFAA precursors, fluoropolymers, and perfluoropolyethers (PFPEs) have not been adequately studied compared to PFAAs, which have been among the most common in use and includes compounds such as PFCA, PFSA, PFPA, PFECA, PFESA, and PFPiAS [26]. Generally, all PFAS compounds are more simply classified as long-chain or short-chain PFAS, noting the substantial behaviroal differences between them. First, short-chain PFAS compounds with less than six carbon atoms [29] do not usually undergo either abiotic or biotic degradation at typical environmental conditions because of the highly stable carbon-fluorine bond. As such, they are often considered to be highly-stable transformation products resulting from degradation of long-chain PFAS compounds [30]. Short-chain PFASs have a greater mobility and lower adsorption partition than long-chain PFASs because of the lower log K_{ow} number, which indicates the fractioning between polar and non-polar liquids [31]. Because of this high mobility and low adsorption, it is difficult to completely remove short-chain PFAS from the environment using typical pollutant treatment technologies [32].

Due to the studies about chemical and physical properties of long-chain PFSA compounds, concern emerged regarding long-chain PFAAs found in the environment. The long-chain PFAAs also has stable carbon-fluorine bond and stronger absorption partition compared with short chain PFAAs. Therefore, the long chain PFAAs shows more bioaccumulation,

bioconcentration and biomagnification ability than short chain PFAAs in the environment. In 2002, the OECD determined that PFOS and related compounds have the potential for toxicity and bioaccumulation in organisms, posing a threat to the environment and human health [33]. Because of these considerations, the 3M Company phased out production of PFOS globally, in response to environmental concerns, and global production dropped significantly, although manufacturing continues in nations outside the U.S. In 2016, the US Environmental Protection Agency (EPA) published the PFOS health counseling level of 70 ng/L total PFSA compound concentration in drinking water [34]. Despite these efforts, PFAS compounds continue to be found in water systems worldwide, increasing concern as one of the most severe public health challenges for the future.

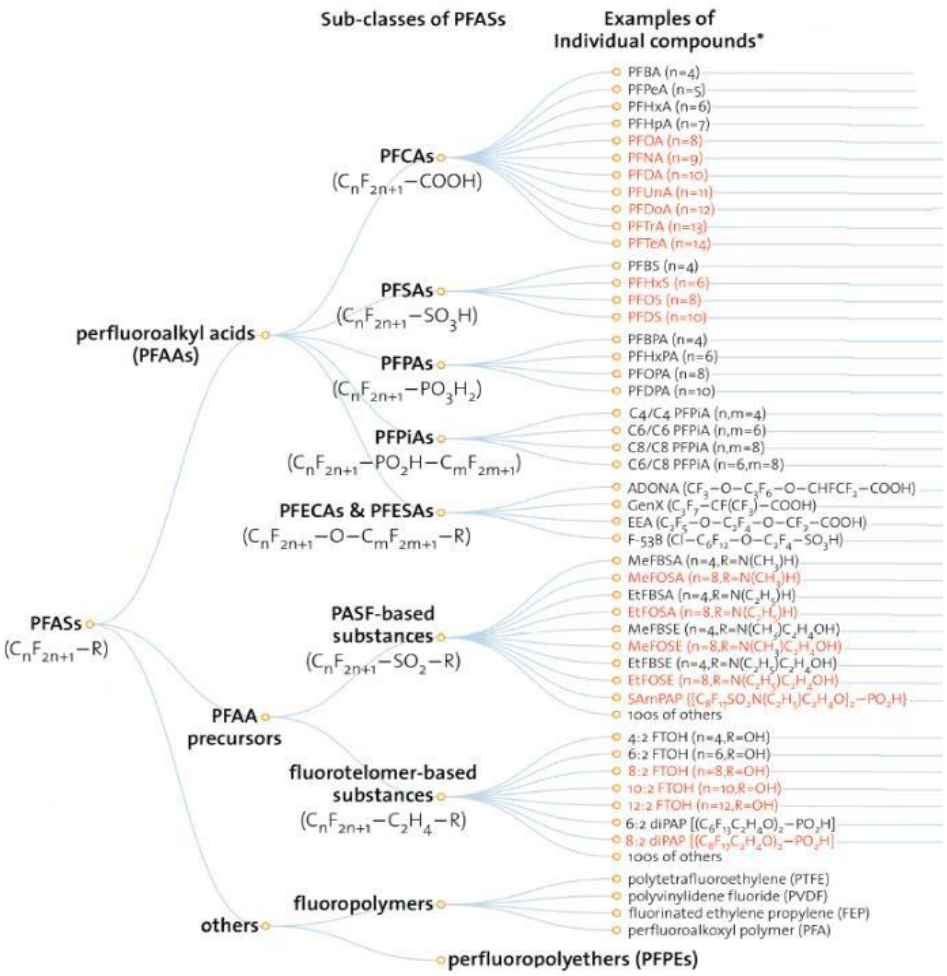


Figure 2. The family tree of PFASs and examples (modified from [26]).

It is generally accepted in the academic community that long-chain PFASs pose a greater inherent environmental hazard than short-chain PFASs, so the international community has gradually eliminated the use of long-chain PFASs in various fields [35]. Perfluorobutanesulfonic acid (PFBS) and Perfluorohexanesulfonic acid (PFHxS) are short-chain PFSAs that are widely used as substitutes for the original long-chain PFASs in a variety of application areas [3]. The compound PFBS, which is known to have a shorter half-life in an organism body than longer-chain compounds, is used as a substitute for the higher homologs [36]. In contrast, PFHxS has a similar or even longer half-life than PFOS in animal and human tests; because of this, PFHxS products may not be the most appropriate substitute for PFOS [37].

1.2.What is Phytoplankton

Phytoplankton are an integral part of the plankton community as autotrophic organisms and are vital as the base of the food chain in aquatic ecosystems. They are also critical to stable productivity and ecological balance in freshwater systems. Although microscopic, as a community they can grow to dominate the aquatic environment and can be seen by their particular color (chlorophyll and pigments) through the water surface. Some phytoplankton algae are considered as indicators of water quality because they play an important role in purifying eutrophic water and are sensitive to environmental changes [38].

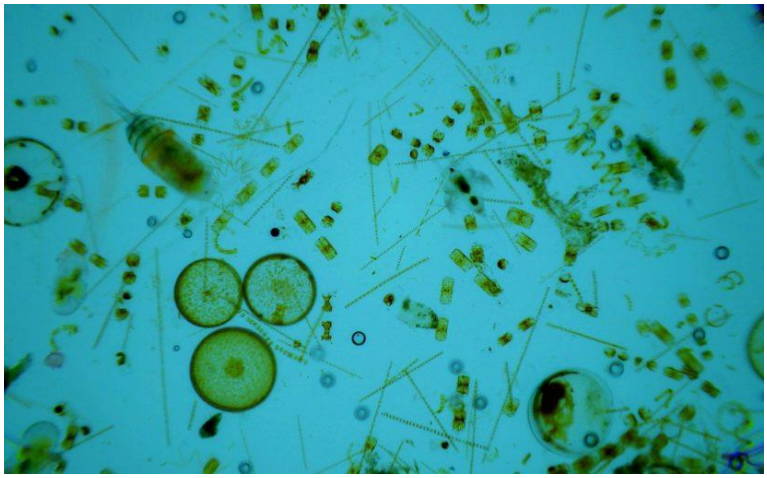


Figure 3. An example of a phytoplankton community from an aquatic ecosystem, showing many different taxa of Phytoplankton [39].

Scenedesmus obliquus is one type of phytoplankton that is found in freshwaters all over the world (Figure 3.). It is a common freshwater plankton that tends to reproduce in nutrient-rich water. *S.obliquus* is often one of the foundation species of the aquatic food chain in freshwater systems. It is sensitive to poisons, easy to obtain, small in size (only 2-3 micrometers in diameter), and fast in reproduction. As such, *S. obliquus* is often used in the laboratory to assess toxicity of substances. Because of the chemical and physical characteristics of PFASs, the influence of these substances on many generations and population levels of *S. obliquus* algae can

be rapidly evaluated. The cell membrane of *S.obliquus* is a critical site for more PFASs compounds to combine with and adhere to because the membrane phospholipids are an amphiphilic substance that can efficiently combine with and accumulate PFASs [40]. Also, the cell membrane surface area of *S.obliquus* is larger than many other phytoplankton in freshwater systems. In response to chemical changes in the water environment, *S.obliquus* populations begin to be dominated by four- or eight-cell colonies instead of a single cell [41], increasing the exposed surface area and amplifying the bioaccumulation and biomagnification ability of PFASs on *S. obliquus* compared to other phytoplankton taxa. *Scenedesmus obliquus* is also easy to grow in batches and has thus been a classic indicator of water quality assessment, having a wide range of applications in the biotechnology and toxicology research areas [42]. Overall, the effect of exogenous pollutants such as PFAS on *S.obliquus* can affect energy transfers within the aquatic food chain, which in turn affects higher organisms and the entire aquatic ecosystem.

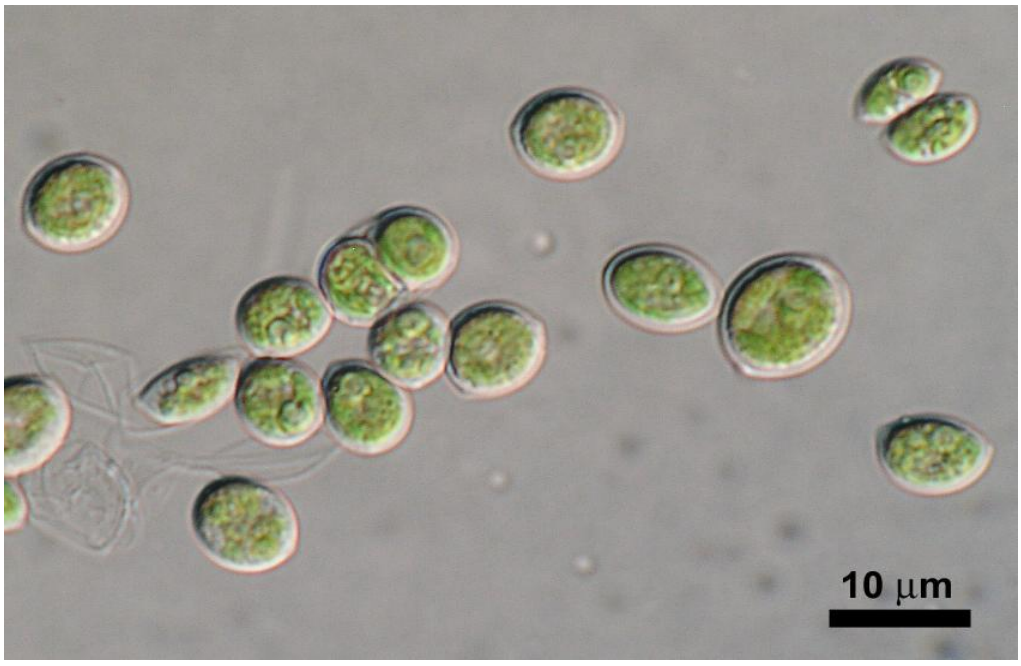


Figure 4. Scenedesmus obliquus under the microscope [43].

Bioaccumulation indicates the ability of organisms to accumulate pollutants relative to their presence in the environment. It is the final result of all ingestion and loss processes (such as breathing and dietary intake), losses due to eating, passive diffusion, metabolism, and transmission to future generations (Figure 5.) [44]. Bioconcentration is the specific process of chemicals transferring from water to organisms, resulting in higher concentrations in organisms than in the water. Biomagnification refers to the transfer to the trophic food chain. Because of transfers through trophic interactions, biomagnification results in an increase in the concentration of chemicals at higher trophic levels of the food web. Many manufactured and industrial compounds, such as PCBs, metals, and some volatile organic compounds (VOCs) have historically been identified as bioaccumulation agents [45]. The bioaccumulation potential of perfluorinated compounds has only recently been identified[46], and there is current interest in studying this process to understand the impact on environmental systems.

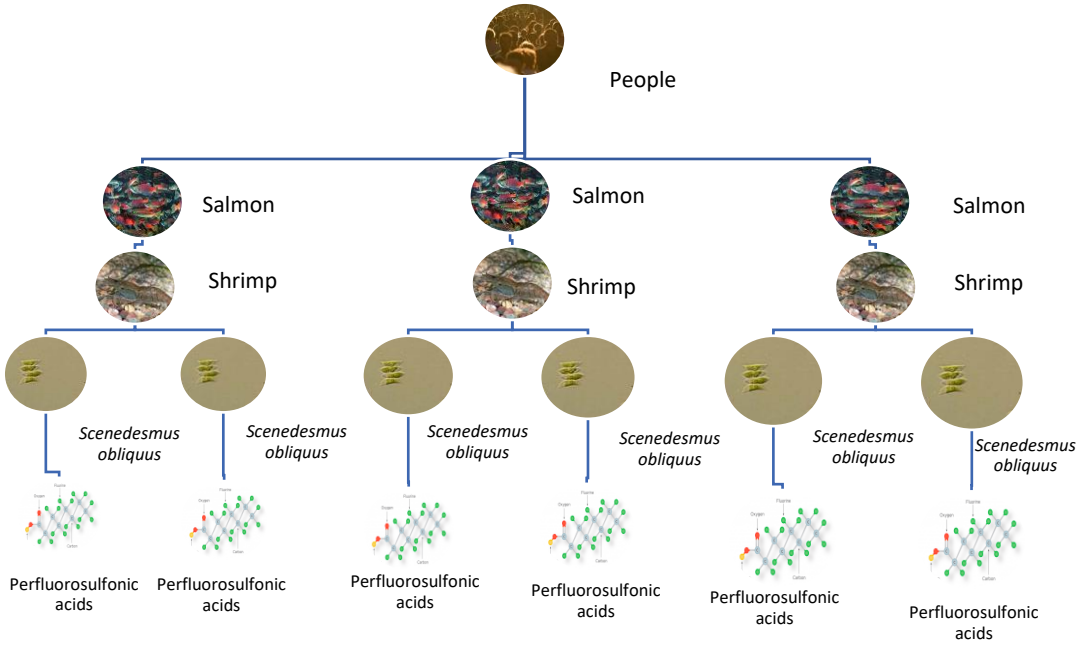


Figure 5. Bioaccumulation of contamination to live organisms in a trophic food web.

1.3. The influence of PFSAs in the environment

Generated through the manufacturing process by industrial factories, consumer applications, and post-use disposals, PFSA compounds are often discharged directly into the environment, where they are of concern as contaminant compounds. However, many PFSAs are found in the environment resulting from the natural breakdown of other industrial polyfluoroalkyl chemical precursors [47] (Figure 6.). Studies of PFOS show their pathway into the environment, through the emission from human-generated sources, and have demonstrated near-global dispersion of PFSA compounds, which have been found in open seas around the world [48], and in tissues of Arctic animals with no known human contact [49]. There are many risks when PFASs enter the human body through drinking water, as they are suspected to damage fetal development and growth, basal metabolism, immune systems and endocrine function [50]. The high dispersion levels of PFASs result from their relatively non-reactive characteristics, which explains their persistence in the environment and transport routes through environmental advective processes of the global hydrosphere and biosphere. As such, they are classified as emerging pollutants by the US Environmental Protection Agency (EPA), indicating their accumulating concentration in specific parts of the environment and emerging awareness of potential toxicity effects at those concentrations [51].

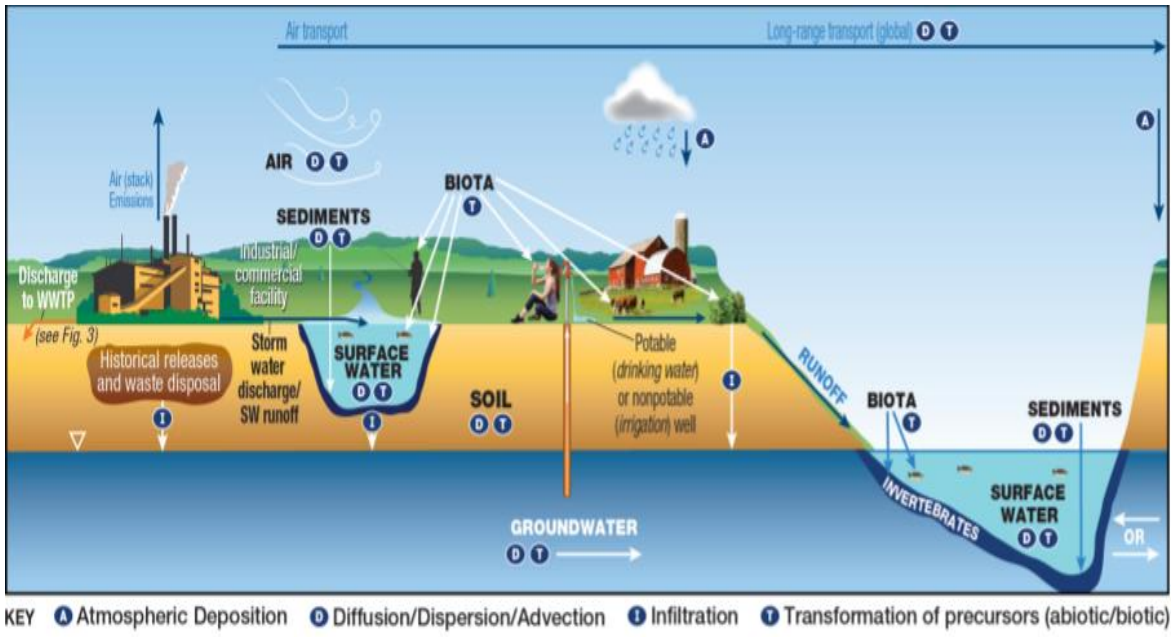


Figure 6. Environmental fate and transport for Per- and Polyfluoroalkyl substances [52]

1.4. Purpose of research

There is emerging concern over PFSA compounds as a novel harmful substance by a lot of environmental organizations. The chemical structures of PFSAs have polycarbonate-fluorine bonds which increased stability. They cause bioconcentration, bioaccumulation and biomagnification through lower-level organisms to higher trophic level organisms in the food web, including humans. *Scenedesmus obliquus* is one basic species of the freshwater food web and also a fundamental model organisms for toxicity studies for PFAAs [13-15]. From the literature, studies have looked at the toxicity levels for single PFAA compounds, such as PFOS or PFOA, and the effects of mixtures of these compounds have been rarely studied. However, in the real environment, PFSA compounds exist in complex mixtures of varying molecular size. In addition, the mechanisms of how PFSAs interacts with biological molecules and genetic matrial in algae is not well known.

Emerging pollutants (EPs) are defined as synthetic or naturally occurring chemicals that are not normally monitored but likely to enter the environment and cause known or suspected adverse ecological and/or human health effects [53]. PFOS and PFBS are two of the emerging pollutants that have been commonly studied in recent years. Due to the inert property of PFSAs and low amount of information about these substances, many industries use these compounds as primary source material in manufacturing [54]. In 2002, the OECD determined that PFOS and related compounds showed potential for toxicity and bioaccumulation in organisms and posed a threat to the environment and human life, including such impacts as acute toxicity and sub-chronic/chronic toxicity to fish, invertebrates and aquatic plants [33]. In 2009, the United Nations Environment Programme (UNEP) officially adopted PFOS and its salts as new persistent organic pollutants through the Stockholm Convention, agreeing to reduce and eventually forbid the use of such substances [55]. The U.S. Food and Drug Administration (FDA) declared that three products containing PFOS analogues would no longer be used as food contact material [56]. Later, the US Environmental Protection Agency (EPA) in 2016 published the PFOS human health threshold level of 70 ng/L overall for drinking water, no matter if it is an individual compound or combined with other PFASs [34].

1.5.Hypothesis

The overall problem for this experiment is to understand the fate and impact of a novel PFSA compound mixture associated with phytoplankton, representing the bottom of the freshwater aquatic food chain. The potential fate of PFSA compounds associated with phytoplankton is that they accumulate in the biomass through various organismal, cellular, and molecular processes. The potential effect of PFSAs on phytoplankton is through toxic effects on the growth and cellular health of the cultures. Therefore, there are two main goals for this study.

The first goal is to investigate the bioconcentration ability of a mixture of four- to eight carbon chain PFSAs (PFBS, PFHxS, and PFOS), in a model phytoplankton culture of *Scenedesmus obliquus*, and determine the fate of these compounds in the cell culture solution. The second goal is to investigate the toxicities of this set of PFSAs (PFOS, PFBS, PFHxS) on a growing culture of *Scenedesmus obliquus*, as determined through measurement of rate of growth and of pigment concentration in the overall mixture.

The overall hypothesis of the toxicity study is that the cell number and chlorophyll concentration of a growing culture of *Scenedesmus obliquus* will be lower with increasing concentration levels of each of PFOS, PFBS, and PFHxS individually, and of a PFSAs mixture of these. The overall hypothesis for the bioconcentration study is that the concentration of PFBS, PFHxS, and PFOS will be greater on the cell surface of *Scenedesmus obliquus* than inside after a prescribed length of exposure to external concentrations because of the lipophilic characteristic and that concentrations associated with the cell will increase with increasing external aquatic concentration.

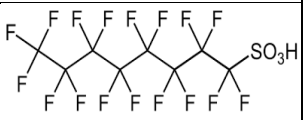
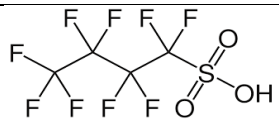
2. Literature Review

2.1. Toxicity of PFSAs in the environment

2.1.1. Physical and chemical aspects in the food chain

Perfluorosulfonic acids (PFSA) are a class of synthetic fluorosurfactant that is globally significant as a pollutant. Studying the structure of these compounds is necessary for determining strategies for treatment and remediation of them in the environment. Perfluorooctanesulfonic acid (PFOS), Perfluorobutanesulfonic acid (PFBS), and Perfluorohexanessulfonic acid (PFHxS) are three chemicals in the PFSA class where the only difference is the length of carbon chain (Table 1.). Because of its widespread use historically, PFOS is the most commonly studied compound within the class. However, concentrations of PFBS and PFHxS have recently been observed in higher amounts in the environment [7, 12, 22]. While much remains unknown about these two chemicals, the known chemical and physical characteristics of PFOS, within the same class, offer a guide for understanding behavior and for finding substitute chemicals for industrial companies. Because of the difference in carbon chain length, each of the chemicals of this class has different chemical characteristics that determine their bioaccumulation and toxicity potential. However, much of that information remains unknown.

Table 1. Chemical structure of some PFSAs

Perfluorosulfonic acid (PFSA)			
Chemical name	Chemical formula	Carbon number	Molecular Structure
Perfluorooctanesulfonic acid (PFOS)	$C_8F_{17}SO_3^-$	8	
Perfluorohexanessulfonic acid (PFHxS)	$C_6F_{13}SO_3^-$	6	
Perfluorobutanesulfonic acid (PFBS)	$C_4F_9SO_3^-$	4	

The molecular structure of perfluorooctanesulfonic acid (PFOS) makes it both hydrophobic and lipophobic; however, the sulfonic acid or sulfonate group increases the overall polarity of the molecule [57], determining its hydrophobicity and possible transport dynamics. The polycarbonate-fluorine bonds increase the stability of the PFOS molecule, thereby making it useful in many industrial applications. PFOS can reduce the water surface tension due to its fluorosurfactant properties, similarly to and in place of hydrocarbon surfactants. Perfluorobutanesulfonic acid (PFBS) is a compound having a tetracarbon fluorocarbon chain and a sulfonic acid functional group. As an anion, it acts as a stable fluorosurfactant due to the strength of the carbon-fluorine bond. Perfluorohexanesulfonic acid (PFHxS) has the same sulfonic acid function as PFOS and PFBS but has six carbons in a fluorocarbon chain. Recent studies show that PFBS has a shorter half-life in organisms' bodies compared to longer chain PFSAs. In contrast, PFHxS has the same or even longer half-life than PFOS, which has a longer

carbon chain [3]. Generally, the longer the carbon chain, the more hydrophobic the molecule; alternately, the more soluble the compound, the more transportable it is in aquatic systems. The smaller the carbon chain, however the less lipophilic the molecule, and the less potential for biomagnification in living tissue through binding with fatty acid molecules. Table 2 shows that PFBS is very different in many of these characteristics from the others, being more hydrophilic (the lower K_{ow}), having a higher solubility, and lower boiling point. In contrast, the larger molecule of PFOS is less hydrophilic, has a lower solubility, and higher boiling point. Some of the properties of PFHxS are in the middle condition compared with PFOS and PFBS, as shown in Table 2 [52].

Table 2. Physical and chemical properties of PFOS, PFBS, and PFHxS

Compound	Half-life constant (years)	log Kow number	Henry's law constant (atm cu m/mol)	Boiling point (mmHg)	Solubility (in water @ 25℃/mg*L)
PFOS	0.019	6.3	4.1*10-4	145 ℃ @ 10[58]	3.2*10-3
PFHxS	5.3 [59]	5.17	4.0*10-4	130-134℃ @11[60]	6.2
PFBS	0.07	3.19	1.44*10-5	76-84℃@ 0.1[61]	344

2.1.2. Toxicity of PFSAs in a Phytoplankton community

Many studies are ongoing regarding the toxicity characteristics of Perfluorooctanesulfonic acid (PFOS), Perfluorobutanesulfonic acid (PFBS), and Perfluorohexanessulfonic acid (PFHxS). According to Henry's Law constants, these compounds do not easily volatilize from water to the atmosphere under standard environmental conditions. This property contributes to the potential accumulation of these compounds in aquatic

environments. Because phytoplankton is a significant component of primary production in an aquatic system, it is crucial to understand the potential toxicity of these compounds to primary producers.

The toxicity effects of PFAS compounds on algae have been observed in reduced growth rates in laboratory experiments with *S. obliquus*. Past studies had found that the growth rate of *S. obliquus,* measured over 27-hour exposure trials, was significantly inhibited when the concentration of PFOS was increased in micromolar concentrations (Figure 7) [62]. This result shows that PFOS can decrease the algal growth rate by inhibiting the cell division rather than increasing the cell apoptosis. The PFOS exposed algae cells also showed an increase in size under microscopic analysis, also suggesting that PFOS may interrupt the process of cell division. However, PFBS did not show any inhibiting effects during this same experiment, suggesting it does not present toxicity affection related to the molecular characteristics.

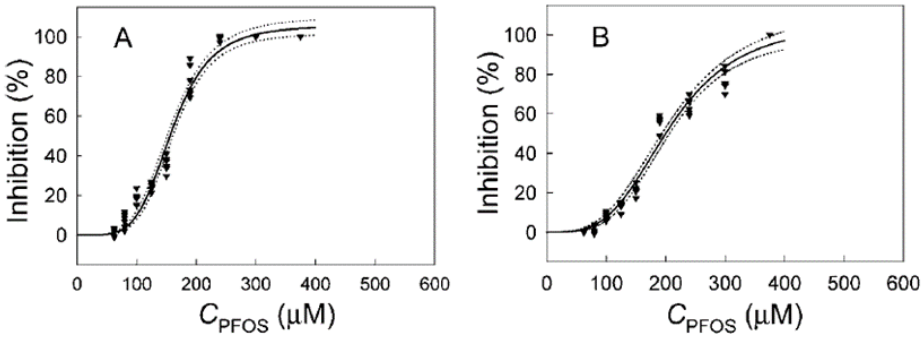


Figure 7. Inhibition of growth rate for the alga Scenedesmus obliquus under PFOS exposure (A. fluorescence; B. optical density) [62].

Photosynthesis and cell division are essential processes in algae growth. The algae growth rate can be hindered by interference and destruction of the photosynthetic processes. Hence, the algae growth rate can indicate cell stress under any particular PFSA concentration. Another indicator of algae condition in culture is the chlorophyll concentration. Investigating the

relationship between chlorophyll and PFOS, one study showed that chlorophyll is very sensitive to PFOS exposure, and thus its measurement can be used as an indicator of the stress or cellular health of a *Scenedesmus obliquus* culture [63]. When *S. obliquus* was exposed continually under high PFOS concentration, the change in chlorophyll concentration indicated the growth trend of algae cells. Moreover, the growth rate of algae also reflected the toxicity level of aquatic PFOS concentrations [64]. This is confirmed by other follow-on studies, which showed the content of chlorophyll decreased when the PFOS concentration increased, such that overall presence of PFOS threatened the health of the *S. obliquus* culture [38].

Some biological indicators, such as some enzymes, reactive oxygen species (ROS) and malondialdehyde (MDA), are intra-cellular essential mechanisms that suggest environmentally-induced organelle injury [65]. When the cell growth condition changes, it causes cells to produce a large number of reactive oxygen species (ROS) from oxidative damage. Environmental variables such as temperature, humidity, salt concentration, atmospheric pollution (SO^{2-}, ozone), ultraviolet rays, and certain pesticides and pathogens all have an effect on cells and their intra-cellular conditions [66]. The active oxygen-containing compounds inside cells include reactive compounds such as superoxide anion (O^{2-}), hydroxide ion (OH^{-}), hydroxyl radical (`HO) hydrogen peroxide (H_2O_2), and others. Active oxygen compounds such as these can advance the destruction of proteins, membrane lipids and other protein compounds. To mitigate these potentially damaging compounds, various enzymes are formed in the cell to detoxify photochemistry or photochemical products. For example, catalase (CAT) can convert H_2O_2 to H_2O and O_2. Superoxide dismutase (SOD) is a group of metal ion-containing enzymes whose function is to deprotonate O^{2-} into H_2O_2 and O_2 [67]. When present in algae cells, the algae will enhance the expression ability of antioxidation defense through increasing enzymes such as

superoxide dismutase (SOD) and catalase (CAT) [66]. Malondialdehyde (MDA) is one of the unsaturated fatty acid peroxidation products, and its content represents the degree of membrane lipid peroxidation. PFOS exposure in the environment may affect the antioxidant defense system of algal cells, causing oxidative damage in cells according to the level of catalase (CAT) and malondialdehyde (MDA) [64].

Liu et al. [68] studied the algae cell uptake capability of a mixture of PFOS and pentachlorophenol, atrazine and diuron. The results show that Perfluorooctane sulfonate (PFOS) affects the cellular absorption and toxicity of compounds with different molecular structures in different ways, and it may also increase the bioavailability of more hydrophobic compounds to *S. obliquus*, such as pentachlorophenol. Another study also confirmed that many PFAS compounds may coexist in the same environment or area and have toxicological interaction with each other to the cells [69]. Furthermore, it appears that the effect of PFOS on the toxicity of other compounds is related to the effect of PFOS on cellular uptake of these compounds, possibly due to the destruction of cell membrane properties by PFOS. The toxicity ability of PFOS may also vary because of cell structure, surface properties and membrane compositions of the exposed cell [65].

2.2 The fate and effects of PFSAs in phytoplankton

The phytoplankton community is the autotrophic component in the plankton community, composed of algae of different classes. Phytoplankton is also the entry point for PFSAs into the aquatic food chain, and they play a key role for PFSAs circulating throughout the water system. Understanding the fate of PFSAs in phytoplankton is key to understanding the transport and sequestration of them in ecosystems. Ismael Rodea-Palmares et al. [70] studied the toxicity effects of PFOS/PFOA combined with herbicides to microorganisms. After culturing *Anabaena* in

PFOS solution at a concentration of 25 mg/ml for 72 hours, the permeability of the cell membrane increased significantly to propidium iodide (PI), showing that PFOS exposure can damage the cell membrane. Also, it is known that the mitochondrial disfunction is a vital factor in the cytotoxic process leading to cytotoxicity and death. The potential hyperpolarization of mitochondrial transmembrane is one of the early molecular events indicating cell death. Pre-exposure of algae to PFOS in lab-level experiments has been shown to trigger this mitochondrial hyperpolarization, causing death of the algae cell [71]. Again, many studies have reported PFOS concentrations in phytoplankton in many areas, such as rivers, lakes, and open ocean water [72-74]. It is generally expected that small microorganisms, with a large surface area to volume ratio, have faster uptake kinetics and accumulation factors, overall affecting absorption to the cell membrane. This phenomenon may happen in some other small-diameter species in the open sea, such as *Prochlorococcus* and *Synechococcus*, with n-PFOS [16].

The characteristics of both the PFSA molecules and the phytoplankton species exposed are determinants of the bioconcentration aspects of the compound. Yuan XJ. et al. [75] studied the bioaccumulation of PFASs in three different phytoplankton strains inclusive of *Scenedesmus obliquus, Spirulina platensis,* and *Chlorella pyredoidosa*. These three algae are often studied as model algae in the laboratory. In bioaccumulation studies, the bioconcentration factor (BCF) is used as a laboratory-level indicator of bioaccumulation, and is expressed as the ratio of the concentration of a pollutant in an organism to the concentration of that pollutant in water [76]. In these studies, it was found that the bioconcentration factor of PFSAs in all species was positively correlated with the carbon chain length of the molecule, from high to low being PFOS> PFHxS> PFBS. This suggests differential effects of each of the components of a PFSA mixture on aquatic organisms to it.

2.3 The fate of PFSAs in the food chain

Perfluorooctanesulfonic acid (PFOS) is one of the most researched of the PFSAs because of its ubiquity and common historical usages. Currently, the environmental behavior of persistent lipophilic compounds is an insight into the understanding of the bioaccumulation of organic compounds. The equilibrium distribution paradigm assumes that organic molecules in an environmental system are transferred from non-biological to biological components at chemical equilibrium [77]. Since the lipids of most hydrophobic chemicals have highly variable properties, persistent lipophilic compounds are concentrated in the lipids of the organism [78]. Generally, the PFAS compounds with higher lipophilicity ability are more likely to accumulate in the organisms as described by the octanol-water partition coefficient (K_{ow}), which represents the tendency of compounds to migrate from the aqueous phase to lipids [79]. The PFAS compounds more easily migrate from the aqueous phase to lipids when the octanol-water partition coefficient (K_{ow}) value is larger. Previous studies have shown that PFOS and PFOA are more likely to accumulate in the cell membrane of aquatic organisms than other PFAS with a high lipid component, leading to enrichment and amplification in the food chain [80].

PFSA contamination is widespread globally and presents a high risk in aquatic food chains. Freshwater ecosystems are often the first step of PFAS contamination. In recent research in rivers within China, the concentration of PFOS is significantly higher than nearby marine waters. The concentration of PFOS in the Dongjiang River was measured at 0.97 ng/L, among the lower values measured throughout the country. The Huangpu River was measured at 20.5 ng/L of PFOS concentration, much higher than the Dongjiang River, Pearl River, Yangtze River, and Songhua River [73]. Moreover, the PFSAs composition pattern of tap water was measured to be comparable to many rivers in China, where 86% of tap water samples from multiple cities

were found to contain PFOS, 74% contained PFBS, and less than 50% contained PFHxS. The problem is ubiquitous, as PFSA compounds have been detected in tap waters of other countries such as Japan, India, U.S. and Canada [81]. Compared with the rivers and tap water, detectible quantities of PFOS are also found in the ocean. For example, the concentration range of PFOS in the western Pacific Ocean, Indian Ocean, and Antarctic area is 5-71.7 pg/L and 8.6-36 pg/L in the North Atlantic [74].

In recent research, sewage treatment plants (STPs) have been shown to be an important pollutant emitter of PFAS in the water environment [82, 83]. Various studies have confirmed the connection between STP sources of PFAS and measurable concentrations in aquatic biota. For example, over a six-month field survey sampling done in Korea, the concentrations of PFAS in fish near STP outfalls decreased throughout that time, and perfluorohexane sulfonate (PFHxS) was found in the macroinvertebrate population (*E. octoculata*) at concentrations ranging from 1.5 to 19 ng/g, and at lower concentration (< 0.3-8.2 ng/g) in *Hydropsyche sp.* At the same time, these organism concentrations were found to be higher than in the STP wastewater [21]. Typically, the sediment in an aquatic system can be a destination and accumulation reservoir for many pollutants, controlling their migration and transformation to other forms. In the Ariake Sea in Japan, researchers found 0.11 ng/L Perfluorooctanesulfonic acid (PFOS) in the sediment [84]. This puts contaminants in direct contact with benthic organisms, who are susceptible to uptake dynamics and toxicity effects of the substances.

Contaminating pollutants can be adsorbed, absorbed, or swallowed by an animal, and eventually stay in the organisms' body, suggesting bioaccumulation. There are generally two ways for aquatic organisms interact with and obtain pollutant substances. Bioconcentration occurs when the body surface directly absorbs a contaminant and is driven primarily by external

concentrations in the environment. For example, this occurs when fish absorb a contaminating pollutant directly from the water through their gills or skin. Biomagnification occurs when ingestion of contaminated food particles brings the pollutant inside, which binds to and collects in lipid compounds in the body in ever higher concentrations [85]. To account for these processes, the bioconcentration factor (BCF) and bioaccumulation factor (BAF) are two parameters that can be used to describe the ability of contaminants to accumulate in the organism's body. The bioconcentration factor (BCF) is the ratio of the concentration of the substance in a specific biological genus to the exposure concentration at equilibrium. The mathematic model of bioconcentration factor (BCF) can be presented as the following equation when the system at the steady-state [76]:

$$BCF = \frac{C_B}{C_{WD}} = \frac{k_1}{k_2 + k_E + k_M + k_G} \quad (1)$$

where

C_B is the chemical concentration in the organism (g/kg);

C_{WD} is the freely dissolved chemical concentration in the water (g/L);

k_1 is the uptake rate constant from the water at the respiratory surface (L/kg*d);

k_2, k_E, k_M, k_G are the rate constants (per day) representing the removal of chemicals from organisms through the respiratory surface and excretion of feces, metabolic biotransformation, and growth dilution.

The bioaccumulation factor (BAF) is a metric to describe the bioaccumulation of contaminants from water to biota due to absorption from all exposures to persistent organic

pollutions (POPs) [86]. It also can be represented as an equation as follows when the system at the steady-state [41]:

$$BAF = \frac{C_B}{C_{WD}} = \frac{k_1 + k_D(C_B/C_{WD})}{k_2 + k_E + k_M + k_G} \quad (2)$$

where

k_D is the uptake rate constant for chemical in the diet (kg/kg*d).

The bioconcentration and bioaccumulation of PFASs have been studied extensively in the marine food web, but the relevant information in freshwater ecosystems in the food web is limited. When the BAF or BCF is higher than 5000, it means this contamination has bioconcentration effect; when the BAF is in the range of 2000-5000, this contamination shows the potential for bioconcentration effects to occur [87].

The biomagnification effects of contaminants transported along the food chain can be measured by biomagnification factors (BMFs). Also called a trophic magnification factor (TMF), this indicates the contaminant biomagnification for a particular sample within a food chain, indicating that a substance has bioaccumulation ability when the TMF is greater than 1 [88, 89].

The relationship between trophic levels (TLs) (such as phytoplankton, zooplankton, shrimp and fish) and poly- and perfluorinated compounds (PFCs) concentration (Figure 8.) is the key to calculate the trophic magnification factor (TMF) [90, 91], determined graphically on a semi-log concentration vs. trophic level plot by the following formula:

$$\ln concentration\ [wet\ weight\ (ww)\ ] = a + (b + TL) \quad (3)$$

where

a is the y-intercept (constant) of the 1st order regression line;

b is the slope of the 1st order regression line.

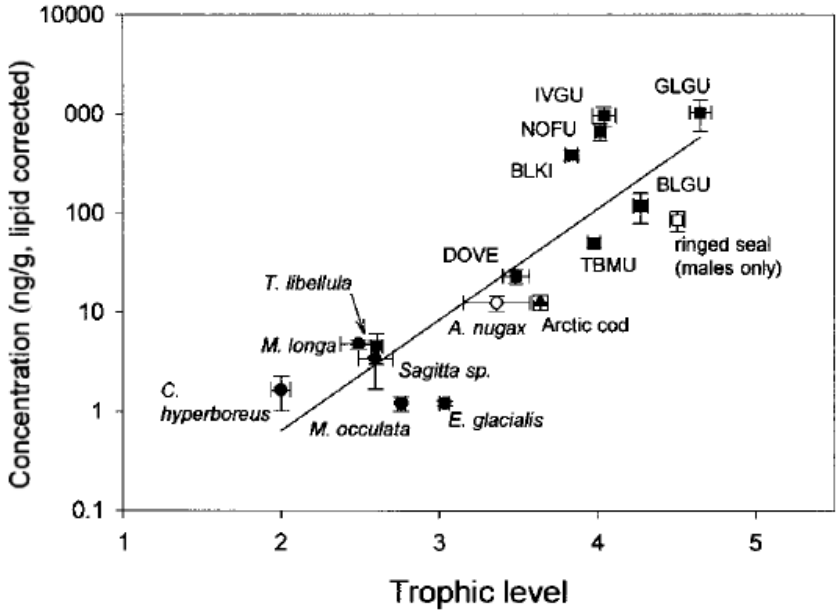


Figure 8. The relationship between Trophic level and PFCs concentration [88].

According to the equation (3), the TMF for each PFC contaminant can be calculated by the slope b using

$$TMF = e^{b} \qquad (4)$$

The bioaccumulation ability of any compound is greater when the TMF number is large. In the Taihu Lake, China, the eutrophic freshwater food web of phytoplankton-shrimp-fish-egrets shows a TMF of 2.9 for PFOS [92]. However, in the food chain of phytoplankton-fish-marine mammals, the TMF of PFOS is 6.3, suggesting a significantly greater biomagnification ability. This conclusion also matches another food chain (Phytoplankton-Bivalve-Fish-Birds-Marine Mammals), where the TMF of PFOS is 3.1 [91]. In another type of food chain

(Phytoplankton-Bivalve-Fish-Birds-Marine Mammals), Kelly et al. [93] measured a TMF of PFOS of 11, confirming the significant ability of PFOS for biomagnification. The tropic magnification factors (TMFs) of PFOS in the planktonic-fish food web that have herbivorous fish and carnivorous fish is greater than 1 in Lake Ontario, Canada [94]. Houde et al. also found that PFOS and PFHxS have a strong biomagnification ability on the Sarasota Bay food network (phytoplankton-invertebrate fish-dolphins), with BMFs in the range of 1.2-35 [95]. Haukas et al. found that, except for herring, the black sea otter and seagull have significant biomagnification effects on PFOS and PFHxS [96]. In general, the higher the trophic level of an organism (such as mammals and birds), the stronger is the ability to internalize the PFSAs and thus the greater propensity to enrich PFSAs in their bodies. In summary, the biomagnification and bioconcentration ability of PFSAs compounds is related to the length of their carbon chain. If the PFSAs compound has a long carbon chain, then its biomagnification and bioconcentration ability will be more prevalent; otherwise, it will not.

3. Material and Method

3.1 General approach

According to the general ideas and hypothesis, the whole experiments had two parts to address the problems. The first part regards toxicity ability of PFSAs mixture on *Scenedesmus obliquus,* including the EC_{50}, the algae growth rate and pigment concentration. All the result from first part of the experiment indicated the effect of PFSAs mixture to algae. The second part of the experiment is the bioconcentration ability of PFSAs mixture on algae. In the bioconcentration test, the contents of PFSAs mixture were detected by Ultra-high performance liquid chromatography- coupled to triple quadrupole mass spectrometry (UHPLC-MS/MS) in the media water, washed algae water, and algae extract solution. The results indirectly pointed out the fate of the PFSAs mixtures in the environment.

3.1.1 Algae culture

The algae isolate *Scenedesmus obliquus* UTEX 393 was purchased from the Culture Collection of Algae at The University of Texas at Austin (Figure 9.). A sample of the original *Scenedesmus obliquus* culture was inoculated into Bold Basal 3N growth medium, and the remainder of the original culture was stored at room temperature (25 deg C) on a 12:12 light: dark cycle. Bold Basal Medium (BBM) has been widely used as a primary culture medium in the cultivation of freshwater green algae [91].

Figure 9. The Scenedesmus obliquus UTEX 393 in the normal lab condition (a) and under 40x microscope (b)

3.1.2 Algae growth parameter and conditions

The growth medium used throughout this set of experiments is Bold's Basal Medium 3N, a common medium for culturing green algae. According to the common recipe of Bold's Basal Medium (BBM) [97], ten stock solutions are prepared first. The full recipe is provided in Appendix 1. To make the stock solutions, every component for the recipe is dissolved in one liter of deionized water at room temperature (Table 3.). All the 3N Bold Basal Medium (BBM) stock solutions were autoclaved after dissolution was complete.

Table 3. The recipe used for Bold Basal Medium (BBM) 3N, providing for three times of nitrate as standard BBM [91]

Component	Solution Number	Stock Solution Concentration (g/L)	Quantity Used in the final solution
Macronutrients Solution			
$NaNO_3$	1	**75 g/L (3×)**	10 mL
$CaCl_2 \cdot H_2O$	2	2.5 g/L	10 mL
$MgSO_4 \cdot 7H_2O$	3	7.5 g/L	10 mL
K_2HPO_4	4	7.5 g/L	10 mL
KH_2PO_4	5	17.5 g/L	10 mL
NaCl	6	2.5 g/L	10 mL
Alkaline EDTA solution			
EDTA	7	50 g/L	1 mL
KOH		31 g/L	
Acidified Iron Solution			
$FeSO_4 \cdot 7H_2O$	8	4.98 g/L	1 mL
H_2SO_4		1 mL	
Boron Solution			
H_3BO_3	9	11.42 g/L	1 mL
Trace Metals Solution			
$ZnSO_4 \cdot 7H_2O$	10	8.82 g/L	1 mL
$MnCl \cdot 4H_2O$		1.44 g/L	
MoO_3		0.71 g/L	
$CuSO_4 \cdot 5H_2O$		1.57 g/L	
$Co(NO_3)_2 \cdot 6H_2O$		0.49g/L	

After preparing the 3N Bold Basal Medium (BBM) stock solutions, a prescribed volume of each stock solution was added to a 1Lvolumetric flask according to the recipe, and the flask was filled to 1 L with deionized water. The 3N Bold Basal Medium (BBM) solution was measured by the pH parameter Orion star A111 (Thermo Scientific) and was adjusted to a pH of 6.80 before transferring into a 1L glass flask. Usually, the pH of the original 3N BBM is around 6.4-6.6 ; pH is adjusted using a 1 mL pipette to slowly add droplets from 1M NaOH solution into the media until the pH is adjusted to 6.79-6.81. Each bottle of 3N Bold Basal Medium (BBM) solution was sterilized via autoclave and stored at constant room temperature (around 25 Celsius degree). Compared to the normal BBM media used for maintenance of the

Scenedesmus obliquus UTEX 393 culture, the nitrate concentration of 3N-BBM is increased by three times [97].

For the formal experiment, 500 mL algae mother culture were mixed with 500 mL 3N BBM and then the mixture was poured into 1L HDPE (High density polyethylene) (VWR® HDPE Multipurpose Containers 16.5dia. x 11.4H cm) buckets. 1g/L PFOS, 1g/L PFBS and 1g/L PFHxS were made as the PFSAs mixture stock solution. Each PFSAs stock solution was pipetted into the HDPE sample bucket according to the experiment PFSAs mixture concentration.

3.1.3 Light configuration

Based on the scale of the experiment and the laboratory space condition, six light fixtures were used during the studies. Two Philips 32W fluorescent Alto II TL 741 fluorescent lamps were used in each light fixture, and each fixture provided an average light intensity of 141 µmol/m^2/s generally in the culture area, measured by using an Apogee MQ-200 PAR meter (Apogee Instrument, Inc.) (Figure 10.). Thus, all experiment containers were illuminated with twelve fluorescent lamps under 12:12 hours light: dark cycle, controlled by an automatic timer. The crisp white fluorescent light bulbs simulate in the lab environment the radiation spectrum as sunlight. Thus, they are widely used to cultivate algae and plants.

Figure 10. Apogee MQ-200 PAR (photosynthetically active radiation) meter

3.1.4 Water temperature control

Experiments were performed in Room 104 of the Swingle Hall at Auburn University, which does not have a heating facility at the time but only air conditioning. The experiment conditions were kept around 21 degree Celsius for the main experiment, cooler than the ambient laboratory temperature, using a cooling system built into 10 L water bath. In total, three water bath containers were constructed from large rectangular HDPE containers, and kept at temperature with a custom-built water cooling system. The water cooling system was built with

two adjustable thermostatic constant temperature chillers CSXC-1 (Chill Solution LLC.), which deliver the water from the water bath containers via a water pump to a reactor heat exchanger, where heat is reduced through the Peltier plate on the chiller, effectively cooling the water (Figure 11.).

Figure 11. The adjustable thermostatic constant temperature chillers CSXC-1, showing the fan and chiller heat exchanger. (Chill Solution LLC.)

Even with the fluorescent lamps that can generate heat, the chilling systems was shown to easily keep the water temperature in the water bath close to 21 within ± 1.5 deg C error (n=576), as measured every 30 min via a data logger (HOBO UA-00x Onset Corporation). However, the disadvantage of the Peltier plate cooling system is the low efficiency of the given cooling capacity. That means it needs a long time to chill the water to the given temperature

(Figure 12. And 13.). Figure 12 shows the water temperature during the bioconcentration test. However, the toxicity test used two water baths to fit in all of the HDPE buckets; one is the bigger water bath which can fit 12 HDPE buckets and another smaller water bath can have 6 HDPE buckets. Figure 13 shown the temperatures in both water baths during the toxicity test.

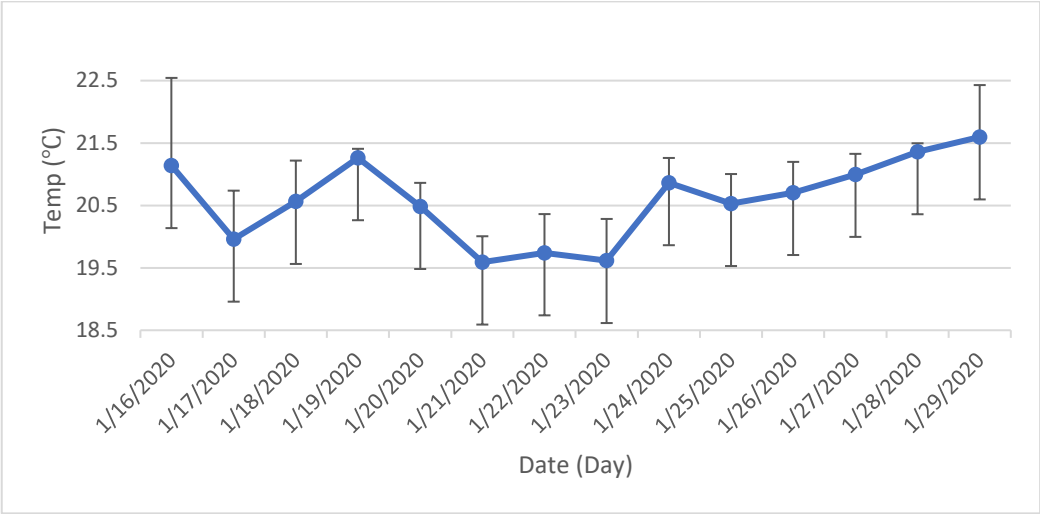


Figure 12. Data logger output shows the water temperature data when the chilling system is working during January 2020. Error bars represent standard deviation of the mean (n=21)

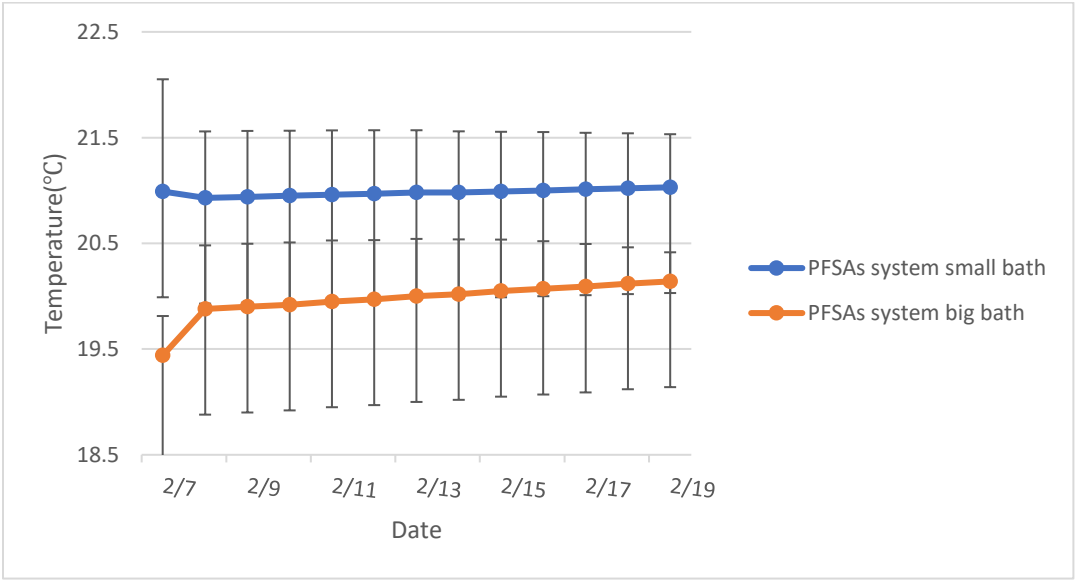


Figure 13. Data logger output showing the water temperature data when the chilling system is working during February 2020. Error bars represent standard deviation of the mean (n=21)

3.2 Preliminary experiment

In order to evaluate some parameters to be used in the main experiment, several preliminary experiments were performed in advance. These pre-experiments were performed mainly to determine how much concentration of *Scenedesmus obliquus* biomass was needed to can detect the presence of PFSAs sorption and ensure the concentration of Perfluoroalkane sulfonates (PFSAs) to be used in the experiment was within the detection range of UHPLC-MS/MS instrument. All data were analyzed using one-way repeated measures ANOVA to detect differences between treatments. Repeated measures analysis of variance (ANOVA) is used to determine whether there was statistically significant difference between the means of three or more independent groups in which the same subject show up in each group.

3.2.1 Growth curve

Growth curve investigation was performed to measure the toxicity effect of PFSAs on *Scenedesmus obliquus.* Growth curve measurements were performed on cultures over a range of concentrations of PFSAs to compare productivity and assess whether the *Scenedesmus obliquus* community followed an exponential growth rate during the sampling time. To determine the growth rate, the algae culture was sampled over time in a known amount of volume (usually 2-3 mL), and the algae cell number for each sample was counted under the microscope using the Sedgwick-Rafter counting chamber [98]. Samples were done in triplicate for each concentration of PFSAs to account for variability in growth rates. Before collecting the algae samples, a polyethylene pipet was used to mix the algae culture by pipetting the algae mixture. This step is to ensure that algae cell is evenly distributed throughout the 1L HDPE (High density polyethylene) bucket. The protocol for sampling is each treatment is sampled three times at every corresponding time point. In addition, every HDPE container was

pseudorandomly reorganized to a different location after each sampling time, such that each subsequent location is different from before.

The sample collection method is introduced as follows. First, a polyethylene pipette is used to mix the algae mixture in each HDPE container. Then, a10 mL translucent graduated polypropylene pipette is used to sample 2-3 mL of the algae culture from each HDPE container. Each sample is pipetted to a 5 mL HDPE tube. After that, 2-3 drops of Lugol's Iodine solution [99] are dropped into the centrifuge tube having samples in it. Lugol's Iodine solution is a widely used fixative and is usually recommended for the preservation of nano-microplankton. The centrifuge tubes were then stored in the centrifuge tube rack in the box at room temperature in the dark environment.

3.2.2 Analysis of growth curves and concentration of PFSAs

Due to the time required to count the cells, samples were preserved using the solution and then stored in a dark environment to count them after the experiment finished. For counting, the Sedgewick-Rafter counting chamber was placed on a three layers of clean paper towel to avoid scratching the bottom surface and contamination. A clean, soft plastic tip was used for pipetting 1 mL (due to the volume of the counting chamber) of the preserved sample onto the counting slide (Figure 14.). The counting slide had 50×20 squares and each square was 1mm which means each square had 1 microliter preserved sample. A cover slip is slid in place, assuring there are no air bubbles trapped in the slide. Once the counting chamber is filled up with sample, it is allowed to settle for 5-10 minutes so that all algae is on the bottom surface under the 10x Nikon microscope. For enumeration, the number of cells per square is counted up to 300 cells in total; this is then divided by the number of counted squares. The algae cell number was

determined as cells per microliter. In the end, the cell number is determined as cells per microliter and converted to the number of cells per milliliter for reporting.

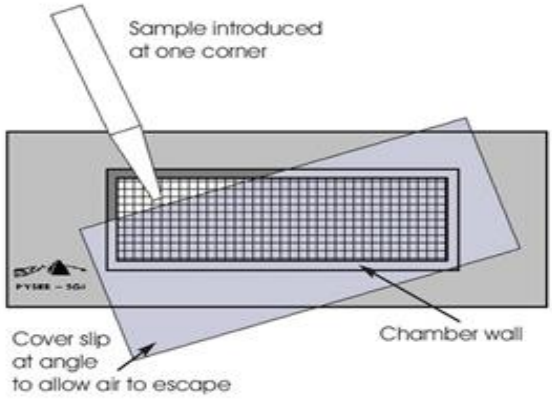


Figure 14. The structure of Sedgwick-Rafter counting chamber[100].

3.3 Exposure study

The primary purpose of study was to evaluate the acute toxicity of Perfluoroalkane sulfonates (PFSAs) at different concentrations to *Scenedesmus obliquus.* In addition, the biococentration of PFSAs on *Scenedesmus obliquus* was investigated through the course of the experiment. For the bioconcentration test, the capability of UHPLC-MS/MS instruments for the experiment of Perfluoroalkane sulfonates (PFSAs) concentration is according to the literatures, ranging from nanogram to milligram concentration. For that reason the range of different PFSAs mixture concentrations were selected for all experiments.

3.3.1 Experimental design

I. Toxicity of PFSAs (PFOS, PFBS, and PFHxS) on *Scenedesmus obliquus*

The goal of this experiment was to find the effect of PFSA mixture on cell growth and pigment level over time of a growing culture of *Scenedesmus obliquus* culture over a range of concentrations of a novel PFSAs mixture (PFOS, PFBS, and PFHxS). HDPE buckets at 1 L were

used for culture vessels, and the lids of the HDPE buckets were used to prevent the evaporation of the media from causing errors in the results. The toxicity was investigated for six trial concentrations, with five different concentrations of PFSAs mixture, and a control test condition at zero concentration of PFSAs compounds. Each trial concentration had three replicates, which provided more data to help to analyze results. Three replicates at each trial condition were treated as a group; two groups each were assigned to each water bath section. Groups were reassigned to a new water bath section pseudo randomly after each sampling time. Meanwhile, each individual bucket was haphazardly floated in its own water bath during each trail. A detailed listing of replicate numbers and concentration conditions is shown in Table 4.

Table 4. The concentration of PFASs mixture in each group for toxicity test

Replicate Number	Concentration (mg/L)
1 - 3	10
4 - 6	20
7 - 9	0
10 – 12	40
13 - 15	80
16 - 18	160

For the two high-density polyethylene (HDPE) plastic containers that were used as the water baths, section 1 and section 2 were in the bigger water bath, section 3 was in the smaller water bath. Initially, baths were filled with DI Water, and a water supply connected to a thermostat chiller was put into each container to maintain the temperature at 21 deg Celsius. Each culture container bucket had its own input airline connected to a 10 ml pipette and output also connected with a 1 ml pipette to generate mixing flow (Figure 15). All water bathes were

put on a platform shaker (VWR, Shaker 5000, ADV 120 V, CAT: 89032-104) to continuously mix the algae culture and PFASs compounds at the speed of 75 rpm/min. The air was pumped through a manifold, at a pressure of 0.15 ±0.05 psi (n=12). While under agitation, individual buckets moved haphazardly in its own water bath.

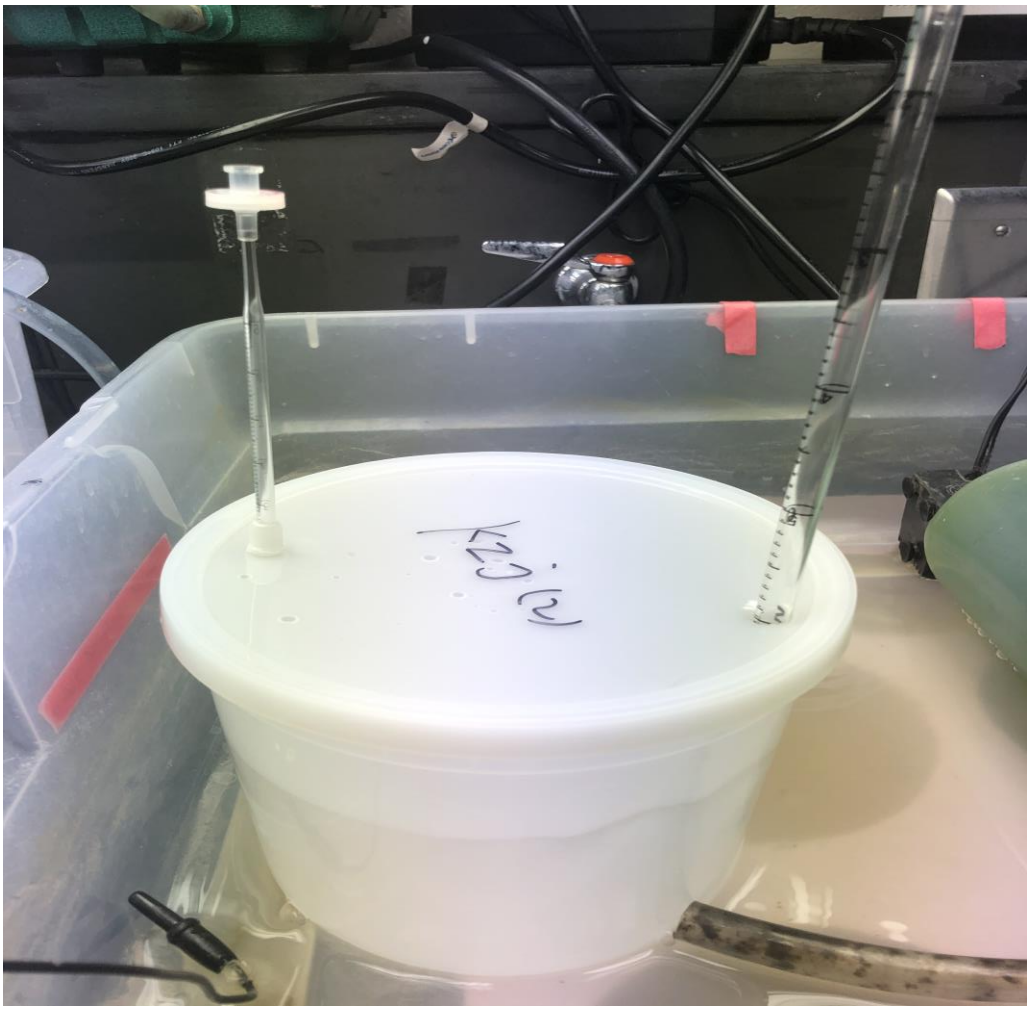
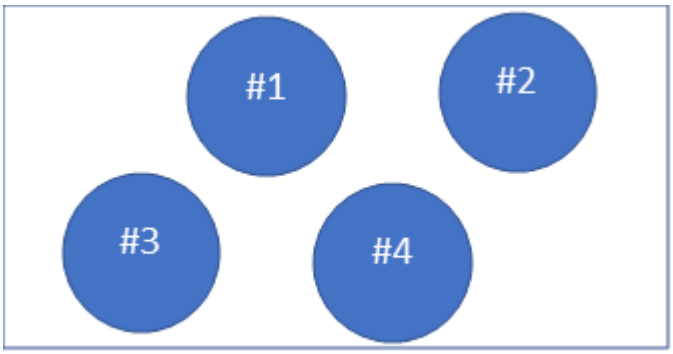


Figure 15. The experimental design for the airline on HDPE buckets.

II. Bioconcentration of PFSAs (PFOS, PFBS, and PFHxS) in *Scenedesmus obliquus*

The goal of this experiment is to find the bioconcentration amount of PFSA compounds in a mixture in a growing culture of *Scenedesmus obliquus* under two different concentrations of

the PFSA mixture. The lids of the HDPE buckets were used to prevent the evaporation of the media from causing errors in the results. Two different concentrations of PFSAs mixture (PFOS, PFBS, and PFHxS) were used as treatments. A tipical experimental setup figure is shown in Figure 16.:



[0.01/0.1 mg/L of PFSAs (# 1-2); Blank (# 3- 4)]

Figure 16. The experimental design for the bioconcentration ability of PFSAs mixture

There were two trial concentrations, where two different concentrations of PFSAs mixture (at 0.1 mg/L and 0.01 mg/L total PFSA concentration) were used in this experiment. 1g/L of PFBS, 1g/L of PFOS, and 1g/L PFHxS stock solutions were made for the experiment and used in equal amounts to make the trial conditions. The control test had *Scenedesmus obliquus* with a zero concentration of the PFSA mixture.

Each trial concentration has three replicates, which will supply more data to help to analyze results. Each replicate culture is tended in 1-L HDPE containers to reduce other influencing factors, such as surface adsorption to the container. A detailed listing of replicate numbers and concentration condition is shown in Table 5.

Table 5 The concentration of PFASs mixture in each group for bioconcentration test

The concentration of PFASs mixture for each group			
Number #	Concentration (mg/L)	Number #	Concentration (mg/L)
1	0.01	5	0.1
2	0.01	6	0.1
3	0.01	7	0.1
4	0	8	0

The water bath from each section was filled with DI Water. The water pump, which connects to a chiller, was put into the container to maintain the temperature at 21 deg Celsius. Each culture container bucket had its own input airline connected to a 10 ml pipette and output also connected with a 10 ml pipette to generate mixing flow (Figure 16). Two of the water baths were put on the shaker at 75 rpm to agitate the algae culture and compounds. All replicates for each PFSAs mixture concentration were in the single water bath and each single HDPE bucket were reassigned to a new location pseudorandomly in the water bath after each sampling. Meanwhile, each individual HDPE bucket moved haphazardly in its own water bath during the trail. Also, the air pressure of the air pump is 0.15 ±0.05 psi (n=12) to each HDPE bucket as well.

3.4 Complimentary study

3.4.1 The toxicity effect of PFSAs on *S. obliquus*

For the PFSAs toxicity test, 20 mL of algae cells solution was sampled from each of the replicates using a pipette according to a sampling schedule throughout the entire growth trial. Algae samples were then filtered on the glass microfiber filter (Whatman GF/F, diameter 47 mm, pore size 0.7 μm) using a 47 mm fritted glass filter funnel clipped to an Erlenmeyer flask and connected to a vacuum pump which makes 0.1 ±0.05 psi (n=5) flow rate to the HDPE buckets. Samples on the glass microfiber filters were wrapped with labeled aluminum foil and preserved in the -20 ℃ freezer for pigment analysis. Approximately 2-3 mL of the sample containing algae were preserved separately for algae cell counting; these were taken into labeled centrifuge tubes with Lugol's iodine solution and stored in a dark environment at room temperatur. Sampling was performed regularly throughout the growth phase of the toxicity experiment; there were 18 samples (one for each replicate) for each sampling time to yield 198 samples total. The sampling schedule is shown in Table 6.

Table 6. The sampling time schedule for PFSAs toxicity experiment

Sampling time	0 mg/L	10 mg/L	20 mg/L	40 mg/L	80 mg/L	160 mg/L
0h						
3h						
6h						
12h						
24h						
36h						
48h						
60h						
72h						
120h						
168h						

Filtered samples were analyzed in the spectrophotometer (Thermo Scientific, Helios Omega UV-VIS) to measure the pigment level at each sampling time. Chlorophyll extraction was performed with 10 ml acetone solution dispensed into 25 mL glass tubes with sample filters. Tubes were vortexed at 1800 RPM by using Analog Vortex Mixer (VWR, Vortexer mini 120V, CAT: 58816-121) to ensure the acetone solution is mixed well with the sample filters. Glass tubes were covered with aluminum foil and kept in the freezer at –20 degree C for at least 24 hours.

The preserved algae cells with Lugo's iodine were used to determine the EC_{50} value (Half maximal effective concentration) by cell counting. The EC_{50} is the half-value response by *Scenedesmus obliquus* between baseline and maximum response in a continuous serial of a toxicant concentration after a particular exposure time. The response in the EC_{50} definition means the algae population changes over time. The cell density of each of the

culture samples was determined using a counting method. A clean, soft plastic pipette tip was used for dispensing 1 mL of culture sample onto a Sedgwick-Rafter counting chamber. Once the counting chamber was filled up with a sample, it was allowed to settle for 5-10 minutes to allow all algae to settle down on the bottom surface under the microscope. Cells were counted per each visible square in the objective (1 mm^2) from the center; counts were made for every 5 squares until 300 cells were counted in the total squares. After recording the algae cell number in each PFSAs mixture concentrations at every sampling time, the EC_{50} calculation of the sample population change at different time points compared with 0 hours was done in Excel. At 168 hours, the algae population changes in the different concentrations was compared again with the control to get the percentage number, which represents the changes in algae population growth rate at the end sampling time point. Following this, the EC_{50} was calculated by the cell numbers using 100% minus the changes of algae population numbers at 168 hours in different PFSAs mixture concentrations.

3.4.2 The bioconcentration of PFSAs on *S. obliquus*

For the bioconcentration experiment, the pipette with disposable pipette tips was used to collect 10 mL mixture samples from each replicate according to a time schedule. Because most of the collection times were focused on the first day, and each concentration requires multiple sampling, for different experimental purposes, the sampling interval for each concentration is 10 minutes. 10 mL samples from each of the HDPE buckets were taken immediately as the 0-hour samples after being mixed well with PFSAs contamination. The samples were all preserved in a -20 deg C freezer for analysis. The samples were processed through the Ultra-high performance liquid chromatography- coupled to triple quadrupole mass spectrometry (UHPLC MS/MS) for analysis. The sampling schedule for the bioconcentration test is shown in Table 7.

Table 7. The sampling time schedule for PFSAs bioconcentration experiment

Sampling time	**0.01 mg/L PFASs mixture**	**0.1 mg/L PFASs mixture**
0h		
3h		
6h		
12h		
24h		
36h		
48h		
60h		
72h		
120h		
168h		

3.5 Quantitative analysis

3.5.1 The UHPLC-MS/MS water sample preparation

After thawing, the mixed *Scenedesmus obliquus* samples with 3N BBM were centrifuged at 13000 rpm, 4 deg C for 15 min, and the supernatant was poured into the centrifuge tubes; these were then called the PFSAs water samples. The water samples were purified through the Oasis WAX column by using the solid-phase extraction (SPE) method. 4 mL of 0.1% ammonium hydroxide in methanol, 4 mL methanol, and 4 mL LC grade water were used to condition the water samples in order by placing the cartridges on a 20-position vacuum manifold unit. The PFSAs water samples were loaded into the prepared cartridges and eluted by adjusting the flow rate. Then, 4 mL LC grade water with 25 mM ammonium acetate

buffer (pH 4.0) was used to wash the cartridges using the 20-position vacuum manifold unit. In the end, the PFSAs water samples were subjected to solid-phase extraction (SPE) and eluted with 4 mL extraction solvent (2 mL methanol and 2 mL 0.1% NH_4OH on methanol).

The mixed *Scenedesmus obliquus* samples with 3N BBM were centrifuged at 13000 rpm, 4 deg C for 15 min, and then the algae pellet was collected into an HDPE bottle.
The *Scenedesmus obliquus* samples were washed with LC grade water and centrifuged at 13000 rpm, 4 deg C for 15 min. The supernatants were algae water samples, which were analyzed for PFSAs adsorption on algae. The algae water samples were subjected to solid-phase extraction (SPE) and eluted finally with 4 mL extraction solvent which has 2 mL methanol and 2 mL 0.1% NH_4OH in methanol.

3.5.2 Biomass sample preparation

3.5.2.1 The UHPLC-MS/MS test preparation

After measuring the wet weight of *Scenedesmus obliquus*, the algae samples were extracted with 15 mL methanol by sonicating them at 60 Hz for two hours. The sample extracts were centrifuged again, and the supernatants were concentrated to 1 mL using a rotary evaporator. The samples were eluted with 3 mL extraction solvent (1.5 mL methanol and 1.5 mL 0.1% NH_4OH on methanol). Concentrated methanol extracts were diluted with 50 mL LC grade water and subjected to solid-phase extraction (SPE) for cleaning up.

All of the samples for the UHPLC-MS/MS analysis were processed according to the method from Mulabagal (2018)[101].

3.5.2.2 Photosynthesis pigment analysis

Pigment concentration was measured using Spectrophotometer method [102]. First, 10 grams of magnesium carbonate was added into 1000 mL of DI water to prepare a saturated $MgCO_3$ stock solution. This solution was settled for 24 hours and only the supernatant was utilized after that period. A 90% (v/v) Buffered Acetone Solution was prepared by adding 100 mL $MgCO_3$ solution into 900 mL of acetone. The 25 ml glass culture tubes were gathered and labeled by numbers. The sample filters were placed in the labeled glass culture tube using tongs. Then, 10 mL Buffered Acetone Solution was dispensed into the glass tubes containing the sample filters. After that, the glass tubes were vortexed to ensure all the filter parts were in contact with the Acetone extract solution. Following this, tubes were stored at -20 deg C for 24-48 hours to allow complete reaction with the solution. On the second day, samples were removed from the freezer and vortexed for 5 minutes to ensure all the pigments are solved in the extract solution. After that, the glass tubes were centrifuged again, to prevent small particles of the filter or algae from causing an error during the reading in the spectrophotometer. The supernatants of pigment extract solution were poured into 10 mm quartz flat cell (VWR, Spectrophotometer cell, 10 mm) and then read by spectrophotometer.

An alternate method for detecting pigment concentration, using an HPLC machine, was used for some sets of samples. New 10 mL falcon tubes were gathered and labeled by numbers. The sample filters were placed in the labeled falcon tube (Falcon, 5 mL Polypropylene Round-Bottom Tube, 12×75 mm) using forceps and the numbers were recorded by order. A pipette was used to insert 2 ml of extract solvent, which was made by acetone, methanol and water in ratio of 80:15:5, into the tubes with the sample filters. Sample tubes were then sonicated with the extract solution for 5 min to make sure all the compounds are mixed well. After that, a sonicator wand

was used to pulverize the samples to ensure that all the filter parts were in the extract solution. Tube were stored at -20 deg C overnight to allow enough time to react with the extract solution. On the second day, the sample were removed from the freezer and vortexed for ten minutes to make sure all the pigments were solved in the extract solution. After that, a 0.20 μm syringe filter (WHEATON Microliter, 13 mm, 0.2 μm Nylon) was used to remove the extracted pigment solution into the HPLC vial, rinsing syringes 3-4 times between each sample, and then samples were loaded into the HPLC tray.

After filtration, the Shimadzu HPLC system was used to analyze the pigment in a 100 μL aliquot of a 3:1 ratio for the ion-pairing agent samples which were in the HPLC tray. The ion-pairing agent was made by a solution of 0.75 g tetrabutylammonium acetate and 7.7 g ammonium acetate in 100 mL HPLC grade water. The method of Leavitt and Hodgson[103] was used to determine the mobile phase and time sequence. Pigments were separated by using a Rainin Model 200 Microsorb C18 column and measured using a photodiode array detector. Pigments were identified based on retention time and peak shape, and integrations were performed by comparing the peak area with a standard of known concentration.

3.5.3 Statistical analysis

All of the statistical analyses for the experiments were calculated by R 3.6.2. For all analyses, differences were determined with a confidence level of $\alpha = 0.05$.

For the high PFSAs mixture toxicity experiment, the repeated measures ANOVA was used in these studies. Repeated measures analysis of variance (ANOVA) was used to determine whether statistically significant differences between the means of three or more independent or uncorrelated groups in the same subjects show up in each group. The mean and

standard deviation of the most experiments is shown in the Appendices. Dunnett's test was used as a post hoc test to determine which groups were significantly different from each other.

One-way repeated measures ANOVA test was also used for the low PFSAs mixture concentrations including toxicity and bioconcentration ability experiment. The Tukey HSD test was used as a post hoc test to determine if pairwise comparison was significant different.

4. Results

4.1.Growth curve of *S. obliquus* after exposure

4.1.1. The population growth rate in preliminary bioconcentration test

Figures 17 and 18 are the results from the pre-test for the PFSAs mixture at two different concentrations. At the lower PFSAs concentration (Figure 17) of 0.01 mg/L, the *Scenedesmus obliquus* culture was affected by the PFSAs mixture slightly. At this concentration, cell density increases in subsequent times, with appreciable difference between treatment replicates and experimental controls at zero PFSA concentration. A one-way repeated measures ANOVA was used to analyze the data, conducted to examine the effects of 0.01 mg/L PFSAs mixture on algae cell density. The algae cell density was not significantly different from the control group ($F = 1.282$, $P < 0.05$). The Tukey HSD test shows the significant difference between treatment and control group (Appendix 3).

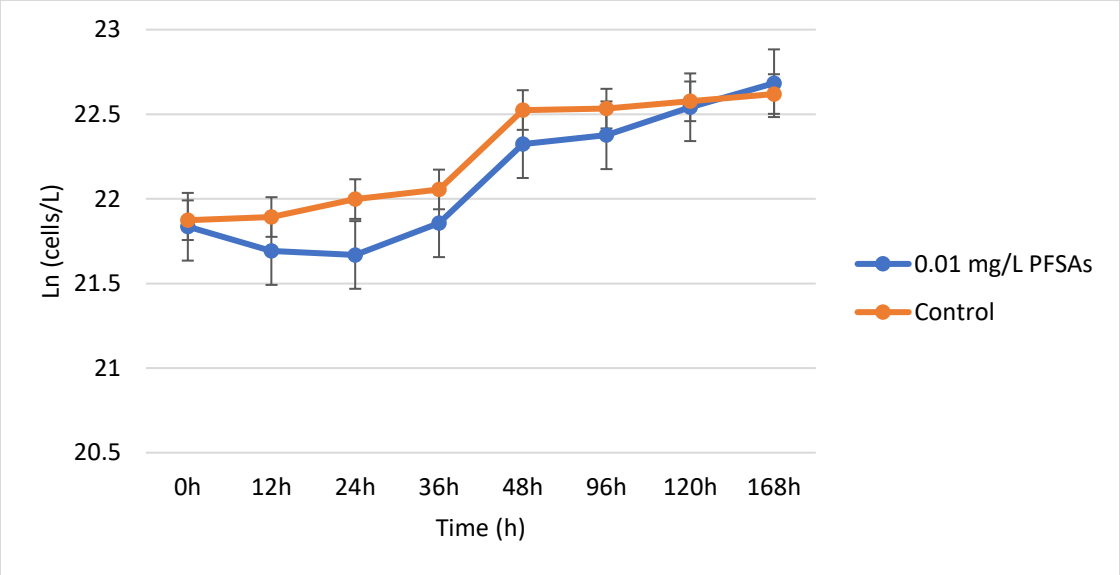


Figure 17. Cell density (in the number of cells per liter) versus time for a culture of S. obliquus exposed to 0.01 mg/L concentration of PFSA mixture. Error bars represent the range of the mean (n=2). 'Control' represents the experimental control at zero PFSA concentration

In addition, results from exposure to higher concentrations (Figure 18) of 0.1 mg/L PFSA shows slightly lower cell density at the final sampling time, suggesting inhibition of algal growth by the presence of PFSAs. At the higher PFSAs concentration, the *Scenedesmus obliquus* culture was also affected by the PFSAs mixture slightly. The one-way repeated measures ANOVA test shows the 0.1 mg/L PFSAs mixture ($F = 0.37$, $P = 0.546$) is not significantly different from the control group. The Tukey HSD post hoc test results shows in the Appendix 3 as well.

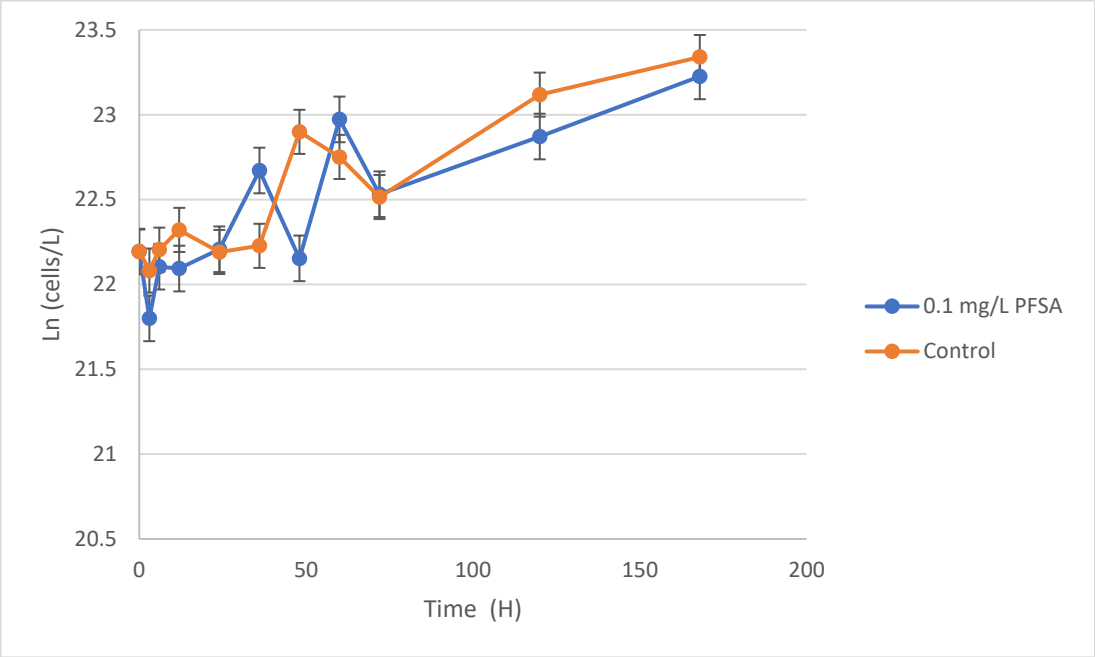


Figure 18. Cell density (in the number of cells per liter) versus time for a culture of S. obliquus exposed to 0.1 mg/L concentration of PFSA mixture. Error bars represent the range of the mean (n=2). 'Control' represents the control at zero PFSA concentration

4.1.2. The population growth rate during toxicity test

Figure 19 shows the results of the seven days exposure experiment for the toxicity effect of PFSAs mixture on *Scenedesmus obliquus*. It displays how the *Scenedesmus obliquus* grows under certain conditions of PFSAs exposure. With a series of increasingly higher concentrations of PFSAs mixture, the algae cell number changes over time. The results show that the cells

of *Scenedesmus obliquus* interact with different concentrations of PFSAs mixture. The amount of inhibition of the algae growth rate increases as well when the concentration of PFSAs mixture increases. The lowest PFSAs mixture concentration tested was 10 mg/L, which shows the cell growth rate increasing over time, but not as much as the control. For the experiment of the *S. obliquus* growth rate experiment, the different PFSAs mixture concentration of algae treatments using repeated measures ANOVA shows significantly different rates of increase of cell number from each other. A repeated measures ANOVA was conducted on eleven different sampling time points to examine the effect that six different PFSAs mixture concentrations had on algae growth rate ($F=3.6136$, $P = 0.0072$). However, after seven days of exposure, the trend in cell number for algae growing in 160 mg/L PFSAs mixture is much lower than any other PFSAs mixture concentrations. The post hoc Dunnett's test results are listed in the Appendix 5.

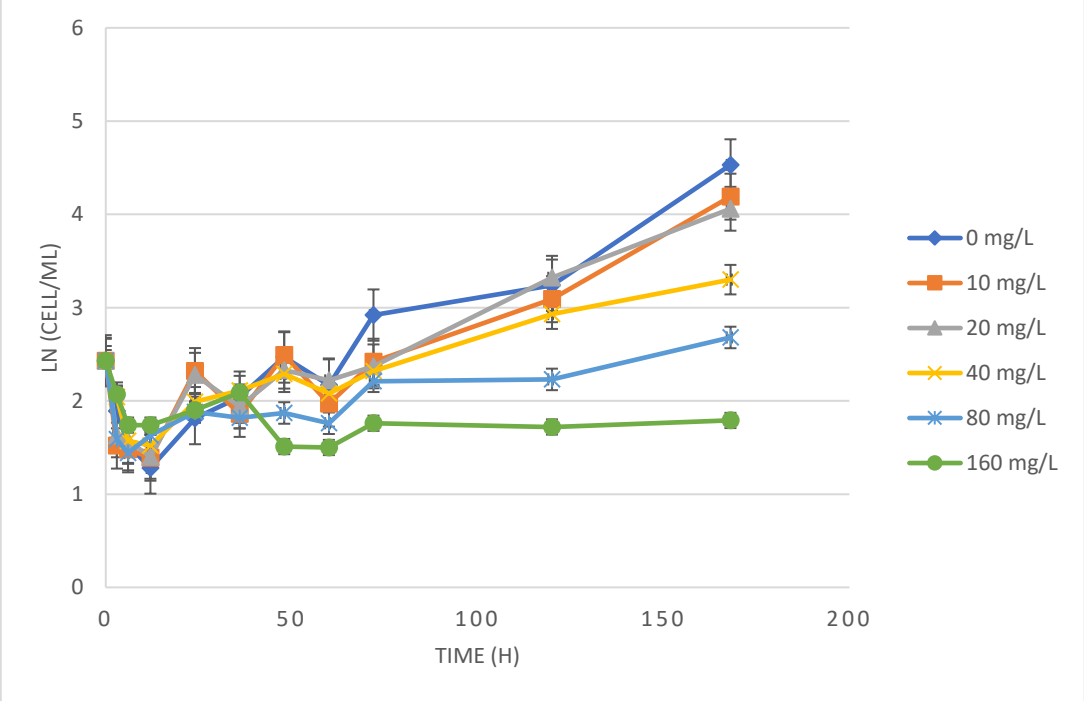


Figure 19 Cell number versus time for a culture of S. obliquus at different concentrations of PFSAs mixture (PFOS, PFBS and PFHxS). Error bars represent the standard deviation of the means (n=3).

4.2 Exposure study for toxicity effect of PFSAs on *Scenedesmus obliquus*

The EC_{50} is calculated through the 50 % change in algae population growth rate at 168 hours from algae cell density. According to the linear regression equation in Figure 20, the EC_{50} of PFSAs mixture concentration for the algae cell is 120.35 mg/L, which indicates that there is 50% of algae cell death when the PFSAs concentration reaches 120.35 mg/L (Table 8. and Figure 21.). From the EC_{50} result, it appears that the higher the PFSAs mixture concentration in the environment, the more the growth of the algae is inhibited, suggesting a toxic effect. The response represents the changes of algae population, as summarized in Table 8 and Figure 20.

Table 8. The cell response of S. obliquus during the experiment time

The *S. obliquus* EC_{50} response (%)											
	3H	6H	12H	24H	36H	48H	60H	72H	120H	168H	Response (%)
0 mg/L	77.8	63.0	52.7	74.5	84.0	101.6	89.3	120.2	133.3	186.4	0
10 mg/L	62.6	60.9	57.2	95.5	76.5	102.5	81.1	99.6	127.2	172.4	7.5
20 mg/L	67.1	64.6	57.6	93.8	79.8	95.9	91.4	97.5	136.6	167.1	10.4
40 mg/L	84.0	64.6	62.6	81.9	86.8	94.2	85.6	95.5	120.6	135.8	27.2
80 mg/L	65.4	59.3	67.1	77.4	74.9	77.0	72.4	90.9	91.8	110.3	40.8
160 mg/L	85.2	85.2	71.6	78.2	86.0	62.1	61.7	72.4	70.8	73.7	60.5

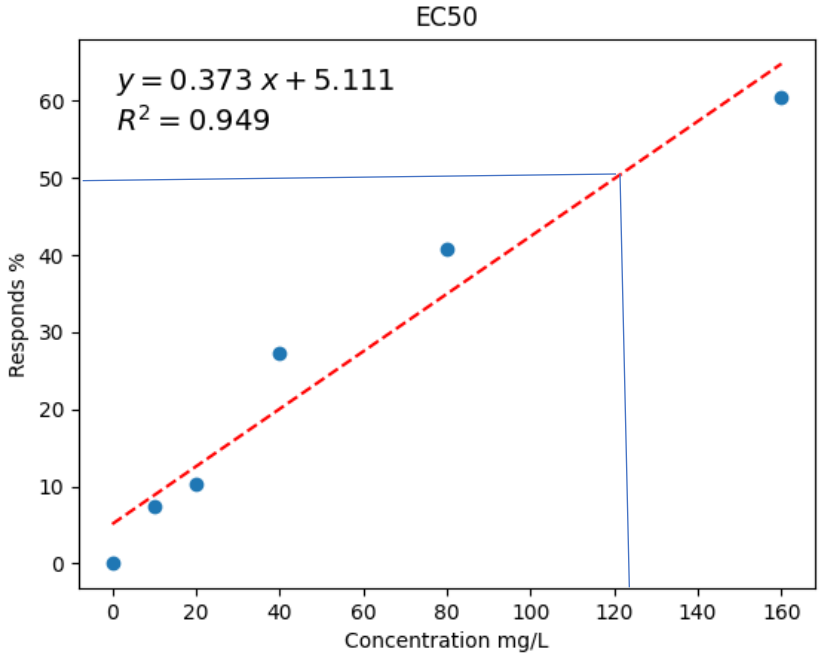


Figure 20. The percentage of cell response vs. PFSAs mixture concentrations for Scenedesmus obliquus under a series of PFSAs concentrations. The Y-ais shows the percent reduction of algae cells after seven days exposure compared with control. The X-axis shows the different concentrations of PFSAs mixture.

4.3 The fate of PFSAs in algal culture

The results of the fate experiments for algae culture in 0.01 mg/L PFSAs mixture concentration are shown in Figures 21 and 22. After seven days of exposure, the concentration of PFBS in the media water stays at the same level. Meanwhile, the concentrations of PFHxS and PFOS in the media water both decreased (Figure 21). For the 0.01mg/L bioconcentration experiment of the *S. obliquus*, the different PFSAs concentration of algae treatments in the media water using one-way repeated measures ANOVA shows PFOS, PFBS and PFHxS were significantly different with time, for which both of them had $P < 0.0001$.

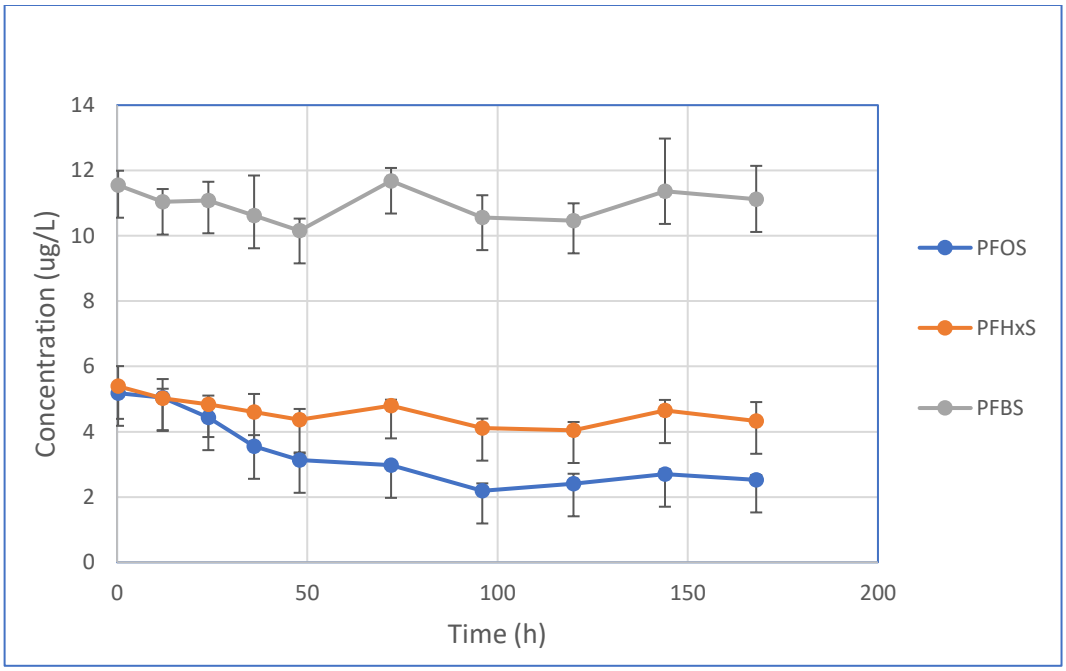


Figure 21. The concentration of PFSA components in a mixture (0.01 mg/L total PFSA starting concentration) in the media water over time in a culture of S. obliquus (n=2).

The measured quantities of each of the PFSA components in the overall mixture inside and on the algae surface are shown in Figure 22. A greater amount of PFOS and PFBS was seen on the algae, as inferred from the concentrations measured in the wash water, with 33.3 %, 29.7 %, and 60.6 % of the total amounts found of PFOS, PFHxS, and PFBS, respectively (Figure 22). Concentrations of PFOS and PFHxS were measured for the overall algae, with 1.13 % and 0.09 % of the total amounts of each found here, respectively. For PFBS, no measurable quantities were found in the internal biomass (Figure 22).

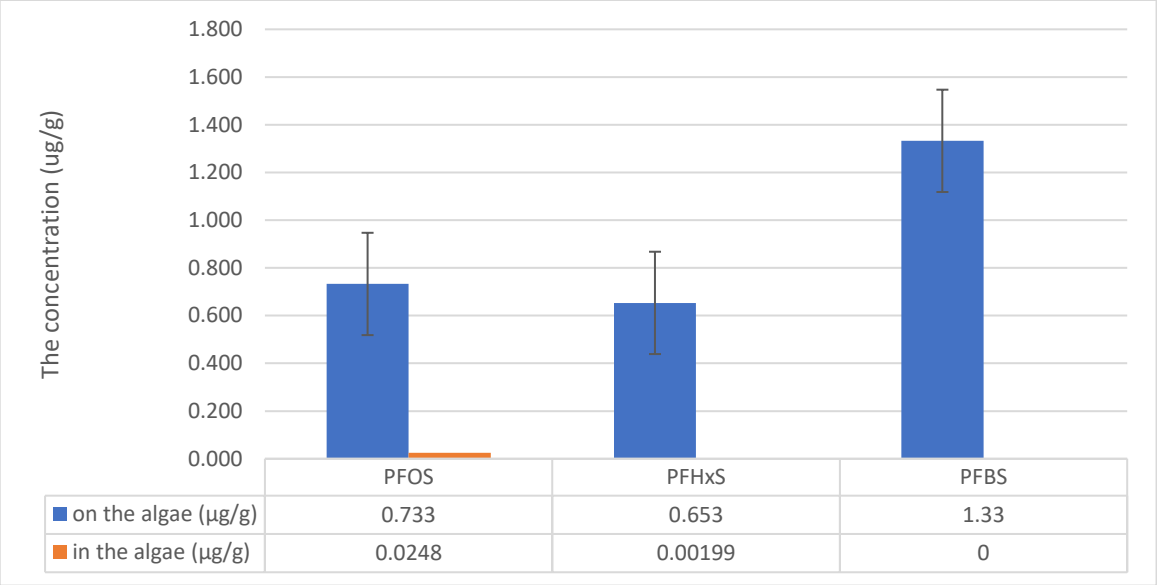


Figure 22. The concentration of 0.01 mg/L PFSAs in the overall algae cells during the whole experiment (n=2).

The result shows that in the 0.01 mg/L PFSAs mixture concentration, PFBS did not go into the algae cell but attached to the exterior of the algae cell. Because of this, the next experiment focused only on the fate of PFOS and PFHxS. Figures 23 and 24 show the results from 0.1mg/L PFSAs mixture, which only had PFOS and PFHxS. The concentrations of PFHxS and PFOS in the media water also decreased, respectively (Figure 23). However, the PFOS in the algae media water decreased over time significantly more compared with PFHxS (Figure 24). A large fraction of the total amount of PFOS and PFHxS was seen on the algae, as inferred from the concentrations measured in the wash water, with 62.7 % and 75.2 % found of PFOS and PFHxS, respectively. At the same time, 0.41% of PFOS and 0.28 % of PFHxS were found in the overall algae cells. For the 0.1mg/L bioconcentration experiment of *S. obliquus*, the different PFSAs mixture concentration of algae treatments were examined using one-way repeated measures ANOVA. The statistical analysis shows PFOS and PFHxS were significantly different from each other with time, for which all of them had $P < 0.0001$. In

addition, the Tukey HSD post hoc test shows PFOS and PFHxS were significant different between each other.

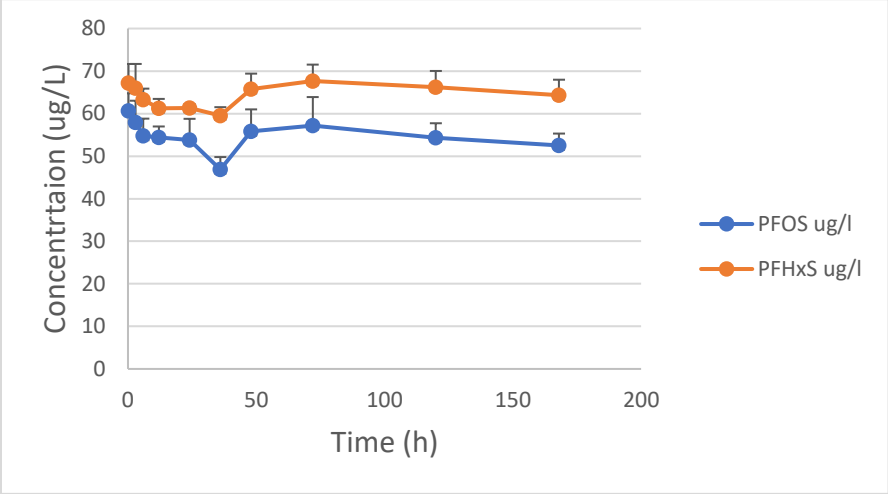


Figure 23. The concentration versus time of PFSA compounds in a culture S. obliquus at initial total concentration of 0.1 mg/L PFSAs mixture in the media water. Error bars represent standard deviation of the means (n=2).

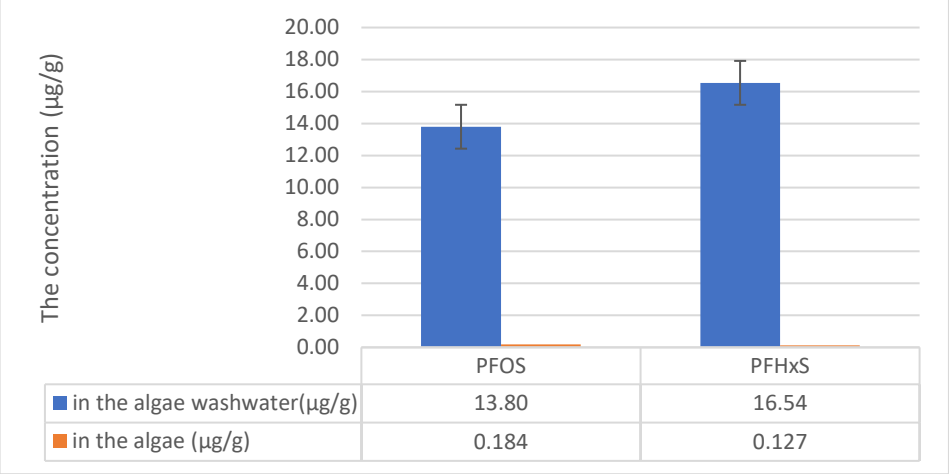


Figure 24. The concentration of 0.1 mg/L PFSAs in the overall algae cells during the whole experiment (n=2).

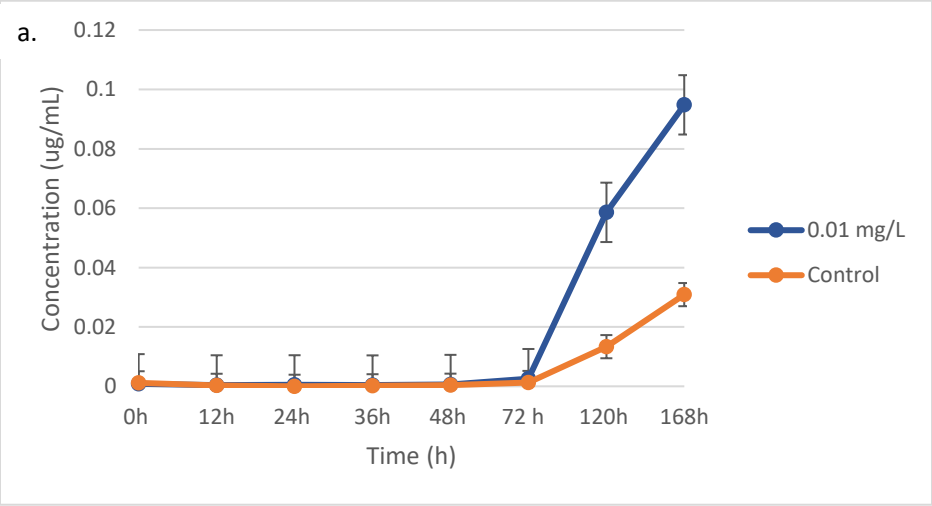
4.4.Photosynthetic pigment analysis

4.4.1 The pigment results of the preliminary bioconcentration test

The results of pigment tests of 0.01 mg/L PFSAs mixture were determined using the HPLC, but the pigment results from 0.1 mg/L PFSAs mixtures were determined using a

spectrophotometer. The results of pigment concentration measurements in 0.01 mg/L and 0.1 mg/L PFSAs mixture experiment are shown in Figure 25, Figure 26 and Figure 27. In this analysis, Chlorophyll-α and Chlorophyll-b were used as indicators to show the algae stress under particular PFSAs mixture concentration. The total pigment, which includes Chlorophyllide, is shown in Figure 28. From all these results, the pigment of *Scenedesmus obliquus* with PFSAs mixture is generally greater than that of the control after 72 hours for the 0.01 mg/L condition, and after 36 hours for the 0.1 mg/L condition; generally, the algae cells show the stress in the PFSAs mixture after 36 h.

A one-way repeated measures ANOVA was used to analyze if each pigment content was significantly different from the control group. For 0.01 mg/L PFSAs mixture concentration, the Chl a, Chl b and total Chl content was not significantly different before 72 hours. Regarding the Chl content in 0.1 mg/L PFSAs mixture, the Chl a, Chl b and total Chl were not statistically different according to the one-way repeated measures ANOVA test.



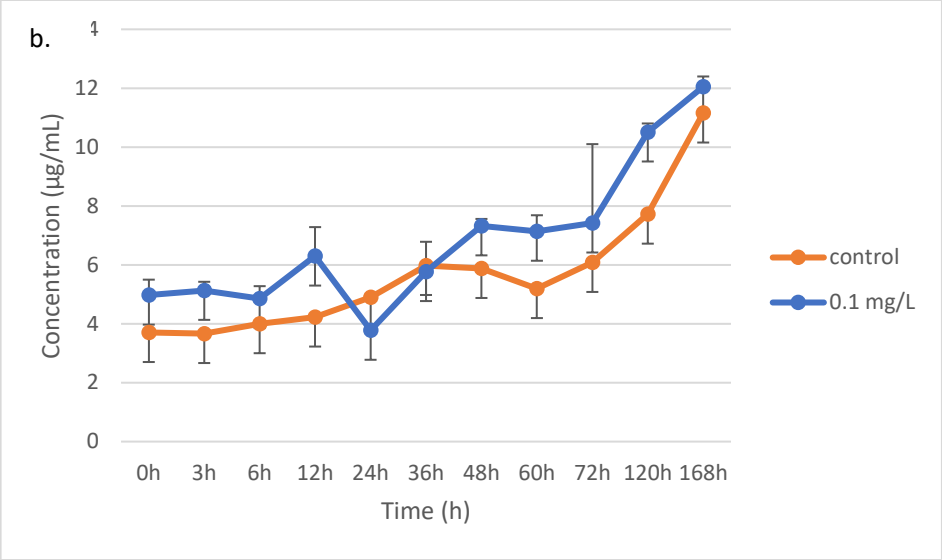
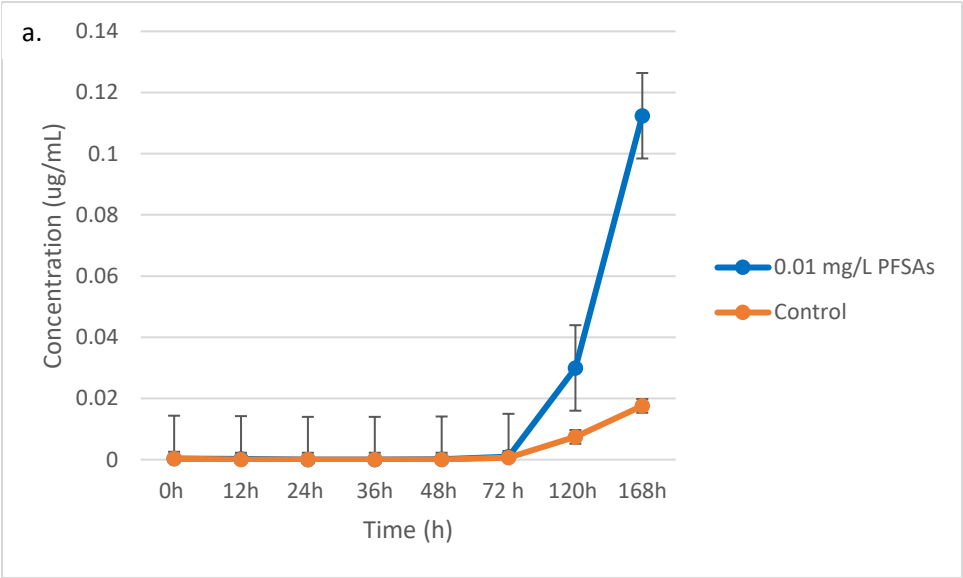


Figure 25. The Chl-a concentration versus time of S. obliquus at the concentrations of 0.01 mg/L PFSAs mixture (a) and 0.1 mg/L PFSAs mixture (b) for bioconcentration test. Error bars represent the range of the means (n=2).



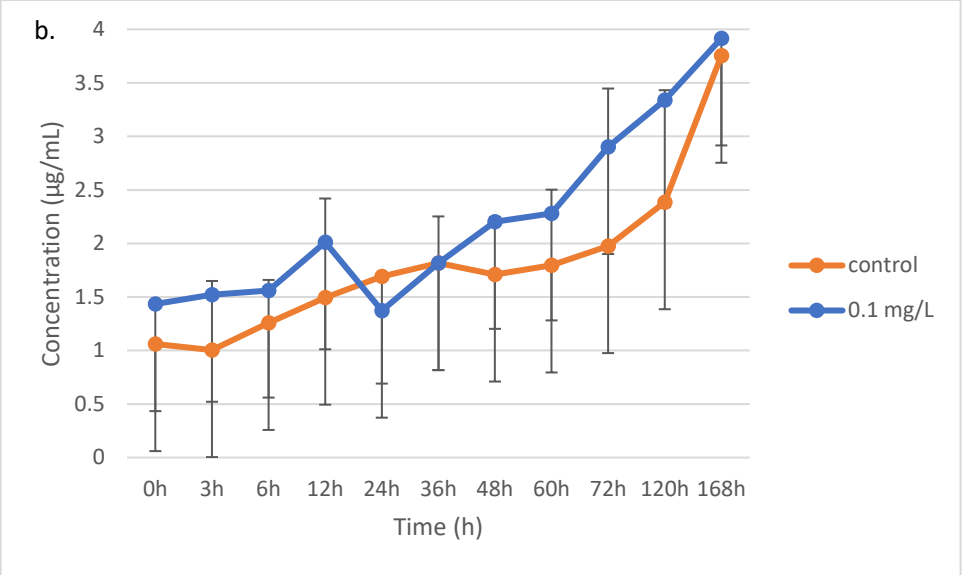


Figure 26. The Chl-b concentration versus time of S. obliquus at the concentrations of 0.01 mg/L PFSAs mixture (a) and 0.1 mg/L PFSAs mixture (b) for bioconcentration test. Error bars represent the range of the means (n=2).

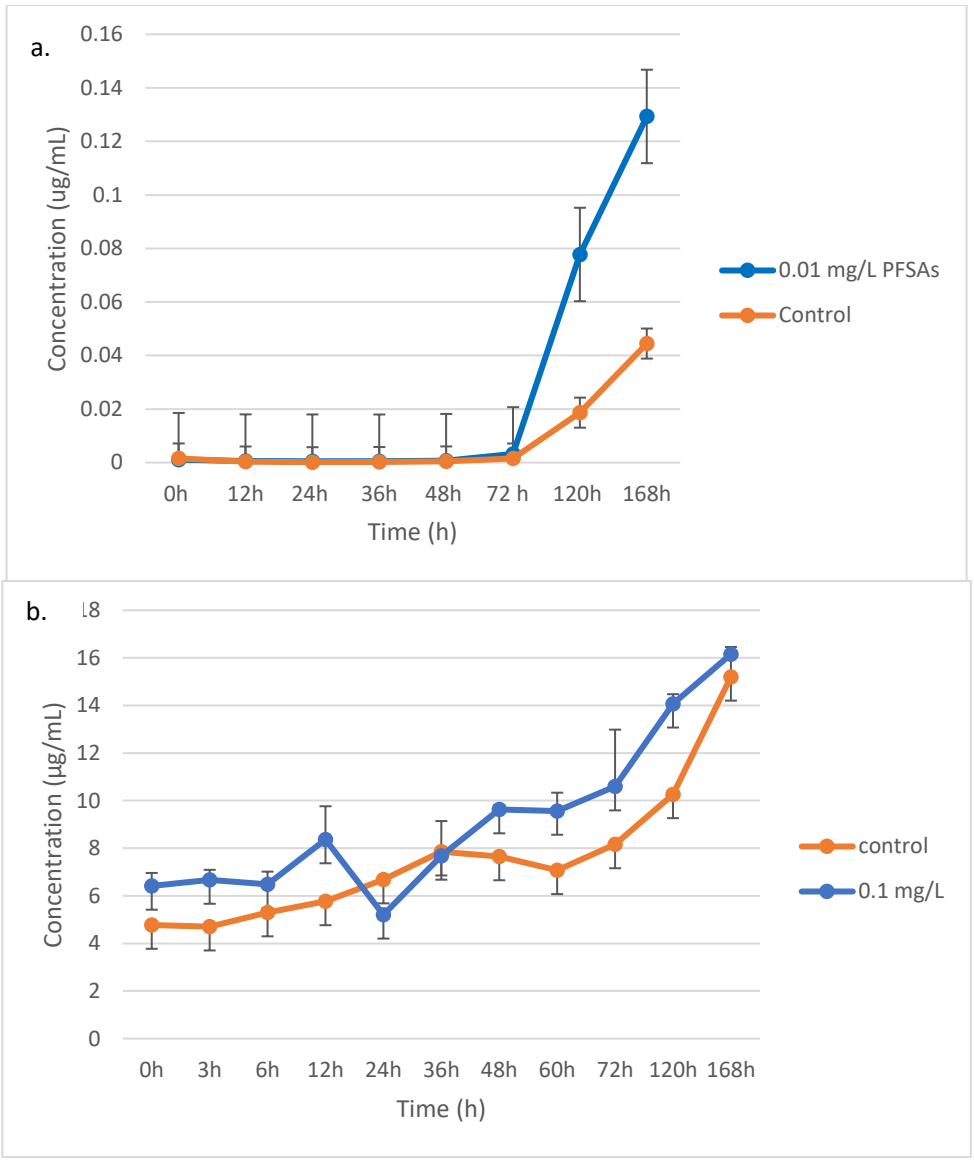


Figure 27. The total pigment concentration versus time of S. obliquus at the concentrations of 0.01 mg/L PFSAs mixture (a) and 0.1 mg/L PFSAs mixture (b) for bioconcentration test. Error bars represent the range of the means (n=2).

4.4.2 The pigment results of the toxicity test

In the different PFSAs mixture concentration groups, a significant difference of Chl-a, Chl-b, and total Chlorophyll was detected between 6 groups of different PFSAs mixture concentrations. A one-way repeated-measures ANOVA was conducted on eleven different sampling time points to examine the effect that six different PFSAs mixture concentrations had

on pigment content. Results showed that the different PFSAs mixture concentrations used lead to statistically significant differences in Chl-a content ($F=12.12$, $p < 0.00001$), Chl-b content ($F=10.18$, $p < 0.00001$) and total Chl content ($F=11.69$, $p < 0.00001$). The pigment concentration results in the toxicity effect of PFSAs mixture experiment using spectrophotometer are shown in Figure 28, Figure 29, and Figure 30. In this analysis, Chlorophyll-α and Chlorophyll-b were measured over time at different particular PFSAs mixture concentration. The total pigment is shown in Figure 30. From Figures 28 and 29, while the Chlorophyll-α and Chlorophyll-b concentration increased over time for all treatments except 160 mg/L, the relative rate of increase was lower for higher PFSAs concentrations. The Chl a and Chl b contents shows statistically significant differenes at 48h and 60h, respectively.

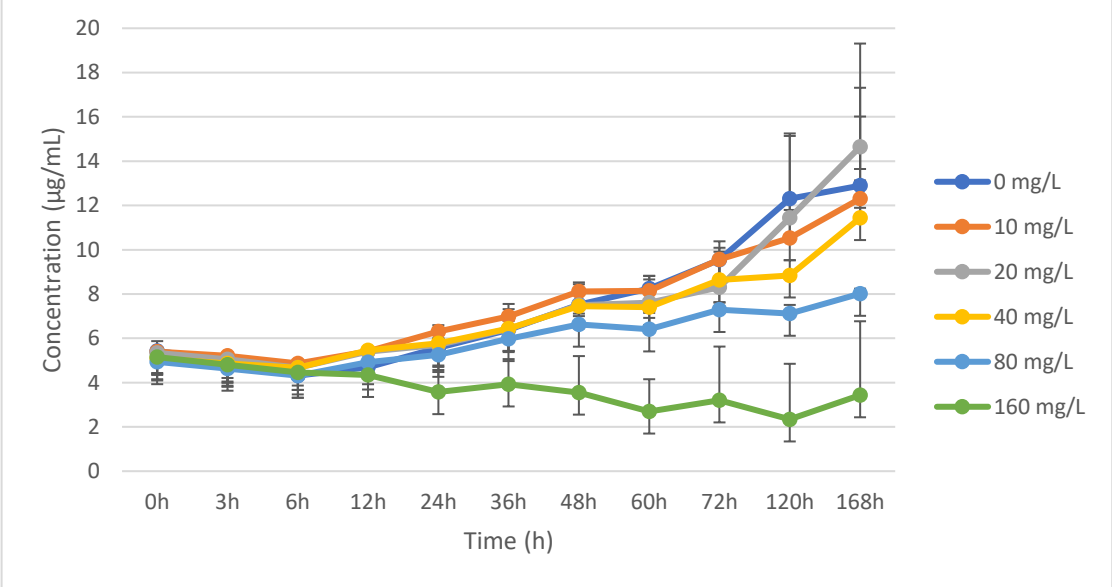


Figure 28. The Chl-a concentration versus time of S. obliquus at different concentrations of PFSAs mixture for toxicity test. Error bars represent the standard deviation of the means (n=3)

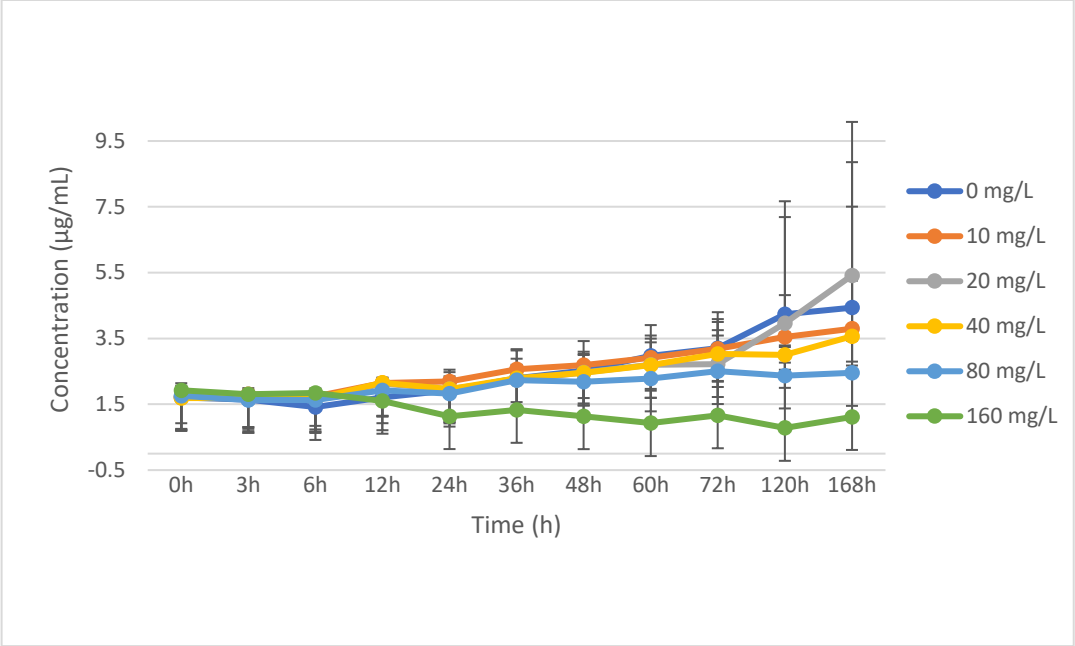


Figure 29. The Chl-b concentration versus time of S. obliquus at different concentrations of PFSAs mixture for toxicity test. Error bars represent the standard deviation of the means (n=3)

Figure 30 shows the effect of the PFSAs mixture on the total chlorophyll concentration of *S. obliquus*. The concentration-dependent reduction of total chlorophyll concentration is observed after 12 hours. The 160 mg/L PFSAs mixture showed a significant decrease starting at 12 hours until 168 hours according to Figure 30. However, the total Chl content shows statistically significant differences at 60h. The total chlorophyll content in the algae also showed the same trend as Chlorophyll-α and Chlorophyll-b, whereas the PFSAs mixture concentration increases, the total chlorophyll content production rate decreases. Since the p-value for PFSAs mixture concentration, time and interaction effect are both less than 0.05, this means that all three factors have statistically significant effect on the algae chlorophyll content.

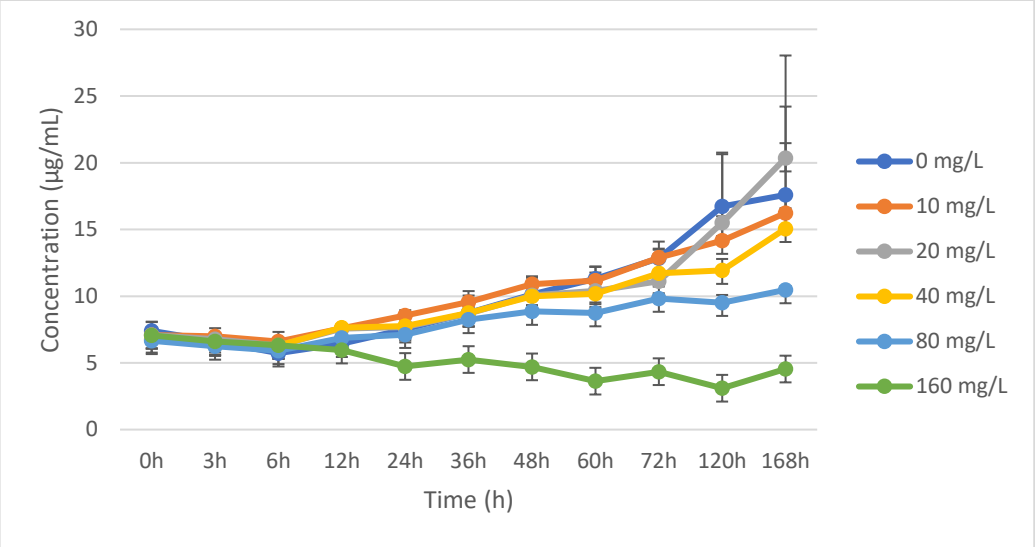


Figure 30. The total pigment concentration versus time of S. obliquus at different concentrations of PFSAs mixture for toxicity test. Error bars represent the standard deviation of the means (n=3).

5. Discussion

5.1. The population growth curve and EC_{50} of *S. obliquus*

For the population growth rate under different PFSAs concentrations in the toxicity experiment, the different PFSAs concentrations start showing the toxicity effect to the population growth rate after the first two days (Figure 19). However, the highest concentration of PFSAs (160 mg/L) significantly inhibited the population growth rate in 7 days more than other PFSAs mixture concentrations. This result indicates the 160 mg/L PFSAs mixture is extremely toxic to the algae cell. The one- way ANOVA shows the PFSAs mixture can significantly affect the algae cell density. Dunnett's test in Appendix 5 displays the statistically significant grouping of variation among PFSAs mixture concentration variables, showing that the 80 mg/L and 160 mg/L concentrations of PFSAs mixture had significant effect on the population growth rate. The EC_{50} value from this study is also confirmed from the result of the population growth rate. The 0.01 mg/L and 0.1 mg/L results indicate that the toxicity effect of the PFSAs mixture to the algae cell is determined by the aquatic concentration of the PFSAs mixture (Figures 17 and 18). The PFSAs mixture is more toxic to algae at the higher concentrations, suggested by the decrease in the population growth rate for cultures exposed to higher concentrations. One suggestion of the population growth rate under different PFSAs concentrations is that the changes in the algae living environment affect the growth rate. The algae need to adjust themselves when they live in a new environment. In this study, the *Scenedesmus obliquus* mother culture was living in the 3N BBM solution in another lab. Figure 19 shows that the algae population was decreasing in the first couple of hours because the algae culture needed to acclimate to the new living environment with different light and temperature. However, after 36 hours, the algal culture started growing again, and the difference between each PFSAs mixture concentrations was demonstrated.

For the concentration of 50% of maximal effect (EC_{50}), the effect of PFSAs mixture on the population growth of *S. obliquus* was analyzed. The algal cells have interacted with the PFSAs mixture in different concentrations; the growth inhibitory effect was noted as the PFSAs mixture concentration increased (Table 8). The EC_{50} of the PFSAs mixture to algae growth, where 50% of the population of algal cells die, was found here to be 120.35 mg/L, while the highest PFSAs mixture concentration had a maximum effect of the growth of *S. obliquus* (Figure 20). This value is similar to those found in other studies published in the literature for pure solutions of PFSA compounds. For example, the EC_{50} of pure PFOS for *S. obliquus* after a 96-hour exposure was found to be 126 mg/L [38]. Similarly, for PFBS, the EC_{50} value for *Pseudokirchneriella subcapitata* after 96 hours of exposure was 5733 mg/L [104]. There are no articles that define the EC_{50} of pure PFBS and pure PFHxS for *S. obliquus.* Although the PFSAs mixture had a definite growth inhibitory effect on the algae, a more significant toxicity effect will show up with higher concentrations and a longer exposure time of the PFSAs mixture on algae. Also, the PFSAs mixture represents a greater toxicity effect than single PFOS in the aquatic environment, in comparison to values determined from the literature review.

5.2. The toxicity effect of PFSAs on *Scenedesmus obliquus*

Some studies [63, 64] have indicated that chlorophyll is very sensitive to PFOS, and declining chlorophyll concentrations can indicate the stress or cell health for algae. The concentration of chlorophyll also can reflect the toxicity level of PFOS. The experimental setup and condition of the pigment test in the toxicity experiment show the same trends compared with the growth rate. In addition, the repeated measures ANOVA showed that the different PFSAs mixture concentrations lead to statistically significant lower Chl-a content ($p < 0.00001$), Chl-b content ($p < 0.00001$) and total Chl content ($p < 0.00001$) than the control group. Dunnett's test

was used to analyze the significance among different groups of PFSAs mixture concentration (Appendix 17). The chlorophyll content in algae at 160 mg/L PFSAs mixture was significantly decreased after 12 hours (Figure 28, 29, and 30). However, there were significant differences in chlorophyll concentration across all five PFSAs mixture concentrations before 60 hours, with decreasing chlorophyll content over time compared with the control group. Compared with the control group, the decreases in chlorophyll suggest that *S. obliquus* was under stress during the whole experiment time of exposure to PFSAs at any concentration. In reflection of these results, however, it may be better to measure the concentration of Chlorophyllide by concentration to confirm the chlorophyll results, as this compound is known to be produced in response to stress conditions. Also, past research has investigated only PFOS effects on chlorophyll; however, this research shows that a PFSAs mixture (PFOS, PFBS, and PFHxS) has a similar effect on chlorophyll. This suggests that complex mixtures of PFSAs, as might be found in impacted natural aquatic systems, can have a continued and persistent effect on the health and vitality of the phytoplankton community in that environment. The pigment results were significant to the alpha= 0.05 level via repeated measures ANOVA analysis, and post-hoc analysis suggested 160 mg/L PFSAs mixture concentration is significantly different from control group. However, to get a better understanding of how chlorophyll content is affected by individual PFSAs, the relationship of chlorophyll content and single PFSAs needs to be studied in the future.

For the lower PFSAs mixture concentration, 0.01 and 0.1 mg/L PFSAs mixture, the PFSAs mixture of toxicity effect for the bioconcentration test shows that the population growth rate under the PFSAs mixture is not significantly lower than the control group. However, the Chl content in the treatment group were higher than the control group, but not significantly. These results may indicate that algae had developed a resistance system to adjust itself under the lower

PFSAs mixture concentration. Thus, the algae need to produce more pigment to do the photosynthesis for supporting its growth under the low PFSAs mixture environment continually. However, this toxicity experiment of low PFSAs mixture concentration needs to have more experiments to confirm this hypothesis.

5.3. Bioconcentration

In the literature review section, some of the field studies showed that PFSAs could be transferred through the aquatic food chain and finally get into the human body. In order to know the fate of PFSAs with different compound's length in the algae cells, media and algae samples were subsampled and analyzed to measure the concentration of PFOS, PFBS, and PFHxS.

The bioconcentration results assumed that all the measured concentration changes for PFSAs mixture are related to biotransformation or bioconcentration that happened in the experiment systems, where reacted or accumulated PFSA molecules became unavailable for measurement in the aquatic media. From the analyzed water samples by UHPLC-MS/MS, declining trends were only observed for PFOS and PFHxS, and not for PFBS. The hypothesis of the bioconcentration experiment was that all of the single PFSAs (PFOS, PFBS, and PFHxS) would be adsorbed and absorbed directly by *S. obliquus* cells. That means the PFSAs mixture concentration should decrease in the media water but increase in the algae washed water sample and algae samples. This would indicate that *S. obliquus* has the adsorption and absorption ability for the PFSAs mixture.

Nevertheless, the mass balance of PFSAs from the UHPLC-MS/MS result shows that the concentration of PFBS did not change in the water samples during the whole experiment time. However, the concentration of PFOS and PFHxS in the media water samples decreased significantly over time. At the same time, none of the PFBS was detected in

the algal cells, even on the cell membrane resulting from the wash water. Additionally, the PFOS shows strong bioconcentration ability compared with PFHxS not only in algae cell membranes but also inside the algae cells.

According to the equation of bioconcentration factors (BCF) in the literature review section, the BCFs of PFOS, PFBS, and PFHxS can be determined based on the results from 0.01 mg/ L and 0.1 mg/L PFSAs mixture bioconcentration experiment. The BCFs for the bioconcentration experiments are list in Table 9. These are within the range determined for other PFSA experiments for BCF. The BCF numbers confirmed the bioconcentration ability of PFSAs mixtures and indicated that the algae uptake and accumulate PFOS and PFHxS on or in the cells. However, PFBS did not accumulate in the algae cell according to BCF and the experiment.

Table 9 The bioconcentration factors of 0.01 and 0.1 mg/L PFSAs mixture bioconcentration experiment

Bioconcentration factor (BCF) (mL/g)			
	PFOS	PFBS	PFHxS
0.01 mg/L PFSAs mixture	2.79	0	0.23
0.1 mg/L PFSAs mixture	2.63	N/A	1.83

Usually, the PFAS compound has bioaccumulation ability when BCF is greater than 5000 or has potential bioaccumulation ability when BCF is around 2000 ~ 5000[105]. According to the results from this bioconcentration experiment, the BCFs do not reach to 5000. It is expected that adsorption is the main way for phytoplankton to remove substances from water because of the lipophilic characteristic of PFSAs. It is also well known that shorter chain of PFSAs, such as PFBS, which has only four carbon chains, has a higher solubility than longer chain PFSAs. Therefore, the analysis did not show any absorption of PFBS in the algae. An

additional experiment about bioconcentration was conducted to obtain more information about PFOS and PFHxS regarding this experiment's result. Due to the bioconcentration result of PFBS from the last experiment, PFBS was not included in this additional experiment.

In recent years, some studies have questioned the applicability of BCF for evaluating the bioaccumulation of PFASs, which are highly water-soluble and easily dissociable[35, 106]. Some works has shown that ionized short-chain PFASs molecules are more likely to bind to the phospholipid layer on cell membranes[107]. Another suggestion to explain the PFBS adsorption behavior in this experiment is because of the kinetic and electric charges on the *S. obliquus* cell membrane. There is no literature indicating the kinetic relationship between PFSAs and algae cell. This remains a good opportunity for future researchers to study.

5.4 Recommendations and future work

This experiment only studied PFOS, PFBS, and PFHxS from Perfluorosulfonic Acid compounds. There are still many other Perfluoroalkyl substances and Polyfluoroalkyl substances distributed in the environment all around the world. Names are known for only some of the PFAA compounds, and studies have not been conducted for most. Most of the PFAAs have toxicity effects on wildlife and humans through the food chain, and are not easily or effectively removed from the environment. There is still a vast unknown area about the fate and behavior of those PFAAs. Also, there is much need to understand the fate of PFAAs in the algae cell and the mechanism of function in algae. Some future work in this regard is suggested as follows:

i. The toxicity effect of a PFSAs mixture compared with single PFSA compounds on algae cells needs to be understood. The effect of a PFSAs mixture to the Chlorophyll in the algae cell, and the investigation into the effects of a PFSAs mixture, to break down Chlorophyll to other pigment types styles, remains to be studied.

ii. The toxicity effect of different molecular lengths of PFSAs to the algae cell, or the same length of PFAAs but from different groups to the algae cells will need to be done in the future.

iii. Future work also needs to find the bioconcentration ability of PFSAs in the different algae species.

iv. The biomagnification ability of PFSAs mixture can be defined through trophic level transfers to animals in the food chain.

v. Future experiment will be designed to study the mechanical effect of PFSAs on the algae cells, looking at enzyme and protein dynamics.

vi. The genetic expression in the algae cells should be studied to indicate the PFSAs mixture toxicity ability in the future.

vii. The PFSAs mixture concentrations in these studies are much higher than typical environmental contamination conditions. More of the environmental relevant concentrations need to be studied to understand the bioconcentration ability of PFSAs mixture.

Lastly, the toxicity and bioconcentration test in this study were limited in scope. Higher replicated studies, and repeated trials of conditions, would be necessary to confirm the results determined here. However, the study results still indicate that the higher concentration of a PFSAs mixture will show more toxicity ability compared with a lower concentration of PFSAs in the long term, and that smaller-sized molecules such as PFBS do not show any bioconcentration ability in algae such as in *S. obliquus*.

6. Conclusions

For 0.01 and 0.1 mg/L PFSAs in the bioconcentration test, both concentrations did not significantly affect the population growth rate compared with control groups. These concentrations are relatively low compared to toxicity levels but are more reflective of concentrations of PFSAs that can be seen in the environment.

For the five different high PFSAs concentration in toxicity test, the population growth results indicate that the PFSAs mixture has a significant inhibition ability on the algae cells growth. Therefore, this indirectly indicates that PFSAs mixture has a toxic effect on algae cells. The toxicity of PFSAs mixture becomes greater with the increases in the concentrations compared with the control group. The growth rate at 160 mg/L PFSAs mixture was significantly decreased than other PFSAs mixture concentrations.

The EC_{50} result was based on the 50 % change in population growth rate under five different PFSAs concentrations at 168 hours sampling point. The EC_{50} indicators gives the relationship between no effects and acute toxicity, but not the chronic toxicity. From the results in the toxicity test, the EC_{50} PFSAs mixture on the algae was determined to be 120.35 mg/L. This is similar in value to results from toxicity studies on individual PFSA chemicals in the mixture. This suggests the equivalent nature of toxicity for mixtures of PFSAs as for individual chemicals.

For 0.1 and 0.01 mg/L PFSAs mixture, the Chl a and Chl b and total Chl were not significantly higher than the control group. These results indicate the PFSAs mixture slightly affected pigment content in a growing algae culture. For the toxicity test, the Chl a and Chl b and total Chl were also significantly different from the control group, respectively. The Chl a, Chl b and total Chl contents decreased when the concentration

of the PFSAs mixture increased. These results together suggest that low concentrations of PFSA mixtures do not have that much effect on algae chlorophyll, but high concentrations show toxicity effects, as reflected through chlorophyll.

For the 0.01 mg/L PFSAs mixture bioconcentration experiment, the individual PFSAs contents were analyzed by UHPLC-MS/MS. According to the results, the PFOS and PFHxS showed significantly decreases in concentration in the algae media water, suggesting the long chain PFSAs has more bioconcentration ability. However, the PFBS did not show significant decreases as with PFOS and PFHxS. Regarding the bioconcentration ability for the PFSAs mixture, 0.733 μg/g PFOS, 0.653 μg/g PFHxS and 1.33 μg/g PFBS were found on the overall algae cell membrane. This result suggests that PFBS has significant adsorption ability on the algae cells than PFOS and PFHxS. Similarly, 0.0248 μg/g PFOS, 0.00199 μg/g PFHxS and 0 μg/g PFBS were detected in the overall algae cells which indicated PFBS did not have absorption ability on algae cells.

Similar testing for bioconcentration with only PFOS and PFHxS at 0.1 mg/L showed accumulation both on and in the algae. Amounts were measurable for both compounds, with 13.80 μg/g PFOS and 16.54 μg/L PFHxS found on the overall algae cell membrane. These results show that PFHxS has significant adsorption capacity with PFOS when there is no PFBS present. In the algae cells, 0.184 μg/g PFOS and 0.127 μg/g PFHxS were detected. This result suggests the PFOS had significantly more absorption than PFHxS on the algae. All the absorption results from the bioconcentration test indicated PFOS had significant absorption ability. Based upon these mass number from the bioconcentration test, the bioconcentration factors (BCF) were also calculated. The BCFs in 0.01 mg/L PFSAs mixture are 2.79 mL/g, 0 mL/g, and 0.23 mL/g for PFOS, PFBS and PFHxS, respectively. The BCFs of PFOS and PFHxS

were also calculated for the 0.1 mg/L PFSAs mixture bioconcentration test, in which PFOS is 2.63 mL/g, and PFHxS is 1.83 mL/g. The PFSAs absorption ability may harm the transport, metabolism and gene expression in the cells and cause cell damage. These results suggest small but present bioconcentration ability of PFSA substances in mixtures in aquatic ecosystems, and concern about the toxicity effect for PFSAs in the environment and whole food chain system remain relevant.

7. References

[1] M. Trojanowicz, A. Bojanowska-Czajka, I. Bartosiewicz, and K. Kulisa, "Advanced Oxidation/Reduction Processes treatment for aqueous perfluorooctanoate (PFOA) and perfluorooctanesulfonate (PFOS) – A review of recent advances," *Chemical Engineering Journal*, vol. 336, pp. 170-199, 2018.

[2] J. M. Conder, R. A. Hoke, W. d. Wolf, M. H. Russell, and R. C. Buck, "Are PFCAs Bioaccumulative? A Critical Review and Comparison with Regulatory Criteria and Persistent Lipophilic Compounds," *Environmental Science & Technology*, vol. 42, no. 4, pp. 995-1003, 2008/02/01 2008.

[3] Z. Wang, Cousins, Ian T, Scheringer, Martin, Hungerbühler, Konrad, "Fluorinated alternatives to long-chain perfluoroalkyl carboxylic acids (PFCAs), perfluoroalkane sulfonic acids (PFSAs) and their potential precursors," *Environment International*, vol. 60, pp. 242-248, 2013.

[4] F. Ye, Y. Zushi, and S. Masunaga, "Survey of perfluoroalkyl acids (PFAAs) and their precursors present in Japanese consumer products," *Chemosphere*, vol. 127, pp. 262-8, May 2015.

[5] J. L. Domingo, "Health risks of dietary exposure to perfluorinated compounds," *Environment International*, vol. 40, pp. 187-195, 2012/04/01/ 2012.

[6] S. Takagi *et al.*, "Fate of perfluorooctanesulfonate and perfluorooctanoate in drinking water treatment processes," *Water Res*, vol. 45, no. 13, pp. 3925-32, Jul 2011.

[7] S. Wild *et al.*, "An Antarctic research station as a source of brominated and perfluorinated persistent organic pollutants to the local environment," *Environ Sci Technol*, vol. 49, no. 1, pp. 103-12, Jan 6 2015.

[8] O. S. Arvaniti and A. S. Stasinakis, "Review on the occurrence, fate and removal of perfluorinated compounds during wastewater treatment," *Sci Total Environ,* vol. 524-525, pp. 81-92, Aug 15 2015.

[9] C. Schroter-Kermani, J. Muller, H. Jurling, A. Conrad, and C. Schulte, "Retrospective monitoring of perfluorocarboxylates and perfluorosulfonates in human plasma archived by the German Environmental Specimen Bank," *Int J Hyg Environ Health,* vol. 216, no. 6, pp. 633-40, Nov 2013.

[10] P. Carlsson, D. Herzke, and R. Kallenborn, "Polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and perfluorinated alkylated substances (PFASs) in traditional seafood items from western Greenland," *Environ Sci Pollut Res Int,* vol. 21, no. 6, pp. 4741-50, Mar 2014.

[11] M. F. Rahman, S. Peldszus, and W. B. Anderson, "Behaviour and fate of perfluoroalkyl and polyfluoroalkyl substances (PFASs) in drinking water treatment: a review," *Water Res,* vol. 50, pp. 318-40, Mar 1 2014.

[12] S. Newton, R. McMahen, J. A. Stoeckel, M. Chislock, A. Lindstrom, and M. Strynar, "Novel Polyfluorinated Compounds Identified Using High Resolution Mass Spectrometry Downstream of Manufacturing Facilities near Decatur, Alabama," *Environ Sci Technol,* vol. 51, no. 3, pp. 1544-1552, Feb 7 2017.

[13] H. Chen *et al.*, "Occurrence, spatial and temporal distributions of perfluoroalkyl substances in wastewater, seawater and sediment from Bohai Sea, China," *Environ Pollut,* vol. 219, pp. 389-398, Dec 2016.

[14] S. Wang *et al.*, "First report of a Chinese PFOS alternative overlooked for 30 years: its toxicity, persistence, and presence in the environment," *Environ Sci Technol,* vol. 47, no. 18, pp. 10163-70, Sep 17 2013.

[15] Q. Cui, Y. Pan, H. Zhang, N. Sheng, and J. Dai, "Elevated concentrations of perfluorohexanesulfonate and other per- and polyfluoroalkyl substances in Baiyangdian Lake (China): Source characterization and exposure assessment," *Environ Pollut,* vol. 241, pp. 684-691, Oct 2018.

[16] P. Casal *et al.*, "Accumulation of Perfluoroalkylated Substances in Oceanic Plankton," *Environ Sci Technol,* vol. 51, no. 5, pp. 2766-2775, Mar 7 2017.

[17] C. Kunacheva *et al.*, "Contamination of perfluorinated compounds (PFCs) in Chao Phraya River and Bangpakong River, Thailand," *Water Science and Technology,* vol. 60, no. 4, pp. 975-982, 2009.

[18] X. Xia, A. H. Rabearisoa, X. Jiang, and Z. Dai, "Bioaccumulation of perfluoroalkyl substances by Daphnia magna in water with different types and concentrations of protein," *Environ Sci Technol,* vol. 47, no. 19, pp. 10955-63, Oct 1 2013.

[19] W. A. Gebbink, L. van Asseldonk, and S. P. J. van Leeuwen, "Presence of Emerging Per- and Polyfluoroalkyl Substances (PFASs) in River and Drinking Water near a Fluorochemical Production Plant in the Netherlands," *Environ Sci Technol,* vol. 51, no. 19, pp. 11057-11065, Oct 3 2017.

[20] C. C.-R. Lam NH, Lee J-S, Soh H-Y, Lee B-C, Lee J-A, Tatarozako N, Sasake K, Saito N, Kannan K, Cho H-S, "Perfluorooctane Sulfonate (PFOS) and Other Perfluorinated Compounds in Water, Sediment, Plankton, and Fish Tissues Collected from Main Rivers and Lake in Korea.," *Organohalogen Compounds,* vol. 75, pp. 1070-1075, 2013.

[21] N.-H. Lam *et al.*, "Perfluorinated alkyl substances in water, sediment, plankton and fish from Korean rivers and lakes: A nationwide survey," *Science of The Total Environment*, vol. 491-492, pp. 154-162, 2014/09/01/ 2014.

[22] E. M. Sunderland, X. C. Hu, C. Dassuncao, A. K. Tokranov, C. C. Wagner, and J. G. Allen, "A review of the pathways of human exposure to poly- and perfluoroalkyl substances (PFASs) and present understanding of health effects," *J Expo Sci Environ Epidemiol*, vol. 29, no. 2, pp. 131-147, Mar 2019.

[23] K. Inoue *et al.*, "Perfluorooctane sulfonate (PFOS) and related perfluorinated compounds in human maternal and cord blood samples: assessment of PFOS exposure in a susceptible population during pregnancy," *Environ Health Perspect*, vol. 112, no. 11, pp. 1204-7, Aug 2004.

[24] X. C. Hu *et al.*, "Detection of Poly- and Perfluoroalkyl Substances (PFASs) in U.S. Drinking Water Linked to Industrial Sites, Military Fire Training Areas, and Wastewater Treatment Plants," *Environ Sci Technol Lett*, vol. 3, no. 10, pp. 344-350, Oct 11 2016.

[25] E. W. Group. (2016, Augest 7). *PFAS Contamination in the U.S.* Available: https://www.ewg.org/interactive-maps/pfas_contamination/map/

[26] Z. Wang, J. C. DeWitt, C. P. Higgins, and I. T. Cousins, "A Never-Ending Story of Per- and Polyfluoroalkyl Substances (PFASs)?," *Environ Sci Technol*, vol. 51, no. 5, pp. 2508-2518, Mar 7 2017.

[27] A. G. Paul, K. C. Jones, and A. J. Sweetman, "A First Global Production, Emission, And Environmental Inventory For Perfluorooctane Sulfonate," *Environmental Science & Technology*, vol. 43, no. 2, pp. 386-392, 2009/01/15 2009.

[28] R. Casson and S.-Y. Chiang, "Integrating total oxidizable precursor assay data to evaluate fate and transport of PFASs," *Remediation Journal,* vol. 28, no. 2, pp. 71-87, 2018/03/01 2018.

[29] R. C. Buck *et al.*, "Perfluoroalkyl and polyfluoroalkyl substances in the environment: terminology, classification, and origins," (in eng), *Integrated environmental assessment and management,* vol. 7, no. 4, pp. 513-541, 2011.

[30] Z. Wang, I. T. Cousins, M. Scheringer, and K. Hungerbühler, "Fluorinated alternatives to long-chain perfluoroalkyl carboxylic acids (PFCAs), perfluoroalkane sulfonic acids (PFSAs) and their potential precursors," *Environment International,* vol. 60, pp. 242-248, 2013/10/01/ 2013.

[31] P. Zhao *et al.*, "Short- and long-chain perfluoroalkyl substances in the water, suspended particulate matter, and surface sediment of a turbid river," *Science of The Total Environment,* vol. 568, pp. 57-65, 2016/10/15/ 2016.

[32] C. Zhang, H. Yan, F. Li, X. Hu, and Q. Zhou, "Sorption of short- and long-chain perfluoroalkyl surfactants on sewage sludges," *Journal of Hazardous Materials,* vol. 260, pp. 689-699, 2013/09/15/ 2013.

[33] (2002). *Co-operation on Existing Chemicals Hazard Assessment of Perfluorooctane Sulfonate (PFOS) and its Salts*.

[34] (2016). *Drinking Water Health Advisory for Perfluorooctane Sulfonate (PFOS)*.

[35] L. Vierke, C. Staude, A. Biegel-Engler, W. Drost, and C. Schulte, "Perfluorooctanoic acid (PFOA) — main concerns and regulatory developments in Europe from an environmental point of view," *Environmental Sciences Europe,* vol. 24, no. 1, p. 16, 2012/05/07 2012.

[36] C. K. Yang Baiqin, Xing Hang, Xiao Jinxin, "Perfluorobutyl-Based Fluorinated Surfactant with High Surface Activity," *Acta Phys. -Chim. Sin.*, vol. 25(12), pp. 2409-2412, 2009.

[37] H. Iwai, "Toxicokinetics of ammonium perfluorohexanoate," *Drug and Chemical Toxicology*, vol. 34, no. 4, pp. 341-346, 2011/10/01 2011.

[38] D. Y. Zhang, X. L. Xu, Y. Lu, H. Y. Xu, and H. M. Yan, "The Effects of Perfluorooctane Sulfonate (PFOS) on Physiological Status and Proliferation Capacity of Scenedesmus obliqnus," *Applied Mechanics and Materials*, vol. 209-211, pp. 1131-1135, 2012.

[39] A. N. R. FILTERS. (2018, Augest 6, 2020). *Aquarium Phytoplankton*. Available: http://www.aquariumfiltersetup.com/aquarium-microfauna/aquarium-phytoplankton/

[40] J. M. Armitage, J. A. Arnot, F. Wania, and D. Mackay, "Development and evaluation of a mechanistic bioconcentration model for ionogenic organic chemicals in fish," *Environmental Toxicology and Chemistry*, vol. 32, no. 1, pp. 115-128, 2013.

[41] X. Zhu, J. Wang, Y. Lu, Q. Chen, and Z. Yang, "Grazer-induced morphological defense in Scenedesmus obliquus is affected by competition against Microcystis aeruginosa," (in eng), *Scientific reports*, vol. 5, pp. 12743-12743, 2015.

[42] K.-C. Lin, Y.-L. Lee, and C.-Y. Chen, "Metal toxicity to Chlorella pyrenoidosa assessed by a short-term continuous test," *Journal of Hazardous Materials*, vol. 142, no. 1, pp. 236-241, 2007/04/02/ 2007.

[43] C. C. o. A. Organisms. (2020, Augest 6). *Scenedesmus obliquus (Turpin) Kuetzing*. Available: https://ccala.butbn.cas.cz/en/scenedesmus-obliquus-turpin-kuetzing-2#:~:text=Algae%20/%20Chlorophyceae%20/%20Sphaeropleales

[44] R. R. Goulet, C. Fortin, and D. J. Spry, "8 - Uranium," in *Fish Physiology*, vol. 31, C. M. Wood, A. P. Farrell, and C. J. Brauner, Eds.: Academic Press, 2011, pp. 391-428.

[45] A. f. T. S. a. D. Registry. (2000). *Toxicological Profile for Polychlorinated Biphenyls (PCBs)* Available: https://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=142&tid=26

[46] G. Y. WU Jiangping, LI Mingyuan, JIN Juntao, MAKOTO Yoshijima, ZHANG Xihui, "Recent research advances on the bioaccumulation potentials of perfluorinated compounds," *Ecology and Environmental Sciences,* vol. 19 (5), pp. 1246-1252, 2010.

[47] J. Liu and S. Mejia Avendano, "Microbial degradation of polyfluoroalkyl chemicals in the environment: a review," *Environ Int,* vol. 61, pp. 98-114, Nov 2013.

[48] S. Nakayama *et al.*, "Distributions of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) in Japan and their toxicities," (in eng), *Environmental sciences : an international journal of environmental physiology and toxicology,* vol. 12, no. 6, pp. 293-313, 2005 2005.

[49] G. T. Tomy *et al.*, "Fluorinated organic compounds in an eastern Arctic marine food web," *Environ Sci Technol,* vol. 38, no. 24, pp. 6475-81, Dec 15 2004.

[50] S. Zhang *et al.*, "Relationship between perfluorooctanoate and perfluorooctane sulfonate blood concentrations in the general population and routine drinking water exposure," *Environ Int,* vol. 126, pp. 54-60, May 2019.

[51] (2012). *Ecological Effects Test Guidelines*.

[52] (2018). *Environmental Fate and Transport for Per- and Polyfluoroalkyl Substances*.

[53] V. Geissen *et al.*, "Emerging pollutants in the environment: A challenge for water resource management," *International Soil and Water Conservation Research,* vol. 3, no. 1, pp. 57-65, 2015.

[54] T.-H. Kim, S.-H. Lee, H. Y. Kim, K. Doudrick, S. Yu, and S. D. Kim, "Decomposition of perfluorooctane sulfonate (PFOS) using a hybrid process with electron beam and chemical oxidants," *Chemical Engineering Journal,* vol. 361, pp. 1363-1370, 2019.

[55] (2009). *Stockholm Convention on Presistent Organic Pollutants (POPs)*.

[56] A. Cordner, L. Richter, and P. Brown, "Can Chemical Class Approaches Replace Chemical-by-Chemical Strategies? Lessons from Recent U.S. FDA Regulatory Action on Per- And Polyfluoroalkyl Substances," *Environmental Science & Technology,* vol. 50, no. 23, pp. 12584-12591, 2016/12/06 2016.

[57] S. Rayne and K. Forest, "Congener-specific organic carbon-normalized soil and sediment-water partitioning coefficients for the C1 through C8 perfluoroalkyl carboxylic and sulfonic acids," *Journal of Environmental Science and Health, Part A,* vol. 44, no. 13, pp. 1374-1387, 2009/10/20 2009.

[58] S. Laboratories, "Perfluorooctanesulfonic acid," ed, 2012.

[59] Y. Li *et al.*, "Half-lives of PFOS, PFHxS and PFOA after end of exposure to contaminated drinking water," *Occupational and Environmental Medicine,* vol. 75, no. 1, pp. 46-51, 2018.

[60] S. Laboratories, "Perfluorohexanesulfonic acid," ed, 2012.

[61] S. Laboratories, "Nonafluorobutanesulfonic acid," ed, 2012.

[62] W. Liu, S. Chen, X. Quan, and Y.-H. Jin, "Toxic effect of serial perfluorosulfonic and perfluorocarboxylic acids on the membrane system of a freshwater alga measured by flow cytometry," *Environmental Toxicology and Chemistry,* vol. 27, no. 7, pp. 1597-1604, 2008.

[63] D. Xu, C. Li, H. Chen, and B. Shao, "Cellular response of freshwater green algae to perfluorooctanoic acid toxicity," *Ecotoxicol Environ Saf,* vol. 88, pp. 103-7, Feb 2013.

[64] C. B. Fang Linna, Shen Qiong, Zhou Huili, Dong Youdi, Xu Dongmei, "Toxic Effect of Perfluorooctane Sulfonate on Scenedesmus Obliquus," *Journal of Zhe Jiang Shuren University,* vol. 14 (2), pp. 30-34, 2014.

[65] G. Liu, S. Zhang, K. Yang, L. Zhu, and D. Lin, "Toxicity of perfluorooctane sulfonate and perfluorooctanoic acid to Escherichia coli: Membrane disruption, oxidative stress, and DNA damage induced cell inactivation and/or death," *Environ Pollut,* vol. 214, pp. 806-815, Jul 2016.

[66] D. Xu, X. Chen, and B. Shao, "Oxidative Damage and Cytotoxicity of Perfluorooctane Sulfonate on Chlorella vulgaris," *Bull Environ Contam Toxicol,* vol. 98, no. 1, pp. 127-132, Jan 2017.

[67] T. A. X. YU Juan, ZHANG Pei-Yu, DONG Shuang-Lin, "Effects of CO2 enrichment on growth, photosynthesis and activities of antioxidant enzymes of two marine micro-green-algae," *ACTA ECOLOGICA SIN ICA,* vol. 25 (2), 2005.

[68] W. Liu, Y. B. Zhang, X. Quan, Y. H. Jin, and S. Chen, "Effect of perfluorooctane sulfonate on toxicity and cell uptake of other compounds with different hydrophobicity in green alga," *Chemosphere,* vol. 75, no. 3, pp. 405-9, Apr 2009.

[69] I. Rodea-Palomares, F. Leganes, R. Rosal, and F. Fernandez-Pinas, "Toxicological interactions of perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) with selected pollutants," *J Hazard Mater,* vol. 201-202, pp. 209-18, Jan 30 2012.

[70] I. Rodea-Palomares, M. Makowski, S. Gonzalo, M. Gonzalez-Pleiter, F. Leganes, and F. Fernandez-Pinas, "Effect of PFOA/PFOS pre-exposure on the toxicity of the herbicides

2,4-D, Atrazine, Diuron and Paraquat to a model aquatic photosynthetic microorganism," *Chemosphere,* vol. 139, pp. 65-72, Nov 2015.

[71] W. Liu *et al.*, "Bioaccumulation and effects of novel chlorinated polyfluorinated ether sulfonate in freshwater alga Scenedesmus obliquus," *Environ Pollut,* vol. 233, pp. 8-15, Feb 2018.

[72] S. Fang *et al.*, "Trophic magnification and isomer fractionation of perfluoroalkyl substances in the food web of Taihu Lake, China," *Environ Sci Technol,* vol. 48, no. 4, pp. 2173-82, Feb 18 2014.

[73] H. G. Zheng Hai, Xu Zhencheng, Li Huashou, Chen Laiguo, Peng Xiaowu, Ren Mingzhong , Guo Shu, Liu Yun, "Contamination Characteristics of Perfluorooctanoic Acid and Perfluorooctane Sulfonate in Surface Sediments from Dongjiang River, South China," *Journal of Agro-Environment Science,* vol. 32 (4), pp. 778-782, 2013.

[74] N. Yamashita *et al.*, "Perfluorinated acids as novel chemical tracers of global circulation of ocean waters," *Chemosphere,* vol. 70, no. 7, pp. 1247-55, Jan 2008.

[75] Y. X. T. Y. S. Hongwen, "Bioconcentration of Perfluoroalkyl Substances in 3 Microalgae," *Asian Journal of Ecotoxicology,* vol. 12 (3), pp. 391-397, 2017.

[76] J. A. Arnot and F. A. P. C. Gobas, "A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms," *Environmental Reviews,* vol. 14, no. 4, pp. 257-297, 2006.

[77] J. Spitzer, G. J. Pielak, and B. Poolman, "Emergence of life: Physical chemistry changes the paradigm," (in eng), *Biology direct,* vol. 10, pp. 33-33, 2015.

[78] D. Mackay and A. Fraser, "Bioaccumulation of persistent organic chemicals: mechanisms and models," *Environmental Pollution,* vol. 110, no. 3, pp. 375-391, 2000/12/01/ 2000.

[79] E. Voutsas, "Chapter 11 - Estimation of the Volatilization of Organic Chemicals from Soil," in *Thermodynamics, Solubility and Environmental Issues*, T. M. Letcher, Ed. Amsterdam: Elsevier, 2007, pp. 205-227.

[80] Z. L. Huang Chushan, Hu Guocheng, Li Liangzhong, Chen Mianbiao, "Research Progress of Perfluorooctane Sulfonate and Perfluorooctanoic Acid Accumulation in the Aquatic Environment and Aquatic Organisms," *Environmental Science and Technology,* vol. 27 (5), pp. 66-71, 2014.

[81] S. T. Yim Ling Mak, Leo W. Y. Yeung, Guohui Lu, Ling Jin, Yongliang Yang, Paul K. S. Lam, Kurunthachalam Kannan, and Nobuyoshi Yamashita, "Perfluorinated Compounds in Tap Water from China and Several Other Countries," *Environ. Sci. Technol.*, vol. 43, pp. 4824–4829, 2009.

[82] M. Sun *et al.*, "Legacy and Emerging Perfluoroalkyl Substances Are Important Drinking Water Contaminants in the Cape Fear River Watershed of North Carolina," *Environmental Science & Technology Letters*, vol. 3, no. 12, pp. 415-419, 2016.

[83] J. Yu, J. Hu, S. Tanaka, and S. Fujii, "Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) in sewage treatment plants," *Water Res,* vol. 43, no. 9, pp. 2399-408, May 2009.

[84] H. Nakata, K. Kannan, T. Nasu, H.-S. Cho, E. Sinclair, and A. Takemura, "Perfluorinated Contaminants in Sediments and Aquatic Organisms Collected from Shallow Water and Tidal Flat Areas of the Ariake Sea, Japan: Environmental Fate of Perfluorooctane Sulfonate in Aquatic Ecosystems," *Environmental Science & Technology*, vol. 40, no. 16, pp. 4916-4921, 2006/08/01 2006.

[85] A. V. Weisbrod *et al.*, "Workgroup report: review of fish bioaccumulation databases used to identify persistent, bioaccumulative, toxic substances," *Environ Health Perspect,* vol. 115, no. 2, pp. 255-61, Feb 2007.

[86] K. Borgå, "Ecotoxicology: Bioaccumulation☆," in *Reference Module in Earth Systems and Environmental Sciences*: Elsevier, 2013.

[87] E. Sinclair and K. Kannan, "Mass Loading and Fate of Perfluoroalkyl Surfactants in Wastewater Treatment Plants," *Environmental Science & Technology,* vol. 40, no. 5, pp. 1408-1414, 2006/03/01 2006.

[88] A. T. Fisk, K. A. Hobson, and R. J. Norstrom, "Influence of Chemical and Biological Factors on Trophic Transfer of Persistent Organic Pollutants in the Northwater Polynya Marine Food Web," *Environmental Science & Technology,* vol. 35, no. 4, pp. 732-738, 2001/02/01 2001.

[89] H. Hop, K. Borgå, G. W. Gabrielsen, L. Kleivane, and J. U. Skaare, "Food Web Magnification of Persistent Organic Pollutants in Poikilotherms and Homeotherms from the Barents Sea," *Environmental Science & Technology,* vol. 36, no. 12, pp. 2589-2597, 2002/06/01 2002.

[90] E. I. Loi, L. W. Yeung, S. Taniyasu, P. K. Lam, K. Kannan, and N. Yamashita, "Trophic magnification of poly- and perfluorinated compounds in a subtropical food web," *Environ Sci Technol,* vol. 45, no. 13, pp. 5506-13, Jul 1 2011.

[91] G. T. Tomy *et al.*, "Fluorinated Organic Compounds in an Eastern Arctic Marine Food Web," *Environmental Science & Technology,* vol. 38, no. 24, pp. 6475-6481, 2004/12/01 2004.

[92] J. Xu, C. S. Guo, Y. Zhang, and W. Meng, "Bioaccumulation and trophic transfer of perfluorinated compounds in a eutrophic freshwater food web," *Environ Pollut,* vol. 184, pp. 254-61, Jan 2014.

[93] B. C. Kelly *et al.*, "Perfluoroalkyl Contaminants in an Arctic Marine Food Web: Trophic Magnification and Wildlife Exposure," *Environmental Science & Technology*, vol. 43, no. 11, pp. 4037-4043, 2009/06/01 2009.

[94] J. W. Martin, D. M. Whittle, D. C. Muir, and S. A. Mabury, "Perfluoroalkyl contaminants in a food web from Lake Ontario," *Environ Sci Technol,* vol. 38, no. 20, pp. 5379-85, Oct 15 2004.

[95] M. Houde *et al.*, "Biomagnification of Perfluoroalkyl Compounds in the Bottlenose Dolphin (Tursiops truncatus) Food Web," *Environmental Science & Technology*, vol. 40, no. 13, pp. 4138-4144, 2006/07/01 2006.

[96] M. Haukås, U. Berger, H. Hop, B. Gulliksen, and G. W. Gabrielsen, "Bioaccumulation of per- and polyfluorinated alkyl substances (PFAS) in selected species from the Barents Sea food web," *Environmental Pollution*, vol. 148, no. 1, pp. 360-371, 2007/07/01/ 2007.

[97] R. A. Andersen, "Algal Culturing Techniques," *Elsevier Academic Press* pp. 426, 437-438, 2005.

[98] P. Optics, "Counting Chambers Brochure," *Pyser Optics* pp. 1-14, 2017.

[99] L. Zarauz and X. Irigoien, "Effects of Lugol's fixation on the size structure of natural nano–microplankton samples, analyzed by means of an automatic counting method," *Journal of Plankton Research,* vol. 30, no. 11, pp. 1297-1303, 2008.

[100] S. Biological. (2020, 15 Feb). *Sedgewick Rafter cell*. Available: https://www.southernbiological.com/microscopes-and-supplies/m7-32-sedgewick-rafter-cell/

[101] V. Mulabagal, L. Liu, J. Qi, C. Wilson, and J. S. Hayworth, "A rapid UHPLC-MS/MS method for simultaneous quantitation of 23 perfluoroalkyl substances (PFAS) in estuarine water," *Talanta*, vol. 190, pp. 95-102, Dec 1 2018.

[102] R. J. Ritchie, "Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents," *Photosynth Res*, vol. 89, no. 1, pp. 27-41, Jul 2006.

[103] P. R. Leavitt and D. A. Hodgson, "Sedimentary Pigments," in *Tracking Environmental Change Using Lake Sediments*, vol. 3, J. P. Smol, Birks, H. J. B. & Last, W. M., eds, Ed. (Developments in Paleoenvironmental Research, Dordrecht: Kluwer Academic Publishers, 2001, pp. 295-325.

[104] S. Valsecchi *et al.*, "Deriving environmental quality standards for perfluorooctanoic acid (PFOA) and related short chain perfluorinated alkyl acids," *J Hazard Mater*, vol. 323, no. Pt A, pp. 84-98, Feb 5 2017.

[105] UNEP, "Final act of the conference of plenipotentiaries on the Stockholm Convention on Persistent Organic Pollutants " Stockholm: UNEP2001.

[106] Z. Wang, I. T. Cousins, M. Scheringer, and K. Hungerbuehler, "Hazard assessment of fluorinated alternatives to long-chain perfluoroalkyl acids (PFAAs) and their precursors: Status quo, ongoing challenges and possible solutions," *Environment International*, vol. 75, pp. 172-179, 2015/02/01/ 2015.

[107] J. M. Armitage, J. A. Arnot, and F. Wania, "Potential role of phospholipids in determining the internal tissue distribution of perfluoroalkyl acids in biota," (in eng), *Environ Sci Technol*, vol. 46, no. 22, pp. 12285-6, Nov 20 2012.

8. Appendices

Appendix 1: The chemicals for BBM solution

CAS number	Name	Company	Lot number
7631-99-4	Sodium Nitrate	VWR Life Science	20A1356387
10035-04-8	Calcium Chloride dihydrate	Amresco	2734C431
10034-99-8	Magnesium Sulfate	Fisher Science	No.965727
7758-11-4	Potassium Phosphate (Dibasic)	Bioscience	141410
7778-77-0	Potassium Phosphate Monobasic	BDH	2784C183
7647-14-5	Sodium Chloride	Fisher Chemical	065737
00138	Ethylenediaminetetraacetic	CHEM-IMPEX INT'L INC	002251-00113
1310-58-3	Potassium hydroxide	BDH	3014C524
7782-63-0	Iron (II) sulfate heptahydrate	Fisher Science	N/A
7664-93-9	Sulfuric Acid	T.J Baker	87832
10043-35-3	Boric acid	EMPROVE	N/A
7446-20-0	Zinc sulfate heptahydrate	Amresco	3234C098
1313-27-5	Molybdenum (VI) oxide	Alfa Aesar	A15Z038
7758-99-8	Copper (II) sulfate pentahydrate	VWR chemical	18C2256744
10026-22-9	Cobalt (II) nitrate hexahydrate (Crystalline)	Alfa Aesar	N11A022
7773-01-5	Manganese (II) chloride	BDH	2757C509

Appendix 2: Chemicals and reagents used

Perfluorooctanesulfonic acid (PFOS) (purity is 97%, CAS number 1763-23-1), Nonafluorobutanesulfonic acid (PFBS) (purity is 97%, CAS number 375-73-5), Perfluorohexanesulfonic acid (PFHxS) (purity is 95%, CAS number 355-46-4) were obtained from Synquest Laboratories, Japan.

The company of analytical grade ammonium hydroxide is Sigma-Aldrich, St. Louis, MO. The methanol, acetonitrile, and water for LC grade solvents were procured from VWR International, Suwanee, GA. The LC grade ammonium formate and ammonium acetate reagents, Captiva Glass fiber nylon syringe filters (0.2µm), UHPLC analytical column (Agilent ZORBAX RRHD Eclipse Plus C18, 2.1×100mm, 1.8µm, Part No. 959758-902) and guard column (Agilent ZORBAX Eclipse Plus C18, 2.1, 1.8µm, Part No. 821725-901) were procured from Agilent Technologies, Wilmington, DE. The LC/MS analysis level of grade standards (purity > 98%) was obtained from Wellington Laboratories (Ontario, Canada).

Oasis WAX (6cc, 150 mg) extraction cartridges and 20-Position vacuum manifold were bought from Waters Corporation, Milford, MA, USA.

Appendix 3: The one-way repeated measures ANOVA test for the growth rate at 0.01 mg/L and 0.1 mg/L PFSAs mixture (Time means different sampling time and groups means different PFSAs mixture concentrations)

		df	Sum sq	Mean sq	F	PR (>F)
0.01 mg/L	**groups**	1.0	3.27	3.271	1.282	0.263
	residual	46.0	117.41	2.552		
0.1 mg/L	**groups**	1.0	3.6	3.629	0.37	0.546
	residual	42.0	411.9	9.808		

The post hoc Tukey HSD test for groups				
	diff	lwr	Upr	P adj
0.01 mg/L PFSAs mixture-control	-0.5220833	-1.450408	0.4062412	0.263485
0.1 mg/L PFSAs mixture-control	-0.5743636	-2.479955	1.331227	0.5462844

Appendix 4: The mean and standard deviation value of the growth rate for *S. obliquus*

THE GROWTH RATE OF ALGAE (10^6 CELLS/ ML)

TIME (H)	0 mg/L		10 mg/L		20 mg/L		40 mg/L		80 mg/L		160 mg/L	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
0	2.43	0.07	2.43	0.07	2.43	0.07	2.43	0.07	2.43	0.07	2.43	0.07
3	1.89	0.32	1.52	0.22	1.63	0.30	2.04	0.11	1.59	0.02	2.07	0.35
6	1.53	0.12	1.48	0.17	1.57	0.12	1.57	0.10	1.44	0.17	1.74	0.09
12	1.28	0.05	1.39	0.25	1.4	0.26	1.52	0.05	1.63	0.20	1.74	0.47
24	1.81	0.26	2.32	0.17	2.28	0.22	1.99	0.18	1.88	0.12	1.9	0.06
36	2.04	0.15	1.86	0.10	1.94	0.05	2.11	0.18	1.82	0.30	2.09	0.37
48	2.47	0.24	2.49	0.28	2.33	0.38	2.29	0.40	1.87	0.14	1.51	0.26
60	2.17	0.38	1.97	0.10	2.22	0.11	2.08	0.16	1.76	0.27	1.5	0.14
72	2.92	0.04	2.42	0.12	2.37	0.12	2.32	0.16	2.21	0.13	1.76	0.18
120	3.24	0.30	3.09	0.28	3.32	0.22	2.93	0.29	2.23	0.23	1.72	0.27
168	4.53	0.24	4.19	0.07	4.06	0.37	3.3	0.38	2.68	0.39	1.79	0.28

Appendix 5: The ANOVA test and post hoc Dunnett test of the growth rate for *S. obliquus* in the toxicity test

The repeated measures ANOVA test				
	F value	**Num DF**	**Den DF**	**Pr > F**
Different PFSAs mixture concentrations	3.6136	5.0000	50.0000	0.0072

The post hoc Dunnett test					
	10 mg/L	**20 mg/L**	**40 mg/L**	**80 mg/L**	**160 mg/L**
diff	-0.10484848	-0.06954545	-0.15757576	-0.43484848	-0.55181818
lwr ci	-0.5190504	-0.4837474	-0.5717777	-0.8490504	-0.9660201
upr ci	0.30935342	0.34465645	0.25662615	-0.02064658	-0.13761628
P value	0.9522	0.9917	0.7975	0.0358	0.0040

The one-way ANOVA at 168 hour					
	df	**Sum sq**	**Mean sq**	**F**	**PR (>F)**
Groups	5	16.350	3.270	34.47	1.02e-06
Residuals	12	1.139	0.095		

The post hoc Dunnett test at 168 hour					
	10 mg/L	**20 mg/L**	**40 mg/L**	**80 mg/L**	**160 mg/L**
diff	-0.3400000	-0.4733333	-1.2333333	-1.8500000	-2.7400000
lwr ci	-1.069840	-1.203174	-1.963174	-2.579840	-3.469840
upr ci	0.3898404	0.2565071	-0.5034929	-1.1201596	-2.0101596
P value	0.54873	0.27007	0.00165	0.00032	1.5e-06

Appendix 6: The repeated measures ANOVA test of 0.01 mg/L pigment test

		df	Sum sq	Mean sq	F	PR (>F)
Chl a	Groups	1	106.4	106.37	7.171	0.0102
residual		46	682.3	14.83		
Chl b	Groups	1	125.4	125.41	10.43	0.00229
residual		46	553.2	12.03		
Total Chl	Groups	1	2095	2095.0	5.492	0.0235
residual		46	17546	381.4		

Appendix 7: The Tukey HSD post hoc test of 0.01 mg/L pigment test

The post hoc Tukey HSD test for groups				
	diff	lwr	Upr	P adj
Chl a: group-control	-2.977243	-5.215135	-0.7393519	0.0102373
Chl b: group-control	-3.232773	-5.247943	-1.217603	0.0022935
Chl total: group-control	-13.21289	-24.56159	-1.864194	0.0234787

Appendix 8: The repeated measures ANOVA test of 0.1 mg/L pigment test

		df	Sum sq	Mean sq	F	PR (>F)
Chl a	Groups	1	29.6	29.57	2.515	0.116
residual		86	1011.4	11.76		
Chl b	Groups	1	3.54	3.539	2.78	0.0991
residual		86	109.49	1.273		
Total Chl	Groups	1	55	54.99	2.59	0.111
residual		86	1826	21.23		

Appendix 9: The Tukey HSD post hoc test of 0.1 mg/L PFSAs mixture pigment test

The post hoc Tukey HSD test for groups				
	diff	lwr	Upr	P adj
Chl a: group-control	1.159434	-0.2940409	2.612909	0.1164616
Chl b: group-control	0.4010831	-0.07713239	0.8792987	0.0990933
Chl total: group-control	1.580989	-0.371948	3.533925	0.1112111

Appendix 10: The repeated measures ANOVA test of 0.01 mg/L PFSAs mixture bioconcentration test

	df	Sum sq	Mean sq	F	PR (>F)
Groups	2	2481.0	1240.5	1677	<2e-16
Residuals	237	175.3	0.7		

Appendix 11: The Tukey HSD post hoc test of 0.01 mg/L PFSAs mixture bioconcentration test

The post hoc Tukey HSD test for groups				
	diff	lwr	Upr	P adj
PFHxS-PFBS	-6.246234	-6.566985	-5.9254826	0
PFOS-PFBS	-7.277327	-7.598078	-6.9565756	0
PFOS-PFHxS	-1.031093	-1.351844	-0.7103416	0

Appendix 12: The repeated measures ANOVA test of 0.1 mg/L PFSAs mixture bioconcentration test

	df	Sum sq	Mean sq	F	PR (>F)
Groups	1	4444	4444	185.5	<2e-16
Residuals	198	4744	24		

Appendix 13: The Tukey HSD post hoc test of 0.1 mg/L PFSAs mixture bioconcentration test

The post hoc Tukey HSD test for groups				
	diff	lwr	Upr	P adj
PFOS-PFHxS	-9.42736	-10.79239	-8.062327	0

Appendix 14: The mean and standard deviation value of the Chl-a for *S. obliquus*

The concentration of Chl-a in PFSAs mixture toxicity test

Hours	0 mg/L		10 mg/L		20 mg/L		40 mg/L		80 mg/L		160 mg/L	
(h)	chl a		chl a		chl a		chl a		chl a		chl a	
	average	SD	average	STD	average	STD	average	STD	average	STD	average	STD
0h	5.43	0.44	5.39	0.15	5.35	0.21	5.10	0.11	4.93	0.15	5.15	0.14
3h	4.99	0.14	5.21	0.12	5.05	0.04	4.86	0.11	4.63	0.05	4.81	0.18
6h	4.32	0.27	4.87	0.16	4.67	0.23	4.67	0.27	4.31	0.09	4.47	0.14
12h	4.69	0.34	5.42	0.02	5.40	0.19	5.45	0.14	4.93	0.14	4.35	0.15
24h	5.57	0.43	6.31	0.29	5.71	0.59	5.77	0.21	5.26	0.19	3.58	0.90
36h	6.38	0.41	6.98	0.57	6.43	0.65	6.43	0.88	5.97	0.17	3.92	1.14
48h	7.52	0.47	8.12	0.41	7.49	0.95	7.45	0.57	6.62	0.38	3.55	1.64
60h	8.24	0.41	8.15	0.68	7.60	1.21	7.41	0.80	6.41	0.51	2.70	1.45
72h	9.54	0.54	9.56	0.81	8.30	1.37	8.64	1.27	7.29	0.32	3.20	2.42
120h	12.30	2.95	10.53	1.27	11.43	3.71	8.84	0.68	7.12	0.39	2.34	2.51
168h	12.89	4.42	12.31	3.71	14.65	4.66	11.44	1.69	8.02	0.23	3.43	3.34

Appendix 15: The mean and standard deviation value of the Chl-b for *S. obliquus*

The concentration of Chl b in PFSAs mixture toxicity test												
Hour(h)	0 mg/L		10 mg/L		20 mg/L		40 mg/L		80 mg/L		160 mg/L	
	chl b		chl b		chl b		chl b		chl b		chl b	
	average	STD	average	STD	average	STD	average	STD	average	STD	average	STD
0h	1.70	0.12	1.72	0.03	1.74	0.05	1.69	0.06	1.75	0.09	1.93	0.08
3h	1.64	0.09	1.80	0.02	1.75	0.02	1.68	0.03	1.63	0.03	1.81	0.14
6h	1.42	0.08	1.74	0.11	1.67	0.08	1.67	0.10	1.63	0.09	1.84	0.19
12h	1.71	0.19	2.14	0.06	2.12	0.09	2.15	0.04	1.93	0.07	1.61	0.02
24h	1.92	0.15	2.19	0.11	1.96	0.21	1.96	0.08	1.82	0.03	1.14	0.32
36h	2.29	0.08	2.57	0.34	2.24	0.41	2.29	0.42	2.23	0.07	1.33	0.50
48h	2.53	0.20	2.69	0.17	2.47	0.38	2.46	0.17	2.19	0.12	1.14	0.63
60h	2.97	0.05	2.91	0.35	2.70	0.60	2.69	0.34	2.28	0.27	0.93	0.55
72h	3.21	0.16	3.19	0.34	2.72	0.52	3.03	0.48	2.51	0.08	1.16	0.95
120h	4.24	1.08	3.55	0.56	3.96	1.40	3.00	0.20	2.37	0.17	0.78	0.92
168h	4.44	2.02	3.80	1.45	5.42	2.81	3.57	0.63	2.45	0.05	1.11	1.11

Appendix 16: The mean and standard deviation value of the total Chl for *S. obliquus*

The concentration of total Chl in PFSAs mixture toxicity test												
Hour(h)	0 mg/L		10 mg/L		20 mg/L		40 mg/L		80 mg/L		160 mg/L	
	chl total		chl total		chl total		chl total		chl total		chl total	
	average	STD	average	STD	average	STD	average	STD	average	STD	average	STD
0h	7.40	0.69	7.09	0.17	7.08	0.26	6.77	0.16	6.66	0.23	7.06	0.20
3h	6.60	0.21	6.98	0.11	6.78	0.01	6.52	0.14	6.24	0.07	6.60	0.33
6h	5.73	0.36	6.60	0.27	6.30	0.31	6.30	0.37	5.91	0.20	6.32	0.33
12h	6.44	0.53	7.59	0.04	7.57	0.26	7.65	0.17	6.87	0.19	5.96	0.18
24h	7.53	0.55	8.55	0.40	7.68	0.82	7.77	0.29	7.12	0.22	4.73	1.23
36h	8.73	0.45	9.56	0.82	8.70	1.05	8.75	1.30	8.23	0.19	5.25	1.63
48h	10.13	0.67	10.91	0.58	10.05	1.33	9.99	0.75	8.85	0.48	4.70	2.28
60h	11.32	0.45	11.17	1.02	10.40	1.83	10.18	1.13	8.74	0.79	3.63	2.01
72h	12.85	0.71	12.89	1.20	11.11	1.89	11.72	1.76	9.83	0.39	4.34	3.39
120h	16.72	4.04	14.17	1.82	15.50	5.14	11.92	0.87	9.52	0.57	3.10	3.45
168h	17.59	6.62	16.23	5.24	20.35	7.69	15.06	2.34	10.47	0.29	4.54	4.45

Appendix 17: The ANOVA test of the Chl content for *S. obliquus* in toxicity test

	The repeated measures ANOVA test for pigment content					
		Df	Sum Sq	Mean Sq	F value	Pr>F
The different PFSAs mixture concentration groups	Chl a	5	374.08	77.97	12.12	3.27e-10
	Residuals	192	1187.8	6.19		
	Chl b	5	41.57	8.314	10.18	1.16e-08
	Residuals	192	156.89	0.817		
	Chl total	5	686.4	137.27	11.69	7.12e-10
	Residuals	192	2254.8	11.74		

The post hoc Dunnett test for Chl a					
	10 mg/L	**20 mg/L**	**40 mg/L**	**80 mg/L**	**160 mg/L**
diff	0.08766143	0.01803365	-0.52846009	-1.48992767	-3.77362217
lwr ci	-1.463860	-1.533488	-2.079981	-3.041449	-5.325143
upr ci	1.63918266	1.56955488	1.02306114	0.06159356	-2.22210094
P value	1.0000	1.0000	0.8577	0.0644	1.1e-08

The post hoc Dunnett test for Chl b					
	10 mg/L	**20 mg/L**	**40 mg/L**	**80 mg/L**	**160 mg/L**
diff	0.02098536	0.06266171	-0.17002636	-0.47846559	-1.24150137
lwr ci	-0.5428930	-0.5012167	-0.7339048	-1.0423440	-1.8053798
upr ci	0.58486377	0.62654012	0.39385205	0.08541282	0.67762296
P value	1.0000	0.9988	0.9069	0.1250	2.6e-07

The post hoc Dunnett test for total Chl					
	10 mg/L	**20 mg/L**	**40 mg/L**	**80 mg/L**	**160 mg/L**
diff	0.06183238	0.04297066	-0.76618354	-2.05449672	-5.12051934
lwr ci	-2.075856	-2.094718	-2.903872	-4.192186	-7.258208
upr ci	2.19952124	2.18065953	1.37150533	0.08319214	-2.98283048
P value	1.0000	1.0000	0.8319	0.0641	1.8e-08