Potential Impacts of Per- and Polyfluoroalkyl Substance Contamination in Laboratory Materials and Model Organisms on Laboratory-based Studies

A THESIS

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Dedication

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Abstract

Per- and polyfluoroalkyl substances are persistent chemicals that present risks to human environmental health as a result of their ubiquitous nature. These compounds also impact laboratory based exposure and toxicology experiments through PFAS contamination in common materials used for aquatic laboratory exposure experiments. This thesis reviews literature surrounding PFAS bioaccumulation in aquatic organisms in laboratory exposure studies using several measures such as the bioaccumulation factor, bioconcentration factor, biomagnification factor, rate of uptake, and elimination rate. Lack of standardization in how the bioaccumulation factors were calculated creates challenges in statistical comparisons. This thesis also investigates potential PFAS contamination and impacts on aquatic laboratory based exposure experiments in habitat materials, fish feed, and aquaculture fish. Results suggest that PFAS contamination is present in common fish feed and can also be found in aquaculture fish. Unaccounted PFAS contamination in bioaccumulation and toxicity studies could confound results and ultimately influence environmental health recommendations.

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List of Abbreviations

Abbreviation	Meaning
PFAS	Per- and polyfluoroalkyl substances
PFBA	perfluorobutanoic acid
PFPeA	perfluoropentanoic acid
PFHxa	perfluorohexanoic acid
PFHpA	perfluoroheptanoic acid
PFOA	perfluorooctanoic acid
PFNA	perfluorononanoic acid
PFDA	perfluorodecanoic acid
PFUnA	perfluoroundecanoic acid
PFDoA	perfluorododecanoic acid
PFTriA	perfluorotridecaonic acid
PFTetA	perfluorotetradecaonic acid
PFBS	perfluorobutane sulfonate
PFPeS	perfluoropentane sulfonate
PFHxS	perfluorohexane sulfonate
PFHpS	perfluoroheptane sulfonate
PFOS	perfluorooctane sulfonate
PFNS	perfluorononane sulfonate
PFDS	perfluorodecane sulfonate
4:2 FTS	1H,1H,2H,2H-perfluorohexane sulfonate
6:2 FTS	1H,1H,2H,2H-perfluorooctane sulfonate

8:2 FTS	1H,1H,2H,2H-perfluorodecane sulfonate
NMeFOSAA	2-(N-Methylperfluorocotanesulfonamido) acetic acid
NEtFOSAA	3-(N-Ethylperfluorocotanesulfonamido) acetic acid
FOSA	perfluorooctane sulfonamide
13C4-PFBA	perfluoro-n-[13C4]butanoic acid
13C5-PFPeA	perfluoro-n-[1,2,3,4,5-13C5]pentanoic acid
13C5-PFHxA	perfluoro-n-[1,2,3,4-13C5]hexanoic acid
13C4-PFHpA	perfluoro-n-[1,2,3,4-13C4]heptanoic acid
13C8-PFOA	perfluoro-n-[13C8]octanoic acid
13C9-PFNA	perfluoro-n-[13C9]nonanoic acid
13C6-PFDA	perfluoro-n-[1,2,3,4,5,6-13C6]decanoic acid
13C7-PFUnA	perfluoro-n-[13C7]undecanoic acid
13C2-PFDoA	perfluoro-n-[1,2-13C2]dodecanoic acid
13C2-PFTetA	perfluoro-n-[1,2-13C2]tetradecanoic acid
13C3-PFBS	perfluoro-1-[2,3,4-13C3]butane sulfonate
13C3-PFHxS	sodium perfluoro-1-[1,2,3-13C3]hexane sulfonate
13C8-PFOS	perfluoro-n-[13C8]octane sulfonate
13C2- 4:2 FTS	1H,1H,2H,2H-perfluoro-1-[1,2-13C2]- hexane sulfonate
13C2- 6:2 FTS	1H,1H,2H,2H-perfluoro-1-[1,2-13C2]- octane sulfonate
13C2- 8:2 FTS	1H,1H,2H,2H-perfluoro-1-[1,2-13C2]- decane sulfonate
D3- NMeFOSAA	N-methyl-d3-perfluoro-1-octane-sulfonamidoacetic acid
D5- NEtFOSAA	N-ethyl-d5-perfluoro-1-octane-sulfonamideoacetic acid
13C8- FOSA	perfluoro-1-[13C8]octanesulfonamide
13C3-PFBA	perfluoro-n-[2,3,4-13C3]butanoic acid

13C2-PFOA	perfluoro-n-[1,2-13C2]octanoic acid
13C2-PFDA	perfluoro-n-[1,2-13C2]decanoic acid
13C4-PFOS	perfluoro-n-[1,2,3,4-13C4]octane sulfonate
BAF	Bioaccumulation factor
BCF	Bioconcentration factor
BSAF	Biota-sediment accumulation factor
BMF	Biomagnification factor
P. promelas	*Pimephales promelas* – Fathead minnow
H. azteca	*Hyalella azteca*
Ret. Time	Retention time
DP	Declustering potential
CE	Collision energy
TOP	Total Oxidizable Precursor
IDL	Instrument detection limit
MDL	Method detection limt
AFFF	Aqueous film forming foam
Perfluoroalkyl acids	PFAAs
DoD	Department of Defense
EPA	Environmental Protection Agency
k_u	Rate of uptake
k_e	Rate of elimination
BAF(k)	Kinetically determined BAF
BAF(c)	Concentration determined BAF

1.1 Introduction

Per and poly-fluoroalkyl substances (PFAS) are a class of fluorinated organic molecules that have been highly utilized due to their unique surface active properties. The carbon fluorine bond is the strongest covalent bond known,[1] resulting in heat resistance and lack of degradation for fluorinated molecules, such as PFAS.[2] The general structure of a perfluorinated substance includes a chain of fluorinated carbons with a headgroup of different functionality, commonly carboxylic or sulfonic acids, giving them surface active properties.[3] The general shape and functionality of PFAS give rise to unique properties such as hydrophobicity as well as oil resistance,[3] which can be seen in applications including Aqueous Film Forming Foams (AFFF).[4] In the case of fuel fires, AFFF containing PFAS have been used to effectively put out the fire and limit any potential re-ignition of flammable vapors due to the surfactant properties of PFAS and its ability to create a thin layer of film over the flammable area.[4] In addition to AFFF, PFAS have been found in a number of consumer products ranging from lubricants and polishing agents,[5] wood building materials,[6] carpets,[7] and non stick cookware.[8]

The ubiquitous nature of PFAS presence in consumer products and AFFF leads to increased human and environmental exposure. PFAS have been found in environments such as soil,[9] drinking and groundwater,[9,10] oceans,[11] lakes,[12] rivers,[9] atmosphere,[13] and biota.[11,14] PFAS most commonly contaminate water,[15] therefore, this is a large exposure route for humans and organisms in the environment. Humans are also exposed to PFAS in high quantities through food packaging, drinking water, and indoor environments (such as dust exposure), however, a lot remains unknown about the mechanisms and exposure sources for PFAS.[6,16–18] It is possible that many small, cumulative PFAS exposures could be as important as understanding a single large point source for PFAS. Evidence of this cumulative PFAS exposure has been observed in results from an NHANES study that found PFAS such as perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), and perfluorononanoic acid (PFNA) in the blood of nearly all Americans tested.[19] While, PFAS exposure to humans have been associated with thyroid disruption,[20] cancer,[21] and reproductive harm,[22] significant information about the toxicity and effects of PFAS on humans remains unknown.[16] In

addition to human exposure, organisms living in aqueous environments are particularly at risk for PFAS exposure considering PFAS have been shown to contaminate water in the environment.[10,15,18] Several laboratory exposure studies have found that certain PFAS are bioaccumulative and persistent in aquatic organisms exposed to PFAS.[23–30] While there are a number of studies investigating PFAS bioaccumulation in aquatic organisms, many of these focus on describing PFAS occurrence in the environment where bioaccumulation values are determined without regard to the influence of external factors, such as pH, salinity, suspended particles, or other environmental chemistry impacts. Considering PFAS can be found in almost any environment, understanding environmentally relevant uptake and bioaccumulation in aquatic organisms is imperative. Furthermore, PFAS exposure has been associated with negative health outcomes in humans.[20–22] Therefore, the threat the PFAS pose to human and environmental health is clear and sparks concern.

An important way to study the threat of PFAS to humans and the environment is to study how PFAS accumulate and affect model organisms. Model organisms are often used as a tool to study the effects of a contaminant on human and environmental health. Laboratory exposure experiments using model organisms have been used to make recommendations for human health and exposure limits for a variety of chemicals. Model organisms can provide insight into the toxicity and bioaccumulation of chemicals, such as PFAS, and, therefore, can inform recommendations for human health. Zebrafish are an example of an aquatic organism that has been commonly used in toxicological studies to model mechanisms in humans.[31,32] Model organisms are useful in toxicity and bioaccumulation experiments because humans have a long lifespan, many additional exposures to a chemical of interest, and it is unethical to purposefully expose humans to a contaminant with unknown bioaccumulative or toxicological effects. Additional toxicology research on aquatic organisms has been conducted to predict the effects of certain chemicals on humans.[33] A PFAS toxicity database demonstrates all toxicology studies published for a set of 24 PFAS.[34] Of these toxicity studies, 240 have been performed in an experimental, controlled laboratory environments, while 62 studies were observational in the natural environment. Common species studied included fish, *Daphnia magna*, a small plankton like organism, and a smaller number of studies looked

at toxicity in other invertebrates. The persistence and ubiquity of PFAS make this class of chemicals important to study from a toxicological perspective, however, these characteristics also pose a threat to laboratory exposure experiments because the materials used in the study could be contaminated with PFAS. Many of the aquatic toxicity studies included in the database mentioned having used laboratory materials, feed, and organisms without necessarily accounting for PFAS contamination.[35–37] Therefore, the results of all of the studies in this database could be confounded by PFAS contamination. Additionally, the hundreds of aquatic toxicity studies for other chemicals that have been used to influence human health recommendations could also be impacted by unknown PFAS interactions with the contaminant being studied. Therefore, understanding PFAS contamination in laboratory exposure experiments is crucial for influencing human and environmental health recommendations.

The objective of this thesis is to 1) review bioaccumulation parameters being used in laboratory exposure experiments and identify a common best practice to calculate bioaccumulation factors for PFAS, and 2) identify the potential PFAS contamination, and its impact, found in common materials used in aquatic laboratory exposure experiments.

The innovation of this work is clear because it recommends a method for calculating the bioaccumulation factor in a way that would account for all PFAS exposures, whereas other studies could be inaccurately estimating the bioaccumulation factor by ignoring these exposures. Another innovative approach is the identification of sources of previously unaccounted PFAS which would influence aquatic laboratory exposure studies for a variety of different chemicals, including PFAS. PFAS are found in most environments as a result of consumer and industrial use, thus, PFAS may contaminate materials used in aquatic laboratory exposure experiments in a similar way. These unaccounted sources of PFAS could be contaminating additional aquatic mesocosms such as freshwater aquaculture farms which are the source for many fish stocks used in laboratory studies. These sources of PFAS could confound bioaccumulation and toxicity results that are the basis for human health recommendations.

Chapter 1. Bioaccumulation of Per- and Polyfluoroalkyl Substances in Aquatic Organisms: A Scoping Review of Laboratory Exposure Experiments

1.1 Introduction

Various per- and poly-fluoroalkyl substances (PFAS) have been linked to lower fertility, increased cholesterol, and increased risk of cancer in humans.[38] In addition, some PFAS such as perfluorooctanesulfonate (PFOS) and perfluorooctanoic acid (PFOA) have been found at low levels in human serum, shown to bioaccumulate in organisms, and persist in the environment.[39–41]

Although PFAS were invented in the 1930s, it was not until the 1980s when they began being used in consumer goods such as water proof clothing, paper products, plastic, cookware, ski wax, adhesives, personal care products, and more due to their ability to repel water, oil, and grease.[42,43] Certain perfluoroalkyl substances, such as PFOS, have been used in AFFF starting in the 1960s.[44] These foams are particularly useful in fire extinguishing because a PFAS create a film to further eliminate flames and prevent the solvent, usually jet fuel, from catching on fire again.[45] In addition, AFFF rapidly cools these fires, further increasing its usefulness as a firefighting agent.[46]

In general, most AFFF formulations are composed of perfluoroalkyl carboxylates, sulfonates, and fluorotelomer sulfonates.[4,45] However, there remain perfluoroalkyl acid (PFAAs) precursors in AFFF and environmental samples which are unknown, challenging to quantify, and/or short-lived.[47] The formulation of such foams has shifted from long chain ($\geq C_8$) PFAS to shorter chain PFAS after the Stockholm Convention on Persistent Organic Pollutants restricted the manufacturing of PFOS.[48] There have been contrasting reports of short chain PFAS having low fire extinguishing capabilities,[49,50] causing long chain AFFF formulations to be phased out in favor of short chain AFFF formulations. However, much remains unknown surrounding the environmental and health effects of the short chain conformers.[51] In addition, the Department of Defense's (DoD) gave a recent report to congress indicating a replacement of PFOA/PFOS AFFF to non-flourine AFFF formulations, however, the health effects of these non-fluorine formulations also remain unknown.[52] PFAS have contaminated the environment from

AFFF and other consumer and commercial sources.

A recent study by Lam et al., found PFAS in water, sediment, and biota in a Vietnam river ecosystem downstream of wastewater discharges.[53] Wastewater can be a large source of the widespread PFAS contamination in many tiers in the environment. Boone et al found PFAS contamination in all 50 samples of source water and treated drinking water studied.[54] The legacy of PFAS is further exacerbated by the resistance to filtration between source water and treated drinking water. A study conducted by Chen et al examined PFAS in an urban catchment system and found that the distribution of PFAS were dependant on chain length; long chain PFAS tended to sorb more to sediments, whereas short chain PFAS were found in the water column.[55] Analytical trends for PFAS determination in air, water, sediment, biota and the associated methods were recently reviewed in 2019.[56] Nakayama et al concluded that robust analytical methodologies are necessary for the determination of original and new PFAS in aqueous samples, further demonstrating that PFAS persist and change within the aquatic environment.

There are a number of useful measurements in understanding the bioaccumulative potential of various chemicals. Some of the parameters documented in this paper include the biota-sediment bioaccumulation factor (BSAF), bioaccumulation factor (BAF), bioconcentration factor (BCF), and biomagnification factor (BMF). The equation for BMF is described in equation (1).[57]

$$BMF = \frac{Concentration\ in\ Predator}{Concentration\ in\ Prey} \quad (1)$$

The BMF relates the concentration in organisms to their predator-prey relationship. If this value is greater than one, then the compound of interest can be biomagnified up the food chain. It is important to note that with BMF, exposure to the chemical of interest is only from dietary exposure. Another useful bioaccumulation parameter is the BCF which is described in equation (2).

$$BCF = \frac{Concentration\ in\ Organism}{Concentration\ in\ Water} = \frac{k\ uptake}{k\ elimination} \quad (2)$$

The BCF relates the concentration within an aqueous organism to the concentration of the water. The BCF can be calculated using the concentration from the whole fish or from

tissue, however, the only exposure route here is aqueous exposure (passive exposure) at equilibrium. This measure can provide information about respiratory filtration within an organism: uptake through gills or skin. The BCF can also be calculated as the uptake rate constant over the elimination rate constant as long as the only exposure route is through the water. In this case the system does not necessarily need to be at equilibrium. The BCF and BAF are very similar where the BAF can be calculated as described in equation (3).

(3) $$BAF = \frac{Concentration\ in\ Organism}{Concentration\ in\ Water} = \frac{k\ uptake}{k\ elimination}$$

The BAF is calculated similarly to the BCF, however, the BAF considers exposures from all aspects of the surroundings: dietary, aqueous, and other unknown exposures, however, the calculation only takes the *dissolved* water concentration into account, not any contaminant that is sorbed to a solid or settles out. The BAF can be calculated using concentrations (similar to the BCF), however, in this case, the BAF functions more closely to a partition coefficient and the system must be at steady state in order to calculate the BAF this way. This measure can also be calculated using the rate of uptake over the rate of elimination, however, exposure from all pathways are considered here. The BAF is considered at steady state, while the BCF can only be calculated at equilibrium. Some studies will indicate that the organisms were exposed through spiked water, but report a BAF to account for any additional exposure to PFAS that may be in the feed, sediments, or habitat material.

Finally, the equation to calculate the BSAF is described in equation (4):[58]

(4) $$BSAF = \frac{Concentration\ in\ Organism}{Concentration\ in\ Sediments} = \frac{k\ uptake}{k\ elimination}$$

The biota-sediment bioaccumulation factor is similar to the BAF, however, is often used for organisms living at the sediment water interface. It may be challenging to differentiate the BSAF from the BAF for a couple reasons: 1) the sediment and water may not be in equilibrium therefore the organisms are likely exposed to contaminated water in addition to contaminated sediment, and 2) the organisms are exposed to water as well as sediments, however, only the concentration in the sediments is considered. Therefore, in this study, BSAF values were included as summary measures in addition to BAF values.

Fish and aquatic invertebrates have been shown to bioaccumulate and transform various chemicals found in their aquatic environment. The bioaccumulation of heavy metals in Carp was quantified by Vinodhini and Narayanan in 2008 who found that cadmium had the greatest accumulation while chromium had the least accumulation.[59] Various chemicals within a specific class have different bioaccumulation potentials in each part of the organism (i.e. liver, tissue, etc) which can make modeling the effects of one chemical based on an entire class of chemicals challenging. Gobas and Lo demonstrated that the respiratory uptake rate of a number of environmental pollutants in rainbow trout were regulated by the gill ventilation rate of the fish.[60] Therefore, uptake rates for fish may be significantly different from invertebrates considering that invertebrates often lack gills as a mode of regulating exposure. Volta et al. found that polychlorinated biphenyl accumulation increased with the age of the fish, likely due to biomagnification factors; however, birds who ate these fish had a higher ability to biotransform these contaminants.[61] For PFAS, there have been contrasting reports of bioaccumulative properties of short chain PFAS; studies have shown that short chain PFAS are not as likely to bioaccumulate, but others have indicated that short chain PFAS may have higher biomagnification factors.[53–55]

System interactions that limit PFAS availability in different matrices (i.e. environments) remain unknown. For example, the interaction between PFAS and invertebrates, fish, sediment, and the PFAS partitioning within various aqueous environments is complex and often unaccounted for, in addition to water chemistry. A study conducted by Gobas et al. found that uptake rates were regulated by gill ventilation rate, therefore, bioaccumulation factors for fish cannot necessarily be related to invertebrates or other organisms lacking gills.[60] In addition, there are species and chemical specific uptake and elimination kinetics that may further complicate comparisons.

Factors that determine the rate of PFAS uptake are complex, species and chemical specific that can lead to large uncertainties. It is possible that fish can have a high bioconcentration factor, but low biomagnification factor due to biotransformation within the fish.[60] To combat this uncertainty, many studies combine the individual compounds

into a total PFAS body burden, which can leave out important chemical specific differences when it comes to bioaccumulation in hopes of being able to compare to other studies. Brandsma et al. found that long chain fluorotelomer carboxylic acids (FTCAs) had a higher bioaccumulation factor in rainbow trout than shorter chain FTCA compounds. However, Chen et al. found that the longer chain precursor, polyfluoroalkyl phosphate diesters (diPAP), had a smaller bioaccumulation factor in Carp than the short chain precursor hypothesizing that the compound was too large to pass through the cell membrane.[55,62] The functionality of a compound, in addition to the organism of interest, must also be taken into consideration when assessing the bioaccumulation of compounds within a class of chemicals, such as PFAS, which can create complicated bioaccumulation models.

In addition, there have been contrasting reports of biomagnification of PFAS. Several studies have indicated that PFOS was found at higher concentrations with increasing trophic levels indicating biomagnification of this compound, however, this could be because PFOS is the result of many biotransformation processes from other PFAS precursors.[63,64] Lescord et al concluded that there was no biomagnification of PFAS with increasing trophic level, but that the horizontal position, or the species an organism belongs to *within* a trophic level determines PFAS concentrations within organisms.[65] Gebbink et al suggested that PFAS transformation plays a small role in PFOS concentration and instead concluded that direct PFOS exposure from a contaminated airport site accounted for the majority of PFOS accumulation within the food web.[66] As observed with biotransformation products, there are likely other precursors, such as diPAPs, that degrade into other, more simple, PFAS or PFAAs. These often unknown PFAA precursors can degrade over time and change the composition of various known PFAS. Houtz and Sedlak developed a method to determine the total oxidizable precursors contributing to PFAAs in a sample, further improving the understanding of the composition of AFFF formulations and their degradation products.[47] However, this method does not include sulfonates and the identity of the precursors remains unknown.

The research gaps illustrated above are explored in this review. This scoping literature review will summarize the literature surrounding PFAS partitioning in fish in

various lab exposure studies; compile uptake, elimination, and bioaccumulation kinetics for various PFAS in a variety of aquatic species; and compare BAFs for each PFAS within each class of PFAS as well as within each organism. The potential underlying reasons for differences between BAFs are identified. Finally, this review will demonstrate the need for continued research to create a model for fish and invertebrate species for all twenty-four non-drinking water PFAS, previously outlined by the EPA.[67]

The objective of this scoping review is to identify, compile, summarize, and compare studies with regard to bioaccumulation of PFAS in aquatic organisms in a variety of exposure studies as well as determine a best practice method for calculating the bioaccumulation of PFAS in an organism.

1.2 Methods

All studies were considered including published and any unpublished literature retrieved through each search method, except where the full text was not available through the University of Minnesota library. Only English papers were considered due to limited time and resources to translate the materials. The timeline chosen was 2000-2020 due to large quantities of literature and relevance of study. Attention regarding PFAS persistence occurred in 2001 after the Stockholm Convention on Persistent Organic Pollutants (POPs) international treaty was signed.[48] To be included in this review, the initial source must have included at least one perfluoroalkyl compound from the EPA non-drinking water list (Table 1) directly studied or focused on with regards to aquatic invertebrates or fish within an aquatic environment in a lab exposure setting.

Table 1. List of PFAS studied, originally from the EPA non-drinking water list.

Acronym	Name
PFBA	perfluorobutanoic acid
PFPeA	perfluoropentanoic acid
PFHxa	perfluorohexanoic acid
PFHpA	perfluoroheptanoic acid
PFOA	perfluorooctanoic acid

PFNA	perfluorononanoic acid
PFDA	perfluorodecanoic acid
PFUnA	perfluoroundecanoic acid
PFDoA	perfluorododecanoic acid
PFTriA	perfluorotridecaonic acid
PFTetA	perfluorotetradecaonic acid
PFBS	perfluorobutane sulfonate
PFPeS	perfluoropentane sulfonate
PFHxS	perfluorohexane sulfonate
PFHpS	perfluoroheptane sulfonate
PFOS	perfluorooctane sulfonate
PFNS	perfluorononane sulfonate
PFDS	perfluorodecane sulfonate
4:2 FTS	1H,1H,2H,2H-perfluorohexane sulfonate
6:2 FTS	1H,1H,2H,2H-perfluorooctane sulfonate
8:2 FTS	1H,1H,2H,2H-perfluorodecane sulfonate
NMeFOSAA	2-(N-Methylperfluorocotanesulfonamido) acetic acid
NEtFOSAA	3-(N-Ethylperfluorocotanesulfonamido) acetic acid
FOSA	perfluorooctane sulfonamide

Studies regarding microbial degradation of PFAS in solids (such as activated sludge, etc) were not included, except for background information about transformation products. No seabirds, aquatic mammals, or rats were included due to different anatomy with respect to PFAS kinetics and this study focused on underwater aquatic organisms given that PFAS are a common contaminant found in water.[41,53] Studies surrounding PFAS in water, with no direct organism impact, were not included, however, they were used to inform introductory and background information. The following databases were searched in January of 2020: OVID Medline(R), PubMed, SciFinder, Water Resource Abstracts, and Scopus. A link to an example search method can be found at the following

url: (http://ovidsp.dc2.ovid.com.ezp1.lib.umn.edu/sp-4.04.0a/ovidweb.cgi?&S=ODLEFPPAACEBBLGAIPBKIFPEDIMJAA00&C=_main&tab=search&Main+Search+Page=1). Each search included some or all of the following keywords: bioaccumulation AND PFAS AND Fishes OR Aquatic organisms.

After compiling the citations and deduplicating the remaining 305 sources were uploaded to Rayyan, a free web application that can be used to sort articles as part of a systematic or scoping review. The title was read for every article and either excluded with the reasoning tagged, included, or placed in a maybe category. During the second pass through, the abstracts of each article were read and the sources were further sorted. Ultimately, 296 sources were excluded and 9 sources were included (see results for flow chart).

Data was extracted independently from each study and compiled into a spreadsheet with the following column headings: title, authors, year of publication, country of origin, aims/purpose, study population and sample size, methods, compounds observed/analyzed, bioaccumulation values, duration of study, and outcomes and details relating to the scoping review question (Supporting Information/Appendix). Data was obtained through online databases and was not confirmed with investigators from the respective study. The population for which data was sought was aquatic organisms, including fish and invertebrates. The concept for which data was sought was the impacts of PFAS including, but not limited to, kinetics, bioaccumulation, uptake, elimination, bioconcentration, and biomagnification. The context for which data was sought was laboratory exposure studies in aquatic environments from 2000 to 2020. Risk of bias of individual studies was not assessed in this review and, thus, not used in any data synthesis.

BAF values were compared between studies, however, when BAF was unavailable, the BCF was used for comparisons. However, there remain a number of species differences between studies. In addition, other, non-numerical, comparisons were made. Data was interpreted and extracted into a spreadsheet table and no results were combined across studies. In the case where uptake and elimination rates were provided

instead of BAF values, the BAF was calculated using the formula in equation (3) above.

1.3 Results and Discussion

A total of 615 records were identified from the search strategy identified above (Figure 1). After duplicates were removed, 305 sources remained.

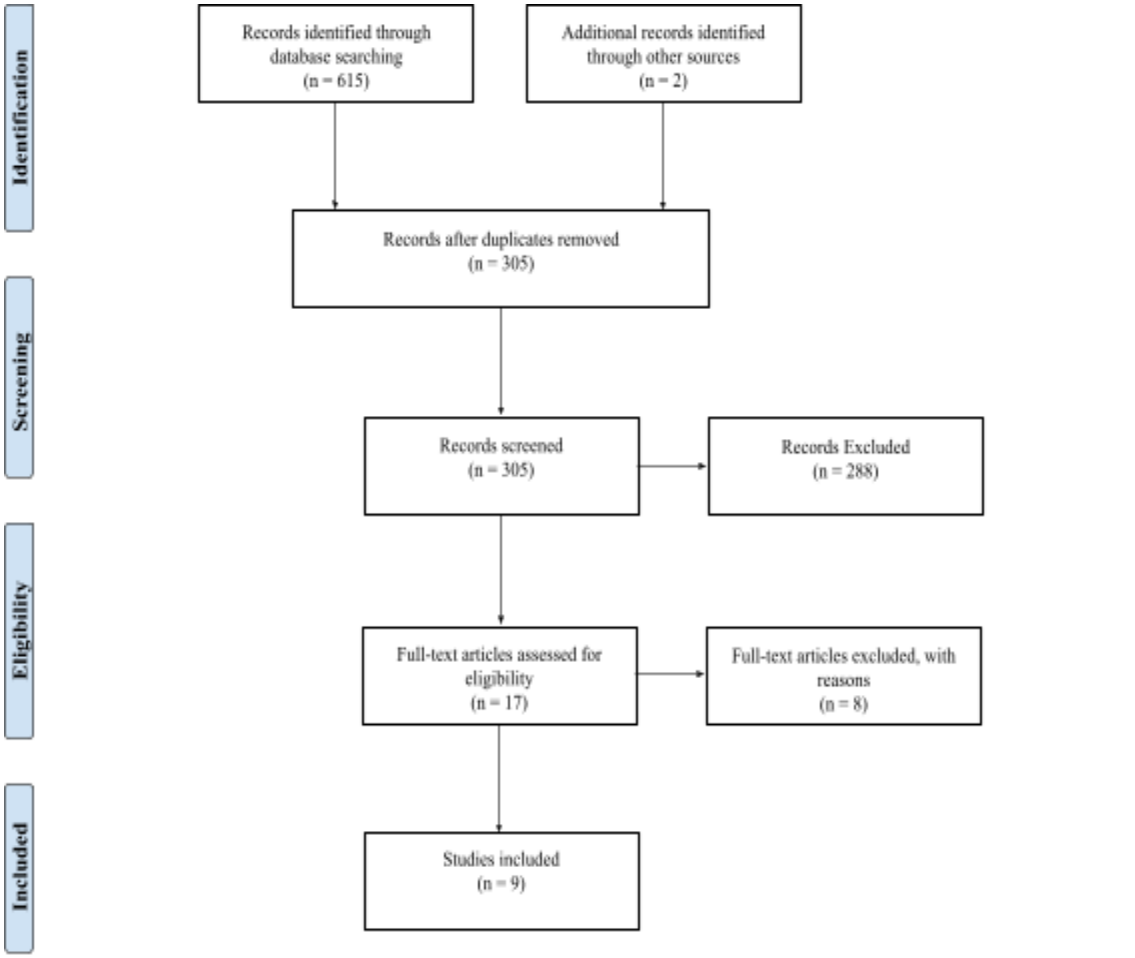

Figure 1. Flow chart detailing study selection adapted from PRISMA.[68]

Once these records were screened, 288 were excluded due to missing a major component of the review question asked. The 17 remaining articles were reevaluated and 8 were excluded. The reasons for all exclusions were documented in Rayyan.[69] In total, 9 studies were included in the analysis of this review. Each study, population sampled, and abbreviations of analyzed compounds can be found in Table 2 below.

Table 2. The study characteristics for each source included in this review. Population and compounds analyzed are included for each study.

Study	Population	Compounds Exposed/Analyzed
Martin et al.[26]	Rainbow trout	PFOA, PFDA, PFUnA, PFDoA, PFTetA, PFHxS, PFOS
Inoue et al.[27]	Common carp	PFOA, PFUnA, PFDoA, PFTetA, PFHxDA, PFODA, PFOS
Dai et al.[25]	Daphnia magna	PFOS, PFOA, PFNA, PFDA, PFUnA, PFDoA
Xia et al.[70]	Daphnia magna	PFOA, PFNA, PFOS, PFDA, PFUnA, PFDoA
Xia et al.[28]	Daphnia magna	PFOS, PFOA, PFNA, PFDA, PFUnA, PFDoA
Hassell et al.[23]	Pseudogobius sp (blue spot gobies)	PFOA, PFOS, GenX
Martin et al.[29]	marine echinoderm-Holothuria tubulosa Gmelin, 1791	PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFOS
Liu et al.[24]	Green mussels (Perna viridis)	PFOS, PFOA, PFNA, PFDA
Higgins et al.[30]	Freshwater Oligochaete-Lumbriculus variegatus	PFOA, PFNA, PFDA, PFUnA, PFDoA, PFOS, PFDS

Bioaccumulation factors (BAF), bioconcentration factors (BCF), biomagnification factors (BMF), and uptake and elimination rates (k_u and k_e) were considered as plausible summary data to compare studies. The comparisons are made below (Table 3). BAF values are most commonly reported and vary widely between studies, likely due to a difference in how various authors calculated the BAF, the concentration and mixture of PFAS that organisms were exposed to, or other differences in water chemistry between studies. For example, Liu et al calculated the BAF as the steady state concentration in the organism divided by the steady state concentration dissolved in the water. While Liu et al exposed the organisms to spiked water, they likely reported a BAF value to incorporate any additional, unknown exposure to PFAS. Therefore, any additional PFAS in the fish

food or habitat materials would not be accounted for in the BAF and would result in a larger BAF than other calculated BAF values. In addition, Higgins et al exposed organisms to spiked sediments and reported a BSAF in lieu of a BAF. However, the BSAF can be compared to the BAF because both the BSAF and BAF are not necessarily in equilibrium and the organisms may be exposed through multiple pathways. Therefore, the values from the Higgins study reported in Table 3 were calculated from the uptake and elimination kinetics which provided BSAFs (reported as BAFs for comparison) close to one. Therefore, these values may be lower than other expected BAF values from studies that did not calculate the BAF this way. If the BAFs from the included studies were calculated as the rate of uptake divided by the rate of elimination, then it would be easier to compare those values to the BSAF values from the Higgins study. However, the BAFs for the majority of the studies were calculated using the concentration in the organism divided by the concentration in the water (Figure 2), with multiple potential exposure routes. Therefore, it can be challenging to compare a BSAF that was calculated kinetically to a BAF partition coefficient. In addition, it can be challenging to compare the BAF values for such a wide range of organisms in a variety of environments that may not have the same exposure routes.

Table 3. Comparisons of bioconcentration factors, both experimentally determined and calculated using equation (3) above.

Compound	Population	BAF (L/kg)	BCF (L/kg)	BMF	Study
PFBA	Holothuria tubulosa Gmelin, 1791	47.9			Martin et al.[a]
PFPeA	Holothuria tubulosa Gmelin, 1791	55			Martin et al.
PFHxA	Holothuria tubulosa Gmelin, 1791	239.9			Martin et al.
PFHpA	Holothuria tubulosa Gmelin, 1791	691.8			Martin et al.
PFOA	Oncorhynchus mykiss		13		Martin et al.[b]

	Cyprinus carpio		6.3	Inoue et al.[c]
	Daphnia magna	91		Dai et al.
	Daphnia magna	197		Xia et al.[d]
	Daphnia magna	136		Xia et al.[e]
	Pseudogobius sp		0.021	Hassell et al.
	Holothuria tubulosa Gmelin, 1791	616.6		Martin et al.
	Perna viridis	15		Liu et al.[f]
	Lumbriculus variegatus	0.95		Higgins et al.
PFNA	Daphnia magna	152		Dai et al.
	Daphnia magna	214		Xia et al.
	Daphnia magna	197		Xia et al.
	Perna viridis	144		Liu et al.
	Lumbriculus variegatus	1.6		Higgins et al.[g]
PFDA	Oncorhynchus mykiss		1,416.70	Martin et al.
	Daphnia magna	175		Dai et al.
	Daphnia magna	349		Xia et al.
	Daphnia magna	310		Xia et al.
	Lumbriculus variegatus	1.02		Higgins et al.
	Perna viridis	838		Liu et al.
PFUnA	Oncorhynchus mykiss		9,300	Martin et al.
	Cyprinus carpio		3,000	Inoue et al.
	Daphnia magna	270		Dai et al.
	Daphnia magna	458		Xia et al.

	Daphnia magna	446			Xia et al.
	Lumbriculus variegatus	0.62			Higgins et al.
PFDoA	Oncorhynchus mykiss		38,000		Martin et al.
	Cyprinus carpio		13,000		Inoue et al.
	Daphnia magna	380			Dai et al.
	Daphnia magna	609			Xia et al.
	Daphnia magna	558			Xia et al.
	Lumbriculus variegatus	0.55			Higgins et al.
PFTriA	--	--	--	--	--
PFTetA	Oncorhynchus mykiss		27,666.70		Martin et al.
	Cyprinus carpio		16,500		Inoue et al.
PFBS	--	--	--	--	--
PFPeS	--	--	--	--	--
PFHxS	Oncorhynchus mykiss		61.9		Martin et al.
PFHpS	--	--	--	--	--
PFOS	Oncorhynchus mykiss		5,400		Martin et al.
	Cyprinus carpio		1,010		Inoue et al.
	Daphnia magna	179			Dai et al.
	Daphnia magna	344			Xia et al.
	Daphnia magna	296			Xia et al.
	Pseudogobius sp			0.261	Hassell et al.
	Holothuria tubulosa Gmelin, 1791	6,456.50			Martin et al.
	Perna viridis	378			Liu et al.

	Lumbriculus variegatus	1.22			Higgins et al.
PFNS	--	--	--	--	--
PFDS	--	--	--	--	--
NMeFOSAA	--	--	--	--	--
NEtFOSAA	--	--	--	--	--
4:2 FTS	--	--	--	--	--
6:2 FTS	--	--	--	--	--
8:2 FTS	--	--	--	--	--
FOSA	--	--	--	--	--

a- BAF was averaged from $BAF_{intestines}$ and BAF_{gonad} at 0.5 mg/L exposure

b- BCF was averaged from $BCF_{carcass}$, BCF_{blood}, and BCF_{liver}

c- BCF was averaged from BCF_{high} and BCF_{low}

d- BAF values for the control group

e- BAF values for the control group (instead of the BAF for each humic substance)

f- This paper defined BAF as the concentration in the organism divided by the concentration in water, used BAF at 1 ppb exposure

g- BAF was calculated using equation (3) above

It is clear from Table 2 that there are some compounds where no BAF, BCF, or BMF values have been reported. This “hole” in the table further demonstrates the need for additional research on many PFAS included in the EPA’s non-drinking water list.

From 2000-2010, only two studies were included, one that used BCF and one that used BSAF as a method of measuring accumulation of PFAS in aquatic organisms. In the following five years, the number of studies estimating accumulation increased, as did the number of ways to calculate bioaccumulative potential. Between 2011 and 2015, the majority of studies utilized the BAF that was estimated through the division of the concentration in the organism by the concentration in the water (BAF(c)). Fewer studies

used the kinetic method of calculating the BAF (BAF(k)). Finally, most recently, studies continued to use the BAF(c) method of calculating the BAF, while including BSAF measures or biomagnification measures (BMF). This trend over time indicates that the field may be shifting interest towards magnification, however, there remains a lot of discrepancies on how to calculate the BAF.

Bioaccumulative models have changed significantly over the last two decades. Arnot and Gobas developed a Quantitative Structure Activity Relationship (QSAR) to determine the bioaccumulative potential of various organic pollutants on aquatic organisms.[71] The authors defined the concentration dependent BAF and BCF as the primary methods of determining bioaccumulative potential. However, over a decade later, Quinn et al created a bioaccumulative model utilizing the uptake and depuration kinetics that outperformed other species specific models and was able to account for more parameters (temperature, body mass, species, etc).[72] Overtime, the field has changed dramatically to better understand bioaccumulative potential, however, it remains challenging to compare values determined via such different models or methods. Therefore, it would be helpful to compare multiple methods of BAF calculation.

Dai et al compared both methods of calculating the BAF.[25] The kinetically determined BAF values ranged from 89 to 474 L kg^{-1}, whereas the concentration dependent BAF values ranged from 91 to 380 L kg^{-1}.[25] These values are quite similar and the BAF(k) values are much larger than one as a result of large rates of uptake compared to the small elimination rate. This is likely organism specific, however, comparisons to other organisms within this study cannot be as easily made because many of the additional studies included calculated the BAF using the concentration method. The results from Dai et al suggest that the rate of uptake influences the concentration in the organism, especially because the system was allowed to reach equilibrium.

The majority of studies performed analyses on whole body homogenates, however, Martin et al (2003 and 2019) and Inoue et al included several important tissues such as the liver, blood, intestines, gonads, and others.[26,27,29] The largest BCF reported in Martin et al (2003) was 40,000 L kg^{-1} for PFDoA in blood.[26] However, Inoue et al reported the largest BCF as 17,000 L kg^{-1} for PFTetA with the highest concentration in

the viscera (internal organs including liver, etc).[27] Martin et al (2019) used the BAF as the measure of bioaccumulative potential and found that the highest log BAF value was 4.39 for PFOS in the intestines.[29] Finally, Martin et al (2019) also reported the highest BSAF value of 2.89 for PFOA in the intestines.[29] Overall, blood showed the highest bioaccumulative potential through the BCF. The blood was not studied in the calculation of the BAF or BSAF in Martin et al (2019), however, this is likely an important value for comparisons. In addition, the BAF was larger than the BSAF indicating the main exposure route may be from water, as opposed to sediments. Between all three studies mentioned, the blood and internal organs of the fish appear to be the most important with respect to bioaccumulative potential.

In addition to documenting the method of calculating the BAF over time for the studies included, a number of analytical consistency measures were noted and summarized to ensure comparisons can be made across different studies. The analytical chemistry measures documented in each paper vary widely likely as a result of improving practices as the field developed between 2003 and 2019. This data is important in creating broader impacts and overall reproducibility. Continued work should be grounded in the best practices with respect to analytical consistency. It should be noted that the analytical consistency measures were not included in the review unless it was specifically mentioned or found in the paper's supporting information. Considering some studies do not mention quality assurance and control measures, trends observed in analytical standards may be misrepresentative. However, it would appear that isotope dilution became more widely used with time likely as a result of growing accessibility to mass labeled standards. In addition, most studies extracted procedural blanks, but found little to no PFAS contamination, therefore, did not subtract the concentration in the blanks from the concentration in the samples. Finally, it is important to note that while trends may seem clear, the number of studies also increased over time, which makes it challenging to draw conclusions from this data. Moving forward, it is just as important that papers document which method of bioaccumulative potential they use as well as various analytical consistency measures to continue improving the practices and comparability between studies in environmental chemistry research.

The results of each meta-analysis performed is presented below. Monitoring environmental concentrations of PFAS in biota is crucial, however, it can be challenging to compare various chemicals across different species in varying lab exposure environments. The bioaccumulation factor may be a more useful metric to compare across studies, however, a consistent method of calculating this measure is necessary. Only two studies reported a bioconcentration factor for various PFAS,[66,69] however, several studies reported calculating a BAF using the PFAS concentration in the organism divided by the concentration in the water. As mentioned above, this method of calculation may be omitting important additional or unknown exposure routes. A single study reported BMF in lieu of BCF or BAF.[25] Studies with no BAF, or BSAF, value were not included in the summary statistics. The BAF values span more than three orders of magnitude for all PFAS including all organisms (Figure 2).

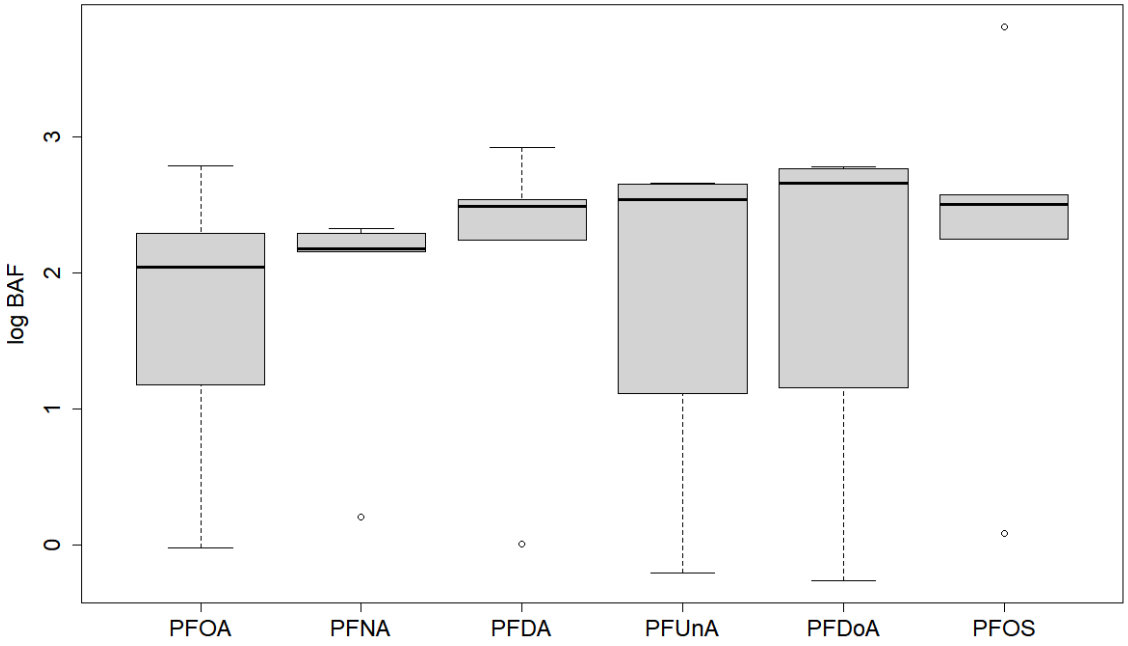

Figure 2. Boxplot of the log BAF values for PFAS that could have statistical analyses performed.

The log BAFs of the perfluoroalkyl carboxylic acids were compared as a function of chain length (Figure 3). As chain length increases, the median log BAF also increases, 2.04, 2.19, 2.54, and 2.66, respectively. This trend is not statistically significant (P <

0.05) via the Mann-Whitney-Wilcox test, likely because of the large variation for each acid chain length as a result of different calculations of BAF and various organisms. This is consistent with previous findings by Martin et al. who found that the log BAF value increases with increasing PFAS chain length.[66]

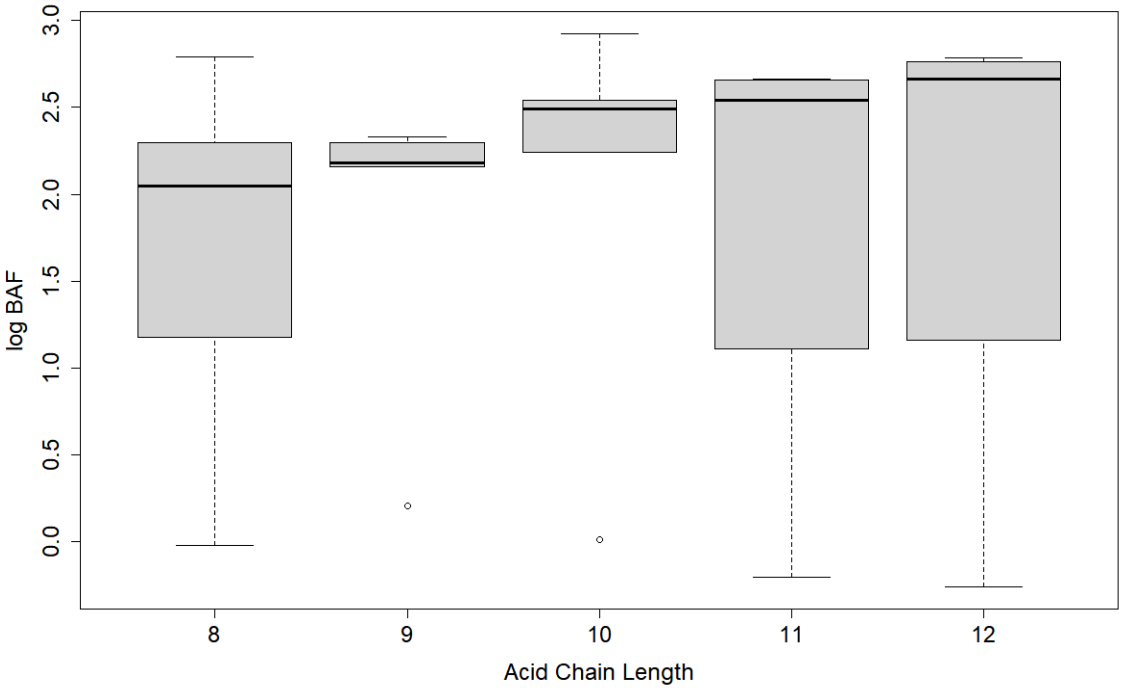

Figure 3. Log BAF for the perfluoroalkyl acids as a function of chain length.

There was only one perfluoroalkyl sulfonate that had enough observations to perform statistics on it and that was PFOS which had two clear low and high outliers indicating a large range of BAF values dependent on the organism and method of calculation. However, the median log BAF value for the eight chain sulfonate (PFOS) was 2.50 which was closest to the log BAF of the ten chain carboxylic acid (PFDA, 2.49). This indicates that the sulfonates may bioaccumulate to a larger extent than the acids due, which is consistent with literature. Zhang et al. found that PFOS has a higher BAF than similarly sized perfluoroalkyl carboxylic acids.[73]

The BAF values for *Holothuria tubulosa* (sea cucumber), *Daphnia magna* (water flea), *Perna viridis* (Aisan green mussel), and *Lumbriculus variegatus* (freshwater worm) were compared in Figure 4. The BAF values were similar for all organisms excluding *Lumbriculus variegatus*, demonstrating that the method of calculation of BAF is crucial

in comparisons of these values. The study that produced these values for *Lumbriculus variegatus* (Higgins et al), exposed the organisms to sediments that were contaminated with PFAS from the environment. *Daphnia magna* had the largest median BAF and *Lumbriculus variegatus* had the lowest median BAF. In addition, Perna viridis has the widest spread of values. This could be attributed to trophic level (i.e. TMF), however, there is not enough data to determine if this is the case.

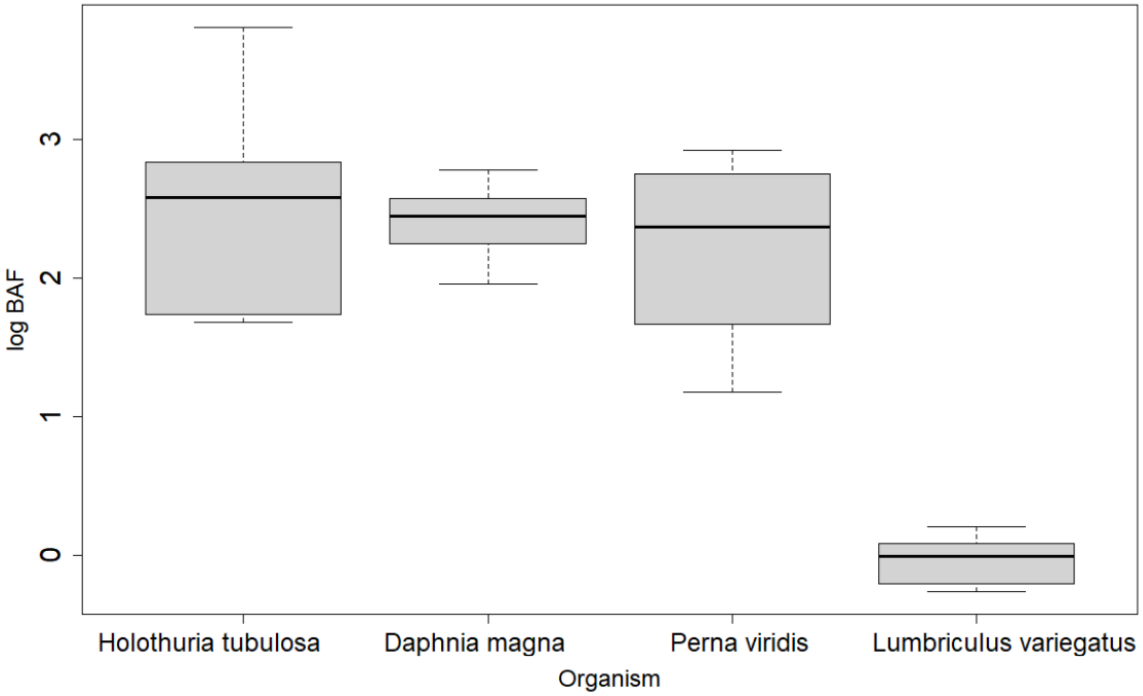

Figure 4. Comparisons of BAF values for perfluoroalkyl carboxylic acids for relevant organisms.

The BAF values for *Lumbriculus variegatus* were several orders of magnitude lower than the other organisms. This could be attributed to three factors: 1) this organism is the only freshwater oligochaete (worms) represented in this dataset, 2) all values for this organism came from a single study by Higgins et al. where BSAF was reported in lieu of a BAF, and 3) the BSAF was calculated using the uptake rate constant divided by the elimination rate constant.[30] While the oligochaetes were the only organism knowingly exposed to spiked sediments, the BAF should account for all exposure routes (sediment, water, etc), therefore, the differentiation between BSAF and BAF is nuanced. In addition, the sediment and water the oligochaetes were exposed to was likely not in equilibrium,

making it even more challenging to discern this value from the BAF. The method through which the BSAF values for this study were calculated (rate of uptake divided by rate of elimination) should be independent of exposure pathway and places the focus on the exposure of the organism and less on the concentration of the surrounding water, therefore, these values should be comparable to other BAF values (calculated using kinetic parameters). While calculating the BAF through the concentration method is meant to represent all potential exposure routes, it normalizes the values to the concentration in the water, therefore, this measure may not be fully independent of exposure route or concentration. Therefore, *Lumbriculus variegatus* was included in Figure 4 despite differing exposures and magnitudes of BAF values.

Martin et al included the concentration dependent BAF value in addition to the BSAF. This may be able to place the BSAF values from Higgins et al (above) in context. The log BAF values ranged from 1.14 to 4.39, whereas the log BSAF values ranged from 0.91 to 2.89.[29] The differences between these values indicates that the organisms in this study more readily uptake PFAS from the water column compared to the sediments, however, sediment exposure still remains an important pathway. Higgins et al reported log BSAF values were much lower, ranging from -0.92 to 0.086.[30] The BSAF values in that study were calculated using the kinetics of uptake and elimination whereas, Martin et al calculated BSAF values from the concentration in the organism compared to the concentration in the sediments which is likely highly dependent on the composition of the sediments.

An important aspect of the Higgins study is the composition of sediments as well as water sediment partitioning. While the BSAF values reported were calculated using the kinetic method, it can be useful to understand the composition of the sediments in addition to the partition coefficients previously determined by Higgins et al. The sediments used to determine the BSAF were from the same sample previously used to determine PFAS partition coefficients by Higgins et al.[74] The sediment was composed of 4.34% organic carbon, 31% sand, 38% silt, and 31% clay.[74] The composition of the sediment, including the fraction of organic carbon, can greatly influence the partition coefficient. The organic carbon normalized sediment distribution coefficient (log k_{oc}) was

previously determined to range from 2.11 for PFOA to 3.66 for PFDS, which is dependent both on the chain length as well as the head group functionality.[74] The importance of this Higgins study was that hydrophobic interactions were deemed more important in directing sorption than ion exchange interactions with the sediment.

These findings are particularly relevant to research groups studying PFAS exposure because there are clearly some inconsistencies in how various parameters are calculated that result in differences of several orders of magnitude. In addition, regulatory agencies may be interested in these findings because many BAF values reported are quite high, even for shorter chain PFAS, therefore, future research and regulation of PFAS is necessary.

Since this review was written, several similar reviews have been published. The challenging nature of comparisons between bioaccumulation studies is confirmed in a recent study by Savoca et al as a result of the different concentrations, water chemistry, and laboratory environments that organisms are exposed to in determination of the bioaccumulation parameters.[75] In addition, Savoca et al mentioned the difficulty comparing bioaccumulation factors determined in the field with those determined in the laboratory simply due to uncontrolled external factors.[75] Burkhard et al further confirmed the findings that higher quality bioaccumulation studies are needed. Specifically, for some lower quality studies where multiple exposure pathways could occur, Burkhard et al used the bioaccumulation factor to represent the bioconcentration factor since the bioconcentration factor could be confounded by additional PFAS exposure.[76] Unknown and unaccounted PFAS contamination could significantly impaction bioaccumulation studies which was concluded in this review and further confirmed by other researchers in the field.

1.4 Limitations

There are a number of challenges surrounding comparing the summary bioaccumulation measures for each study included in this review. This challenge arises from significant differences in species investigated, known and unknown exposure routes, and how the BAF was computed. Another potential limitation was that no risk of

bias was assessed, however, there could be potential bias because the majority of studies calculated the BAF using the concentration method, which provided values much larger than the values calculated from the uptake and elimination kinetics in Higgins et al.[30] In addition, error estimates and confidence measures were challenging to compare and include, which could be an additional source of error.

1.5 Conclusions

When multiple known and unknown exposure pathways are present in laboratory exposure studies, the kinetically determined BAF value is a better estimate of bioaccumulative potential because the values would be closer to one and put the focus on the exposure of the organism, rather than the concentration of the surrounding water. In addition, the concentration dependent BAF acts more similarly to a partition coefficient which may be inaccurate considering the system being studied may not be at equilibrium. This would ease comparisons of BAF values, rather than comparing an organism to an often inconsistent environment across lab exposure studies. Future models are necessary to aid in comparisons of all 24 non-drinking water PFAS within fish and invertebrates in different environments with varying water chemistry. Standardization of BAF calculations is necessary and should be defined as in the second part of equation (3). This study also demonstrates that BSAFs can be compared to BAF values, specifically when calculated using kinetic parameters independent of exposure route. The BAFs for all twenty-four PFAS ranged several orders of magnitude. The organisms with the highest and lowest median BAF values were *Daphnia magna* (water flea) and *Lumbriculus variegatus* (freshwater worm), respectively. In general, as the perfluoroalkyl chain length increased, so did the median log BAF. The eight chain sulfonate (PFOS) had a log BAF value closest to the ten chain carboxylic acid (PFDA) indicating sulfonates have a higher bioaccumulative potential compared to the perfluoroalkyl acids. This scoping review should be considered by regulatory agencies as evidence of high bioaccumulation potential for PFAS, as well as research groups to build a more cohesive field of bioaccumulation study.

Chapter 2: Occurrence and Impact of PFAS Contamination of Materials Used in Aquatic Laboratory Exposure Experiments

2.1 Introduction

Bioaccumulation estimates can clearly be influenced by unknown and unaccounted PFAS contamination, therefore, it is crucial to understand the potential sources of PFAS in laboratory exposure experiments. If PFAS are present in various materials that are used as part of toxicity and bioaccumulation studies, then those results could be confounded and human and environmental health recommendations based on these studies could be inaccurate. In a recent study, Glüge et al found PFAS or fluoropolymers were used in a number of laboratory materials for their hydrophobic and surface active properties.[77] Specific laboratory materials that use PFAS in the manufacturing process are vials, caps, tape, gloves, solvents, and seals/membranes, to name a few.[77] While the study mentioned which materials use PFAS, and when available the suspected function of PFAS in that application, there are few mentions of the concentration of PFAS present as well as which PFAS can be found. This remains a large knowledge gap in the field, especially considering the number of laboratories studying PFAS and using these common laboratory materials. To attempt to address part of the concern with the ubiquity of PFAS in research materials, Rodowa et al found that field sampling materials had little to no PFAS present and were likely not the cause of contamination of field samples.[78] While this finding is useful for field sampling, it is not comprehensive and, therefore, other laboratory materials should not be assumed to not contain or contaminate samples with PFAS.

Since PFAS are resistant to degradation and often contaminate the aqueous environment,[79,80] many laboratory exposure studies focus on organisms that are found in water. Therefore, scientists studying bioaccumulation and toxicity should be particularly cautious of PFAS contamination of materials that interact with the water in experiments and with model organisms being studied. Aquatic laboratory exposure experiments often use organisms that must be housed in tanks, such as fish and invertebrates. As a result, the plumbing for the tanks as well as filters and screens need to be PFAS free (Figure 5). However, there are few, if any, studies specifically indicating the presence, or lack

thereof, of PFAS in these tank materials or the coatings. Unaccounted PFAS from the aquatic housing materials could influence the PFAS concentration in the water and the organisms being studied, thereby confounding bioaccumulation or toxicity results.

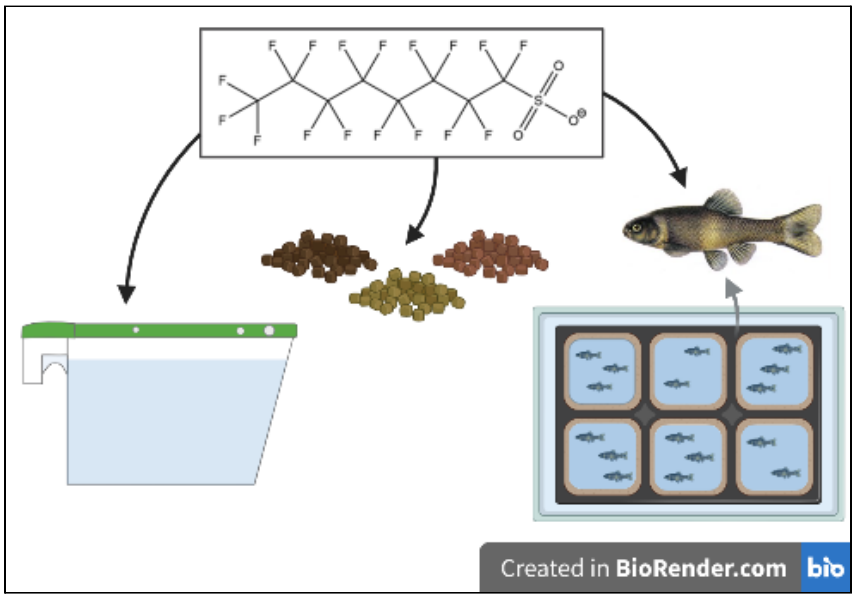

Figure 5. Research scheme to study PFAS presence in tank materials, feed, and aquaculture minnows.

Another potential source of PFAS contamination to aquatic laboratory exposure experiments could be in the feed given to organisms being studied (Figure 5). While many toxicity and exposure studies are specifically spiking the chemical of interest into the organism's food, the baseline concentration of PFAS in the feed is important in order to more accurately determine the contaminant load on the organism. Additionally, considering that the major environmental sink for PFAS is water, there has been a new interest in understanding how organisms uptake and accumulate PFAS from the surrounding water. Therefore, laboratory exposure experiments should be concerned with any additional sources of PFAS, especially from a dietary route that is unintended. PFAS contamination in fish feed could significantly contribute to inaccurate results across a variety of laboratory exposure experiments.

Model organisms are incredibly important for laboratory toxicity, bioaccumulation, and other exposure studies. It is necessary for these organisms to be contaminant free in order to control as many variables as possible to obtain accurate

results. While many laboratories raise their own model organism stocks, others often refer to suppliers and aquaculture farms to provide the organism of interest. These suppliers could have PFAS contaminated water, tank parts, or even fish feed, therefore, the organisms raised there may have accumulated PFAS as a result of the potential contamination (Figure 5). A recent study using Striped Bass from an aquaculture facility as control samples, found 9.41 ng/mL PFOS in the serum of the fish from the aquaculture facility.[81] Although the concentration in the aquaculture fish was lower than the concentration in the fish from the environment,[81] the PFAS present in the aquaculture fish still pose a concern to laboratory exposure experiments. Additionally, Guillette et al analyzed eleven PFAS, however, there are many more PFAS that could be present in fish from aquaculture farms. The background levels of PFAS in the stock organisms could confound PFAS bioaccumulation results or potentially cause interactions between another chemical being studied in a toxicity experiment, for example. Therefore, it is imperative to further investigate and understand the PFAS contamination in stock organisms from aquaculture facilities.

This study aims to understand the PFAS contamination and profile in aquatic tank materials, various brands of fish feed, and *P. promelas* obtained from several different aquaculture locations (Figure 5). *P. promelas* were chosen as they are currently a widely applicable model organism being studied for PFAS bioaccumulation in a collaborators laboratory. Therefore, these organisms are immediately relevant and the results can be applied widely.

2.2 Materials and Methods

2.2.1 Materials

Mixtures of native and mass labeled per- and poly-fluoroalkyl substances, PFAC-24PAR, MPFAC-24ES, and MPFAC-C-IS were obtained from Wellington Laboratories (Ontario, Canada). The PFAS in each mixture are detailed in Table 4.

Table 4. PFAS full names, abbreviations, and standard mixture association.

PFAS	Full Name	Mixture Name
PFBA	perfluorobutanoic acid	PFAC-24PAR
PFPeA	perfluoropentanoic acid	PFAC-24PAR
PFHxA	perfluorohexanoic acid	PFAC-24PAR
PFHpA	perfluoroheptanoic acid	PFAC-24PAR
PFOA	perfluorooctanoic acid	PFAC-24PAR
PFNA	perfluorononanoic acid	PFAC-24PAR
PFDA	perfluorodecanoic acid	PFAC-24PAR
PFUnA	perfluoroundecanoic acid	PFAC-24PAR
PFDoA	perfluorododecanoic acid	PFAC-24PAR
PFTriA	perfluorotridecanoic acid	PFAC-24PAR
PFTetA	perfluorotetradecanoic acid	PFAC-24PAR
PFBS	perfluorobutane sulfonate	PFAC-24PAR
PFPeS	perfluoropentane sulfonate	PFAC-24PAR
PFHxS	perfluorohexane sulfonate	PFAC-24PAR
PFHpS	perfluoroheptane sulfonate	PFAC-24PAR
PFOS	perfluorooctane sulfonate	PFAC-24PAR
PFNS	perfluorononane sulfonate	PFAC-24PAR
PFDS	perfluorodecane sulfonate	PFAC-24PAR
4:2 FTS	1H,1H,2H,2H-perfluoro-1-hexanesulfonate	PFAC-24PAR

6:2 FTS	1H,1H,2H,2H-perfluoro-1-octanesulfonate	PFAC-24PAR
8:2 FTS	1H,1H,2H,2H-perfluoro-1-decanesulfonate	PFAC-24PAR
NMeFOSAA	2-(N-methyl-perfluoro-1-octane-sulfonamido) acetic acid	PFAC-24PAR
NEtFOSAA	3-(N-ethyl-perfluoro-1-octane-sulfonamido) acetic acid	PFAC-24PAR
FOSA	perfluorooctane sulfonamide	PFAC-24PAR
13C4-PFBA	perfluoro-n-[13C4]butanoic acid	MPFAC-24ES
13C5-PFPeA	perfluoro-n-[1,2,3,4,5-13C5]pentanoic acid	MPFAC-24ES
13C5-PFHxA	perfluoro-n-[1,2,3,4-13C5]hexanoic acid	MPFAC-24ES
13C4-PFHpA	perfluoro-n-[1,2,3,4-13C4]heptanoic acid	MPFAC-24ES
13C8-PFOA	perfluoro-n-[13C8]octanoic acid	MPFAC-24ES
13C9-PFNA	perfluoro-n-[13C9]nonanoic acid	MPFAC-24ES
13C6-PFDA	perfluoro-n-[1,2,3,4,5,6-13C6]decanoic acid	MPFAC-24ES
13C7-PFUnA	perfluoro-n-[13C7]undecanoic acid	MPFAC-24ES
13C2-PFDoA	perfluoro-n-[1,2-13C2]dodecanoic acid	MPFAC-24ES
13C2-PFTetA	perfluoro-n-[1,2-13C2]tetradecanoic acid	MPFAC-24ES
13C3-PFBS	perfluoro-1-[2,3,4-13C3]butane sulfonate	MPFAC-24ES
13C3-PFHxS	sodium perfluoro-1-[1,2,3-13C3]hexane sulfonate	MPFAC-24ES
13C8-PFOS	perfluoro-n-[13C8]octane sulfonate	MPFAC-24ES
13C2- 4:2 FTS	1H,1H,2H,2H-perfluoro-1-[1,2-13C2]- hexane sulfonate	MPFAC-24ES

13C2- 6:2 FTS	1H,1H,2H,2H-perfluoro-1-[1,2-13C2]- octane sulfonate	MPFAC-24ES
13C2- 8:2 FTS	1H,1H,2H,2H-perfluoro-1-[1,2-13C2]- decane sulfonate	MPFAC-24ES
D3-NMeFOSAA	N-methyl-d3-perfluoro-1-octane-sulfonamidoacetic acid	MPFAC-24ES
D5- NEtFOSAA	N-ethyl-d5-perfluoro-1-octane-sulfonamideoacetic acid	MPFAC-24ES
13C8- FOSA	perfluoro-1-[13C8]octanesulfonamide	MPFAC-24ES
13C3-PFBA	perfluoro-n-[2,3,4-13C3]butanoic acid	MPFAC-C-IS
13C2-PFOA	perfluoro-n-[1,2-13C2]octanoic acid	MPFAC-C-IS
13C2-PFDA	perfluoro-n-[1,2-13C2]decanoic acid	MPFAC-C-IS
13C4-PFOS	perfluoro-n-[1,2,3,4-13C4]octane sulfonate	MPFAC-C-IS

Water used in mobile phase A was filtered in the laboratory using reverse osmosis filtration. Methanol (Optima grade, purity > 99.9%) and 0.22 µm glass fiber filters were purchased from Fisher Scientific (Hampton, NH, USA). Ammonium acetate (99.999%) and Supelclean ENVI-Carb were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Supelco (Bellefonte, PA, USA), respectively. SecurityGuard Standard guard columns (4 x 2 mm, C_{18}) and Gemini (3 µm, 50 x 2 mm, C_{18}) liquid chromatography columns were purchased from Phenomenex (Torrance, CA, USA).

Fish food was purchased based on common use in aquatic laboratory exposure experiments with *Pimephales promelas* or *Hyalella azteca* (Table 5). Several studies fed their aquatic organisms Tetramin Tropical Flakes,[82,83] freeze dried brine shrimp,[84–86] frozen brine shrimp,[84,87] and freeze dried bloodworms.[88] For this study, Tetramin Tropical Flakes were purchased from Tetra (Blacksburg, VA, USA). Each of the following were purchased from Brine Shrimp Direct (Ogden, UT, USA) and San Francisco Bay Brand (Newark, CA, USA): brine shrimp flakes, freeze dried brine shrimp, frozen brine shrimp,

and freeze dried bloodworms.

In addition to fish food, habitat materials used to house *P. promela* and *H. azteca* in a bioaccumulation study were also analyzed (Table 5). Standard screen, BetterVue, and aluminum screen materials for the Hyalella azteca were purchased from Phifer (Tuscaloosa, AL, USA). Hardware cloth was purchased from EverBilt (Home Depot, Atlanta, GA, USA). Two plastic plumbing fittings used in mesocosms, slip suction screen (½ inch) and standard threaded bulkhead (½ inch), were purchased from Lifegard Aquatics (Santa Fe Springs, CA, USA).

P. promelas and fish feed were collected from four different freshwater fish farms in Texas on November 20 and 21 of 2020. Additional minnows and feed were collected from a fish farm in Arkansas on May 29, 2021 and Minnesota on June 28, 2021 (Table 5). The sampling locations were chosen based on convenience, however, these aquaculture facilities have been used for toxicity studies in the past and often recommend their organisms for use in research laboratories.

Table 5. Sample count and type tested for contamination of laboratory experiment materials.

Material Type	Description	Number of Samples
Habitat Materials	Plastic Fittings - Polypropylene suction screen and bulkhead	2
Habitat Materials	Screens - stainless steel, coated, plastic	4
Feed	Flakes - tropical and brine shrimp	2
Feed	Freeze dried brine shrimp	2
Feed	Freeze dried bloodworms	2
Feed	Frozen brine shrimp	2
Aquaculture	Minnows and feed from TX	3
Aquaculture	Minnows and feed from AR	1
Aquaculture	Minnows and feed from MN	1

2.2.2 Methods

Extraction methods were adapted from previous methods in the Simcik lab and clean up was adapted from Powley.[64,89] Each fish food was homogenized using a mortar and pestle before 2 g was weighed into a 15 mL polypropylene centrifuge tube. Methanol (8 mL) and 19 mass labeled PFAS (MPFAC-ES, 24 ng) were added to each tube before extraction using a PRO 250 and a PRO SC-250 homogenizer and motor (Pro Scientific, Oxford, CT, USA) equipped with Multi-Gen adapter and a Multi-Gen 7XL generator probe at 30,000 rpm for 30 sec. The centrifuge tube was filled with methanol, capped, and inverted before samples were sonicated for 30 min at 38 ℃ then centrifuged at 4,200 RCF for 15 min. The supernatant was decanted into a new 15 mL polypropylene tube and placed under a gentle stream of nitrogen, in a warm water bath, until the volume in the centrifuge tube was about 2 mL. Activated carbon (ENVI-Carb, 10 mg) was added to each extract, before being vortexed for 20 sec. Each sample extract was filtered through a 0.22 μm glass fiber filter into an autosampler vial before being spiked with the extraction efficiency mass labeled PFAS mixture, $^{13}C_3$-PFBA (24 ng), $^{13}C_2$-PFOA (24 ng), $^{13}C_2$-PFDA (24 ng), and $^{13}C_4$-PFOS (22.964 ng).

The total oxidizable precursor (TOP) assay was initially attempted to determine the contribution of unknown precursors in the samples to the total PFAS, as developed previously by Houtz and Sedlak.[47] When this was performed, it was determined that the assay would need a substantial amount of oxidant in order to convert any PFAS precursors in addition to the competing oxidation of organic material from the fish matrix (i.e. tissue). Additionally, there appeared to be about equal total PFAS before and after TOP indicating limited precursor influence on the total PFAS composition in the feed. Finally, the presence of PFAS precursors is expected to be minimal considering there are likely mechanisms to oxidize and transform precursors to perfluoro carboxylic acids during metabolism by an organism, as described by Zhao et al.[90] Therefore, the TOP assay was not used to further account for unknown PFAS precursors in any of the samples studied.

For the aquatic tank materials, reverse osmosis water was added to each beaker containing each material, such as screens and plumbing fittings, until the top of the

material was covered. The samples were sonicated for 30 min at 38 ℃ before being decanted into 50 mL polypropylene centrifuge tubes and stored at 4 ℃ for future analysis. Methanol (150 mL) was then added to each beaker containing the habitat materials before being sonicated for 30 min at 38 ℃. The supernatant for each material was decanted into three 50 mL polypropylene centrifuge tubes, placed under a gentle stream of nitrogen in a warm water bath, and combined to a final volume of 2 mL. The centrifuge tubes were vortexed for 20 sec before the extract was spiked with the 19 mass labeled internal standard PFAS mixture (MPFAC-ES, 24 ng) and filtered through a 0.22 µm glass fiber filter into an autosampler vial for analysis.

The minnows were extracted similarly to the fish feed, however, the minnows were first homogenized in reverse osmosis water at 30,000 rpm using a PRO 250 and a PRO SC-250 homogenizer and motor (Pro Scientific, Oxford, CT, USA) equipped with Multi-Gen adapter and a Multi-Gen 7XL generator probe and then dried in an oven at approximately 80 ℃ until all of the water was removed from the sample. The wet weight and dry weight masses were recorded before adding the homogenate to a 15 mL polypropylene centrifuge tube with methanol. The aquaculture samples were then extracted in the same manner as the fish food. Additionally, $^{13}C_4$-PFOS (30 ng) was spiked in each sample before extraction to determine the extraction efficiency and the 19 mass labeled internal standard mixture (MPFAC-ES, 24 ng) was spiked in the autosampler vial after extraction, but prior to analysis. Extraction efficiencies were determined from method blanks spiked with 24 native PFAS (PFAC-24PAR, 24 ng) prior to extraction. Additional unspiked method blanks were extracted and run with each sample set.

Analyses were performed on a liquid chromatography tandem mass spectrometer (LC-MS/MS). For separation, an Agilent 1200 LC binary pump was used, in addition to an 1100 series column oven and autosampler, coupled to a SciEx API 4000 for quantification. Before each set of analyses, the source cover was removed and the curtain plate was cleaned. Additionally, the guard column (SecurityGuard Standard, 4 x 2 mm, C_{18}) was replaced before each run of 24 samples. A PFAS delay column (5 µm x 50 mm x 2.1 mm, Restek Corporation PA, USA) was utilized to account for any potential PFAS

contamination in the instrument or mobile phases. The Phenomenex Gemini (3 µm, 50 x 2 mm, C_{18}) analytical column was kept at 40°C throughout the entire run. A vial containing methanol was analyzed between every fish or fish food sample, but after every 3 standards or method blank samples. The injection volume was 5 µL. Mobile phase A was composed of 2 mM ammonium acetate in 90:10 reverse osmosis water/methanol and mobile phase B was composed of 2 mM ammonium acetate in 100% methanol. A flow rate of 0.2 mL/min was used for separation of PFAS, including branched and linear PFOS and PFHxS. The mobile phase gradient can be found in the table below (Table 6).

Table 6. Mobile phase gradient program used to elute PFAS where mobile phase A was composed of 2 mM ammonium acetate in 90:10 reverse osmosis water/optima methanol and mobile phase B was composed of 2 mM ammonium acetate in 100% optima methanol.

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0.0	100	0
0.1	55	45
15.0	55	45
15.5	0	100
20.5	0	100
21.0	100	0
29.0	100	0

Analyses were performed in negative ion mode, with a turbo spray ion source. Initial mass spectrometer conditions were set as follows: collision gas (4), curtain gas (20), ion source/gas (35), ion spray voltage (-4200 V), and temperature (400 ℃). The scan time was set at 0.5 sec to achieve at least 10 points across each peak. Scheduled Multiple Reaction Monitoring (MRM) windows were created based on Hunter et al 2010.[91] The expected retention time for each compound was entered with an MRM Detection Window of 800 sec. The Q1 mass, Q3 mass, Declustering Potential (DP), Collision Energy (CE), and expected retention time for each of the 43 perfluoroalkyl

substances are detailed here (Table 7).

Table 7. Native and mass labeled PFAS, the associated internal standard, expected retention time, precursor ion, quantifier ion, quantifier fragment, declustering potential, collision energy, and internal standard or extraction efficiency designation.

Analyte	Q1 Ion (m/z)	Q3 Ion (m/z)	Ret Time (min)	DP	CE	Internal Standard	Extraction Efficiency Standard
PFBA	213.055	169	6.09	-25	-12	13C4-PFBA	13C3-PFBA
PFPEA	262.947	218.8	7.31	-25	-12	13C5-PFPEA	
42FTS	327.015	307	8.7	-30	-25	13C2-4:2FTS	
PFHxA	313.018	269	8.91	-35	-14	13C5-PFHxA	
PFBS	298.94	80	7.46	-50	-52	13C3-PFBS	
PFHpA	362.996	319.1	11.01	-40	-14	13C4-PFHpA	
PFPeS	348.927	79.9	9.06	-80	-68	13C3-PFHxS	
62FTS	426.936	407	12.41	-90	-34	13C2-6:2FTS	
PFOA	412.895	369.1	12.48	-45	-14	13C8-PFOA	13C2-PFOA
PFHxS	398.914	79.8	11.07	-80	-60	13C3-PFHxS	
PFNA	462.949	418.8	13.74	-50	-14	13C9-PFNA	
82FTS	526.961	506.8	14.95	-90	-40	13C2-8:2FTS	
PFHpS	448.831	80	12.48	-100	-62	13C8-PFOS	
PFDA	512.837	469	14.94	-15	-13	13C6-PFDA	13C2-PFDA
PFOS	498.885	80	13.71	-90	-86	13C8-PFOS	13C4-PFOS

PFUnA	562.906	518.9	15.87	-60	-16	13C7-PFUnA
PFNS	548.883	80	14.87	-120	-62	13C8-PFOS
PFDoA	612.92	568.9	16.74	-65	-18	13C2-PFDoA
NMeFOSAA	569.895	418.9	15.44	-90	-30	D3-NMeFOSAA
PFDS	598.895	79.9	15.8	-110	-66	13C8-PFOS
NEtFOSAA	583.9	418.9	15.88	-85	-28	D5-NEtFOSAA
PFTriA	663.014	618.9	17.95	-75	-18	13C2-PFTetA
PFTetA	712.757	670	20.53	-70	-22	13C2-PFTetA
FOSA	498.023	77.9	14.81	-90	-66	13C8-FOSA
PFOS (qual)	498.885	99.1	13.71	-90	-86	13C8-PFOS

Mass labeled internal standards were used to quantify the PFAS in each of the calibration standards and samples (Table 7). In the event that the mass labeled PFAS standard was not available, the mass labeled standard that was used to quantify a sample was the mass labeled standard that provided a relative response factor closest to one.

In order to effectively calculate the concentration of each PFAS in the sample, calibration curves for each PFAS were created at the following concentrations: 0, 2.4, 4.8, 9.6, 16.8, 33.6, and 60 ng/mL. In order to use these calibration curves for quantification, the r^2 for each curve was greater than or equal to 0.99 for either linear or polynomial (n=2) fit. The equation of the polynomial or linear line was used to determine the mass of PFAS in each sample. In cases where the intercept of the line was negative, the intercept was set to zero to be able to accurately quantify the analyte concentrations.

2.3 Quality Assurance and Control

Quality assurance and control measures outlined in Table B-15 the Department of Defense Quality Systems Manual (version 5.3) were followed when applicable. To determine the extraction efficiency of each PFAS, $^{13}C_4$ PFOS, $^{13}C_3$ PFBA, $^{13}C_{10}$ PFDA, and $^{13}C_2$ PFOA, were spiked in each sample (24 ng) just before analysis. Additionally, method blanks, method blank spike (24 ng), and matrix spikes (24 ng) were run with each sample set. Recoveries for the extraction of *Pimephales promelas* are all within 70% and 130% (Table 8).

Instrument detection limits (IDLs) were determined using an eleven point calibration curve ranging from 0 ng/mL to 120 ng/mL. The analyte response was plotted against the mass injected. The IDL for each PFAS was chosen as the lowest mass injected (pg) where the response was lower than the previous, higher concentration standard. IDLs ranged from 0.2244 to 4.8 ng/mL (Table 8). Method detection limits (MDLs) were determined using Title 40 Code of Federal Regulations Appendix B to Part 136 Definition and Procedure for the Determination of the Method Detection Limit.[92] MDLs ranged from 0.086 to 15.063 ng/g in *Pimephales promelas* homogenate (Table 8).

Table 8. Instrument detection limits, method detection limits, solvent, and matrix (using *P. promelas*) recoveries for 24 PFAS.

	IDLs (pg injected)	**MDL (ng/g)**	**Solvent Recovery (%)**	**Matrix Recovery (%)**
PFBA	4.8	0.675	89.18%	93.32%
PFPeA	2.4	0.096	79.04%	86.04%
PFHxA	2.4	0.404	103.48%	93.20%
PFHpA	4.8	0.486	83.83%	80.36%
PFOA	12	15.063	89.02%	114.12%
PFNA	4.8	0.095	84.82%	73.41%
PFDA	4.8	0.827	89.78%	82.02%
PFUnA	4.8	0.454	107.28%	86.33%
PFDoA	4.8	0.442	94.08%	96.83%
PFTriA	4.8	1.156	94.51%	85.68%
PFTetA	4.8	0.086	94.74%	106.75%
PFBS	10.62	0.779	101.41%	92.75%
PFPeS	2.256	0.825	81.94%	126.20%
PFHxS	10.944	0.449	99.72%	91.60%
PFHpS	11.4	2.246	109.28%	81.32%
PFOS	11.106	0.185	101.65%	92.66%
PFNS	11.52	0.909	90.49%	88.64%

PFDS	11.58	0.573	97.20%	91.95%
4:2 FTS	2.244	0.679	84.78%	84.47%
6:2 FTS	11.14	0.606	74.92%	70.98%
8:2 FTS	23.04	1.003	78.57%	87.65%
NMeFOSAA	48	1.280	87.18%	72.38%
NEtFOSAA	48	0.212	94.92%	77.12%
FOSA	4.8	0.641	72.56%	78.12%

To ensure the accuracy of the extraction and analysis methods, Standard Reference Materials were extracted and analyzed from the National Institute of Standards and Technology. Lake Michigan and Lake Superior fish tissue samples were extracted and found to have PFOS within 15.35% and 25.61% of the nominal values provided by NIST in the 1946 and 1947 SRMs, respectively.

Extraction efficiency was ensured to be between 70% and 130% for all samples (Figure 8). In addition, a laboratory control sample was run once per sample set and was created as a blank spiked with all analytes at about 24 ng/mL. A matrix spike was also prepared before extraction and processed with each sample set. In addition, branched and linear PFOS and PFHxS were included in the calibration standards. Two ion transitions (quantifier and qualifier) were determined for PFOS and the quantifier to qualifier ratio was within three standard deviations of the ratios observed in the mid concentration calibration standards.

The retention times of each analyte fall within 0.4 minutes of the predicted retention time (based on the daily calibration curve) and the matching mass labeled internal standard must fall within 0.1 minutes of the native PFAS. Instrument blanks with mass labeled internal standards are run after the highest calibration standard with each sample set to determine the background concentration of PFAS.

2.4 Results and Discussion

2.4.1 Habitat Materials

To understand how habitat material might confound bioaccumulation studies, materials were chosen based on current use for toxicity and bioaccumulation experiments. This would allow for the determination of habitat PFAS contamination in those measurements further demonstrating the possibility for background PFAS contamination to alter the bioaccumulation results. Therefore, it is important to carefully ensure that the environment is PFAS free before performing laboratory exposure experiments. Habitat materials were chosen based on the type of material used in toxicity and bioaccumulation experiments performed by collaborators working under the same grant. Several plastic tank fittings, ½" slip suction screen and ½" standard threaded bulkhead, and four different screen types were extracted in methanol and analyzed for PFAS contamination prior to the toxicity and bioaccumulation experiments to ensure that any resulting bioaccumulation parameters would not be influenced or confounded by background PFAS contamination in the habitat materials.

The ½" slip suction screen had no PFAS contamination, while the ½" standard threaded bulkhead had a total mass of 1.16 ng and 5.49 ng for PFHxA and PFHxS, respectively (Figure 6). The total mass extracted for all PFAS in the standard threaded bulkhead was found to be 6.64 ng. While this mass is relatively small, any additional PFAS exposure could influence the results of bioaccumulation experiments, especially if those experiments are exposing their organisms to concentrations around the level of contamination.

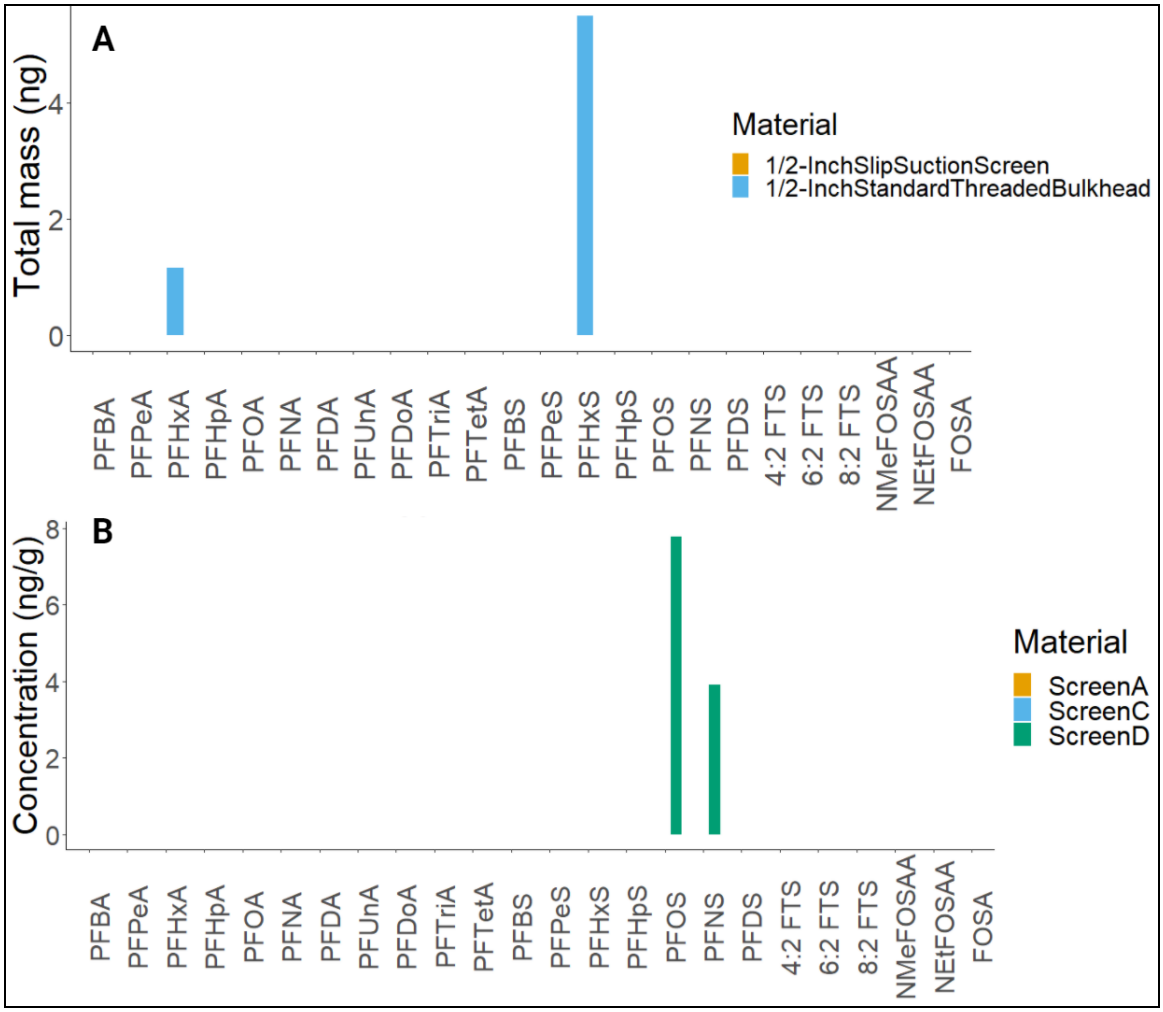

Figure 6. Total mass (ng) of a suite of 24 PFAS present in two polypropylene tank fittings (a) and concentrations of PFAS (ng/g) three screens (b).

The four types of screens were provided by a collaborator currently using those screens in bioaccumulation studies with *H. azteca*. The screens used varied in both size, color, and material. Based on physical appearance, two of the screen types were metal while the other two were some sort of mesh or coated metal screens. Screen B was a section of metal screen that had been cut out in a square, however, upon receiving this sample, there was evidence of marker on the edges that was likely used to draw on the screen before it was cut out. Therefore, when placed in methanol, the extract turned purple and the extract was challenging to analyze. Additionally, Rodowa et al has shown that PFAS can be found in different permanent marker brands (although there are few

ways for this to contaminate samples in the field).[78] Therefore, screen B was excluded from the analysis.

Of the remaining three screen samples, screen D was the only screen containing PFAS above the detection limits. Screen D had a total PFAS concentration of 11.68 ng/g (Figure 6). The composition of PFAS in screen D was 7.77 ng/g of PFOS and 3.90 ng/g of PFNS. This contamination is larger than the total mass extracted from the plastic suction screen and bulkhead fittings. Additionally, these screens are used to feed *H. azteca*, therefore, the PFAS contamination likely poses a larger threat because the organisms are directly interacting and feeding off of the screens.

In a subsequent aqueous extraction (using PFAS free reverse osmosis water), no PFAS were found for any screen type above the limit of detection. Therefore, PFAS contamination can effectively be eliminated from laboratory materials by rinsing the materials with methanol, followed by PFAS free water, before introducing them to organisms in laboratory experiments. However, some materials cannot be simply rinsed with methanol to prevent PFAS contamination in bioaccumulations or toxicity experiments, such as fish feed or the stock organisms themselves.

While habitat materials, that can leach PFAS and confound bioaccumulation studies, can be rinsed with methanol to reduce and prevent PFAS exposure, other materials such as fish feed or the stock organism themselves cannot be rinsed in methanol before use. Therefore, it is important to understand the extent of PFAS contamination coming from these sources in a different way.

2.4.2 Fish Feed

To ensure accurate bioaccumulation results, it is necessary to understand how much, if any, PFAS contamination comes from feed used in these studies. Considering organisms need food in order to be used in toxicity or bioaccumulation experiments, this feed should be investigated for potential contamination. This contamination in feed could unknowingly influence bioaccumulation parameters and toxicity results. To address these questions, four types of fish feed were acquired from three different brands, totaling eight different fish feed samples. The feeds chosen were based on commonly used fish feed in

laboratory experiments and the EPA method for rearing *P. promelas* (as described in *Section 3.3.2*),[82–88] and include flakes (generally made of brine shrimp), freeze dried brine shrimp, freeze dried bloodworms, and frozen brine shrimp. Of the flakes that were tested, food brand B was the only one that was contaminated with PFAS (Figure 7). PFNA was the sole contaminant in brand B flakes at a concentration of 1.25 ± 0.39 ng/g. While low contamination may have been expected, it was unknown whether there would be significant contributions from the seemingly more processed nature of the fish flakes. Since it is still unclear how PFAS bioaccumulate, repeated low level exposure could bioaccumulate and add up to a larger effect in the organism, especially in smaller organisms such as *P. promelas* and *H. azteca*.

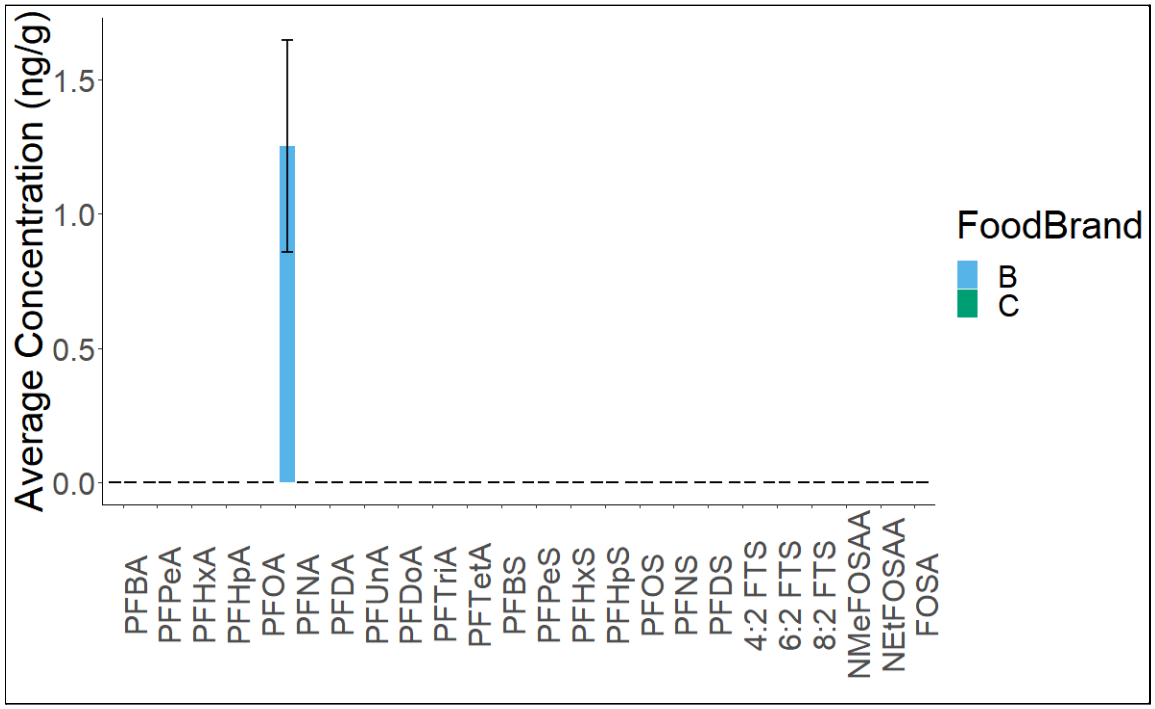

Figure 7. The PFAS composition and concentration (ng/g) profile in two brands of fish flakes where the error bars represent one standard deviation of triplicate extraction.

Since the flake food had little PFAS contamination, another type of fish food was investigated for contamination: freeze dried brine shrimp. Of the two brands analyzed, both were contaminated with PFAS. The total PFAS present for each brand was 6.42 ng/g and 12.63 ng/g for brand A and B, respectively. The largest PFAS contributors were

PFOS for brand A, and PFHpA for brand B (Figure 8). The PFOS contamination was 3.08 ng/g and 2.59 ng/g for brand A and B. Additionally, the percent composition of PFOS for brand A and B were 70% and 88% linear, respectively.

Given that aquatic organisms preferentially uptake the linear isomer of PFOS,[93] the relatively low percent composition of linear PFOS in the freeze dried brine shrimp indicates that there is an additional source of PFAS contributing to this contamination. When PFOS is manufactured through a process called electrochemical fluorination, 70% of the PFOS is linear while 30% of the PFOS created is branched.[94] While brand B has an enriched linear percent composition (88%) compared to the manufactured percent composition, brand A has a percent composition that nearly matches the manufactured percent linear composition. It is possible that the brine shrimp, before being freeze dried, were exposed to a different more branched source, such as the water in the facility where they were raised. However, a small enrichment of the linear isomer (>70%) in the brine shrimp would still be expected to occur given the uptake processes that occur in aquatic organisms.[93] Therefore, this branched PFOS contribution is likely from another source. Specifically, it is possible that this contamination was introduced somewhere in the freeze drying process, although there have been reports that freeze drying does not contribute to PFAS contamination.[95]

Another possibility would be that the higher branched PFOS composition and contamination may come from the packaging process considering previous studies have found many packaging materials have measurable concentrations of PFAS.[96,97] To further study this point, the container seals were rinsed with methanol to determine if PFAS from these materials could be contributing to the higher percentage of branched PFOS contamination observed in the freeze dried brine shrimp. PFAS contributions to the fish feed from the seal were determined to be minimal considering most PFAS were at or below the detection limits and any PFAS that were found were below 4 ng total. This is not considered to be a concern because a) it is unclear whether the small amount of PFAS in the seal was from the feed residue on the seal or the seal itself, and b) the seal was extracted with methanol which could overestimate the actual contamination to the fish feed considering PFAS have a higher affinity for methanol over sorption to a solid (i.e.

the fish food).[98] Since the branched PFOS contamination is likely not a result of the brine shrimp preferential bioaccumulation, freeze drying process, or packaging materials, the further source of this contamination cannot be speculated on at this time. However, the overall contamination of PFOS in either brand of freeze dried brine shrimp is fairly low, therefore, it is possible that there is more variability in the measurement at a low concentration. Branched and linear isomer profiles for PFOS in freeze dried brine shrimp should be further confirmed in future studies.

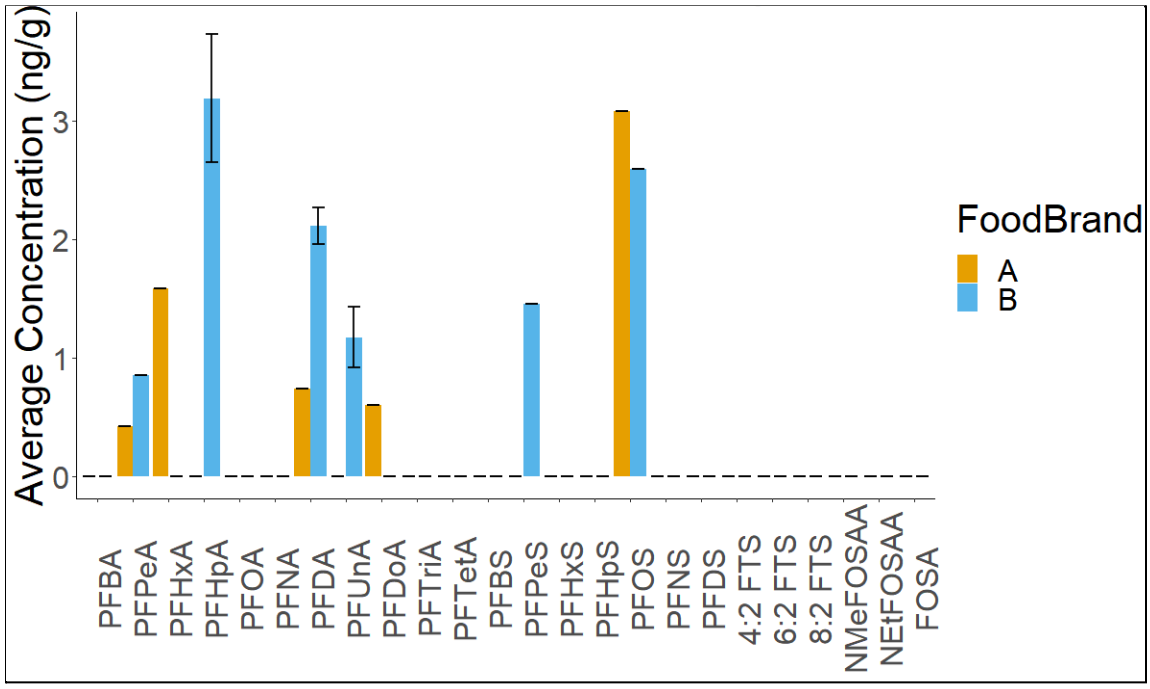

Figure 8. The average concentration (ng/g) and profile of PFAS present in two brands of freeze dried brine shrimp where the error bars represent one standard deviation of triplicate extraction.

The freeze dried bloodworms were examined next for PFAS contamination. The total PFAS present in freeze dried bloodworms brand A was 28.34 ng/g and 5.06 ng/g in brand B, respectively (Figure 9). The PFOA present in brand B was 1.62 ng/g, which is fairly low, but still important considering organisms can readily bioaccumulate PFOA.[99,100] While brand B had no PFOS above the limit of detection, brand A was significantly contaminated with PFOS at a concentration of 23.31 ng/g. The percent composition of PFOS was 91% linear. This highly linear percent composition is

consistent with organisms preferential uptake of linear isomers compared to the more mobile branched isomer.[93] The PFOS contamination could be a result of the processing of the fish feed, however, several sources have indicated limited PFAS contamination from that process.[95] Additionally, if that were the case, there would likely be similar PFOS contamination across brands that have been freeze dried and that is not observed in these results. Freeze dried bloodworms can be very contaminated with linear PFOS, however, this contamination is variable between brands of freeze dried bloodworms.

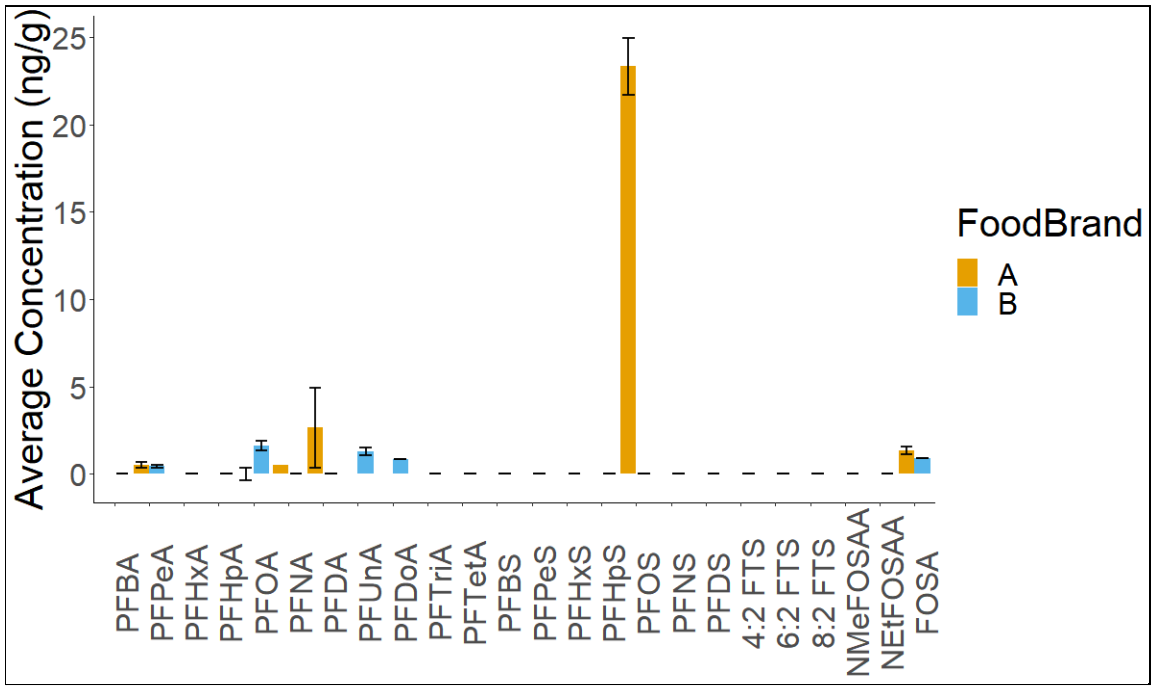

Figure 9. The average PFAS concentration (ng/g) found in two brands of freeze dried bloodworms where the error bars represent a single standard deviation.

Another common type of fish feed is frozen brine shrimp. While the other feeds investigated in this study were dry and kept at room temperature, the frozen brine shrimp was kept frozen and appeared to have a larger water content than other types, like freeze dried brine shrimp for example. Frozen brine shrimp are recommended by the EPA for maintaining *P. promelas* for many laboratory based exposure studies,[84] therefore, it may be hypothesized that the frozen brine shrimp would be the least contaminated feed. However, of two frozen brine shrimp brands sampled, the total PFAS present was 21.19

ng/g and 16.95 ng/g for brand A and B, respectively (Figure 10). Interestingly, there were no perfluorosulfonates contaminating this brand of feed, considering PFOS is a large contributor to PFAS contamination in freeze dried bloodworms. Instead, perfluorocarboxylates made up all of the contamination in frozen brine shrimp. PFOA was the largest contaminant in brand B at 13.86 ng/g, while PFDoA was the highest concentration contaminant in brand A at 8.10 ng/g. The differences between the two brands is unknown, however, some factors that could influence the contamination could be the container materials or the source of brine shrimp to the food manufacturers. The packaging for both brands of frozen brine shrimp was different than that of the other brands and had a foil cover over an array of small plastic squares, each holding a small cube of frozen brine shrimp. There could be PFAS coating on any of these materials, as is common in other food packaging materials.[96] However, as previously mentioned, when the container seals were analyzed for PFAS, it was concluded that a minimal amount of PFAS from the container seal contributed to the PFAS observed in the fish feed. Therefore, the source of frozen brine shrimp could account for the differences between brands as one brand likely obtains shrimp from a large lake nearby their facility while the other brand likely obtains shrimp from nearby coastal waters. The brine shrimp could be exposed to different water sources before being processed and frozen for fish food, potentially accounting for the differences between brands. Regardless, the PFAS contamination present in these common brands of frozen brine shrimp is alarming considering many laboratories rear *P. promela* according to the EPA guidelines, therefore, the toxicity or bioaccumulation results from these studies could be confounded by underlying PFAS that may potentially interact with chemicals being studied. Unknown PFAS contamination in toxicity and bioaccumulation studies could undermine many existing and future results, which are often the basis for regulation and assessment of these contaminants.

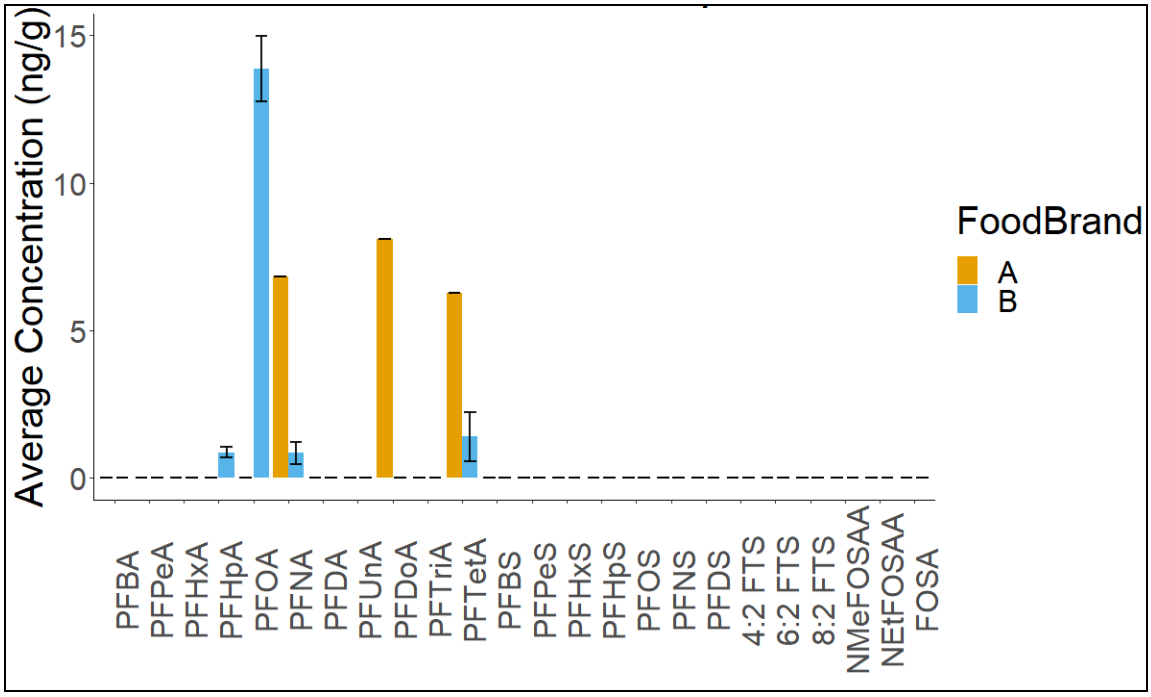

Figure 10. The average PFAS concentration (ng/g) found in two brands of freeze dried bloodworms where the error bars represent a single standard deviation.

From the four types of fish food studied, the most common contaminants were PFNA, PFDoA, PFOS, and PFOA. The average concentration across all types of fish feed were as follows: 1.18 ng/g for PFNA, 1.94 ng/g for PFOA, 1.19 ng/g for PFDoA, and 3.62 ng/g for PFOS (Figure 11). Frozen brine shrimp had the highest concentrations of PFOA, PFNA, and PFDoA for all of the types. However, freeze dried bloodworms were the most contaminated with PFOS at 23.31 ng/g. PFOA and PFOS contamination is perhaps unsurprising considering these two PFAS were manufactured the most and found at high concentrations in many environmental media such as soil and water.[101–103] The PFNA and PFDoA contamination is supported by previous studies that have found the longer chain perfluorinated carboxylates tend to have higher bioconcentration factors.[104] This PFAS profile of contamination is consistent with the overall conclusion that the most often contaminants of fish feed are the perfluorinated carboxylates and PFOS.

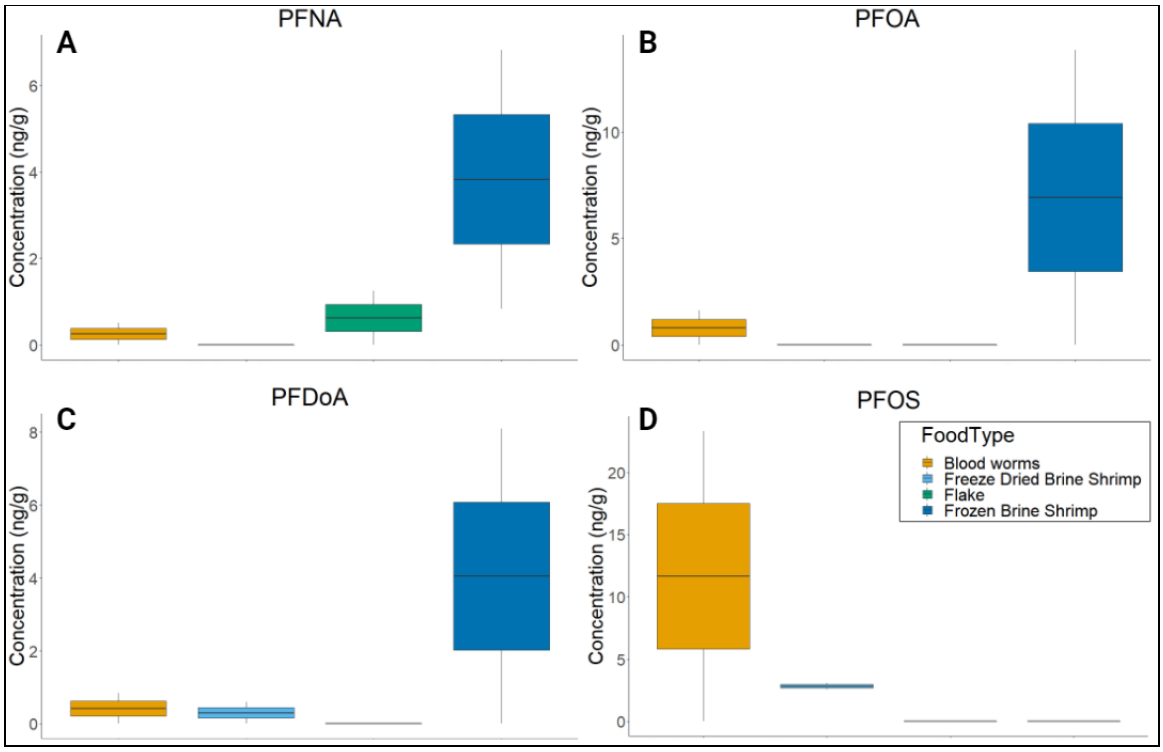

Figure 11. The concentrations (ng/g) of PFNA (a), PFOA (b), PFDoA (c), and PFOS (d) in two brands of freeze dried bloodworms, freeze dried brine shrimp, flakes, and frozen brine shrimp.

Comparisons can be made between the PFAS composition of each type of fish feed. Frozen brine shrimp were the most contaminated with PFAS at a total concentration of 38.14 ng/g which was 36% PFOA (Figure 12). Freeze dried bloodworms were the next most contaminated fish feed with total PFAS at 33.40 ng/g which was 70% PFOS. Interestingly, freeze dried bloodworms also had a small concentration of FOSA present, whereas no other fish feed had FOSA, any of the fluorotelomer sulfonates, or the sulfonamido acids present. Given this profile for freeze dried bloodworms, the large contribution of PFOS may be a result of FOSA biotransformation and breakdown. Houtz and Sedlak have demonstrated that under oxidative conditions, FOSA can be transformed into PFOS.[47] Additionally, there are metabolic processes that have been demonstrated in organisms to biotransform FOSA to PFOS.[90] This information suggests that the higher concentration of PFOS and lower concentration of FOSA in freeze dried bloodworms

could be a result of biotransformation of a larger concentration of FOSA in the bloodworms before they were freeze dried and processed for fish feed.

The next highest contaminated fish feed was the freeze dried brine shrimp at 17.80 ng/g which was 15% PFOS. Surprisingly, the fish flakes were the least contaminated and only had PFNA at 1.25 ng/g. The flakes were often brightly colored and more homogenous than the other types of fish feed, therefore, they were thought to be potentially more processed than the other types of feed leading to more PFAS contamination; however, significant PFAS contamination was not observed for either brand of fish flakes. It is unknown why the flake feed is less contaminated with PFAS, however, it is likely the parent material (i.e. the fish meal) was simply less contaminated or from a different source.

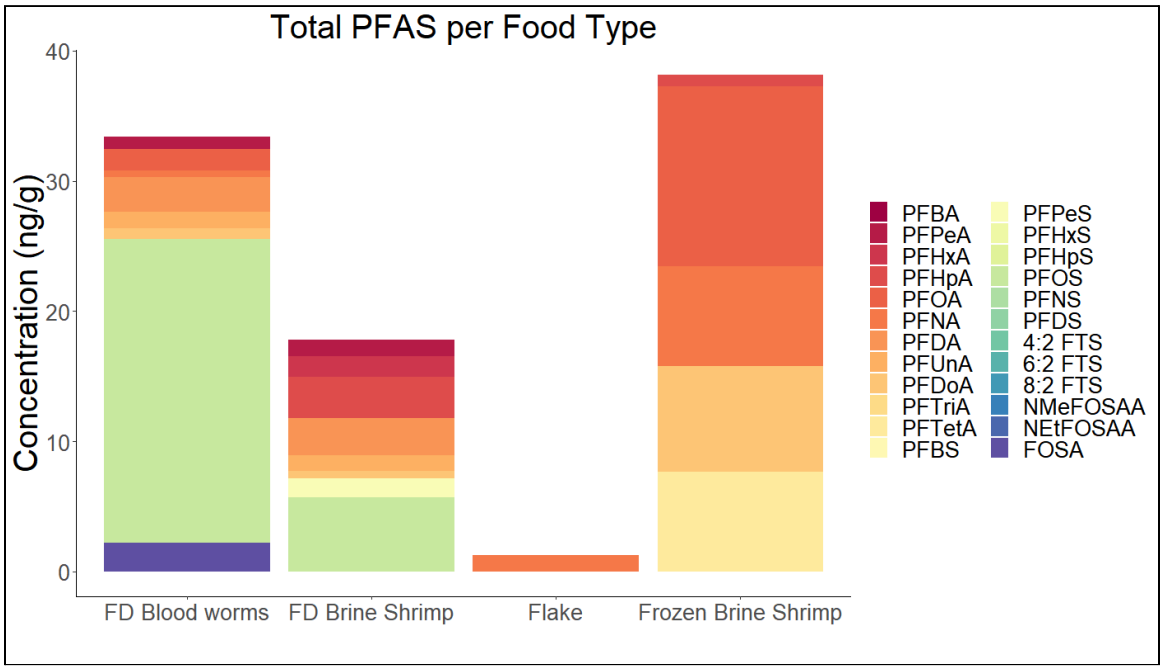

Figure 12. Total PFAS concentration (ng/g) and composition for each type of feed sampled.

The PFAS composition (i.e. the type of PFAS present) looked similar across types of fish feed which consisted of short and mid chain length perfluorinated carboxylates along with PFOS. Only one type, freeze dried bloodworms, had any precursors that were extracted and analyzed for, however, this fish feed also had high concentrations of PFOS

suggesting a potential transformation process from FOSA, which is a precursor, to PFOS. Frozen brine shrimp had the highest concentration of total PFAS which is particularly alarming given its recommendation for raising *P. promelas*. Additionally, the frozen brine shrimp appeared to have a larger water component than other types of dried fish feed. Considering the main environment for PFAS, especially shorter chain carboxylic acids, contamination is water (surface, drinking, etc),[101,103,104] the water present in the frozen brine shrimp could be contributing to the high concentration of PFAS in the sample. This hypothesis is consistent with the results shown in Figure 12 and Figure 10 because the composition of the frozen brine shrimp contamination is mostly short chain perfluorinated carboxylates which have been proven to be more mobile and often contaminate water sources.[105] In short, PFAS contamination varies greatly from 1.25 ng/g to 38.17 ng/g and is dependent on both fish feed type and brand.

Given that fish feed can be highly contaminated with PFAS, organisms fed this food could bioaccumulate PFAS unknowingly. This is of particular importance for bioaccumulation and toxicity studies, however, aquaculture facilities that raise organisms for laboratory experiments must also consider potential PFAS accumulation in organisms as a result of contaminated fish feed. If PFAS can be found in fish feed, then it may also be found in organisms fed this feed, such as in aquaculture facilities, therefore, it is critical to understand if stock organisms, which need to be PFAS free, are contaminated with PFAS as a result of feed or habitat sources.

2.4.3 Aquaculture Minnows

Laboratory toxicity and accumulation studies need to ensure that their testing materials are PFAS free, whether that be in the laboratory supplies, aquatic tanks, feed, or even the stock organisms themselves. Considering the findings that multiple types of fish feed are contaminated with PFAS, fish that eat this food may accumulate PFAS. This is especially important for aquaculture facilities that provide aquatic organisms, such as *P. promelas*, to laboratories for toxicity and bioaccumulation studies because those studies need to ensure that the organisms are PFAS free to avoid any potential interaction between chemicals being studied (these could be PFAS or another chemical) and PFAS,

themselves, that could confound toxicity or bioaccumulation results. Therefore, it is crucial to understand potential PFAS contamination and sources to aquaculture *P. promelas*.

To understand this, five fish farms were sampled for *P. promelas*, three of which were located in Texas (farms A, B, and C), one was located in Arkansas (farm D), and one farm that was located in Minnesota (farm E). Of these farms, farm A had the largest PFAS contamination with a PFOS concentration of 166.08 ng/g (Figure 13). The percent composition of PFOS was 94 % linear. Farms C and E had small PFOS contributions at 3.19 ng/g and 4.14 ng/g. The percent composition of PFOS for each farm was 97% linear and 91% linear for farms C and E, respectively. There are a number of other acids and sulfonates that were found in the minnows at concentrations much lower than for that of PFOS.

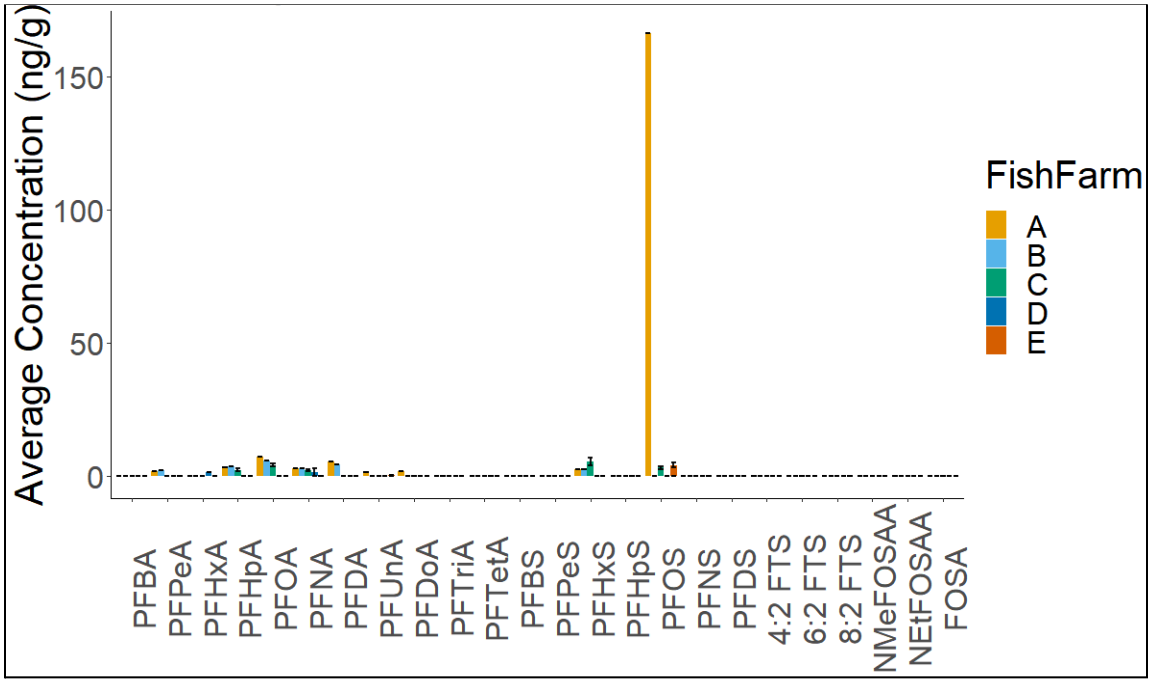

Figure 13. The PFAS concentration (ng/g) found in five aquaculture farm fathead minnow samples where the error bars represent a single standard deviation.

When PFOS is removed from the data, the contributions from other PFAS can more easily be observed (Figure 14). There are more perfluorinated carboxylates present as well as PFHxS at a lower concentration compared to PFOS. Farms A, B, and C have

PFHxS present at 2.57 ng/g, 2.55 ng/g, and 5.44 ng/g, respectively. All three farms contaminated with PFHxS had 90% or greater composition of the linear isomer. Of the PFAS that were analyzed for, no long chain carboxylates or precursors were found. The lack of these specific compounds makes sense when considering the current hypotheses in the field about how PFAS uptake and accumulate; specifically, that when ingested by an organism, many precursors can be biotransformed and that long chain acids may not bioaccumulate due to their large size, and therefore, possibly from a lack of gill membrane permeation.[26,90] The PFAS profile observed for the farms is consistent with what would be expected based on the PFAS profile seen in the fish feed considering the main contaminants in the feed were short chain carboxylates and PFOS. This would indicate that the feed may play a larger role in the PFAS accumulation of *P. promelas* in aquaculture farms.

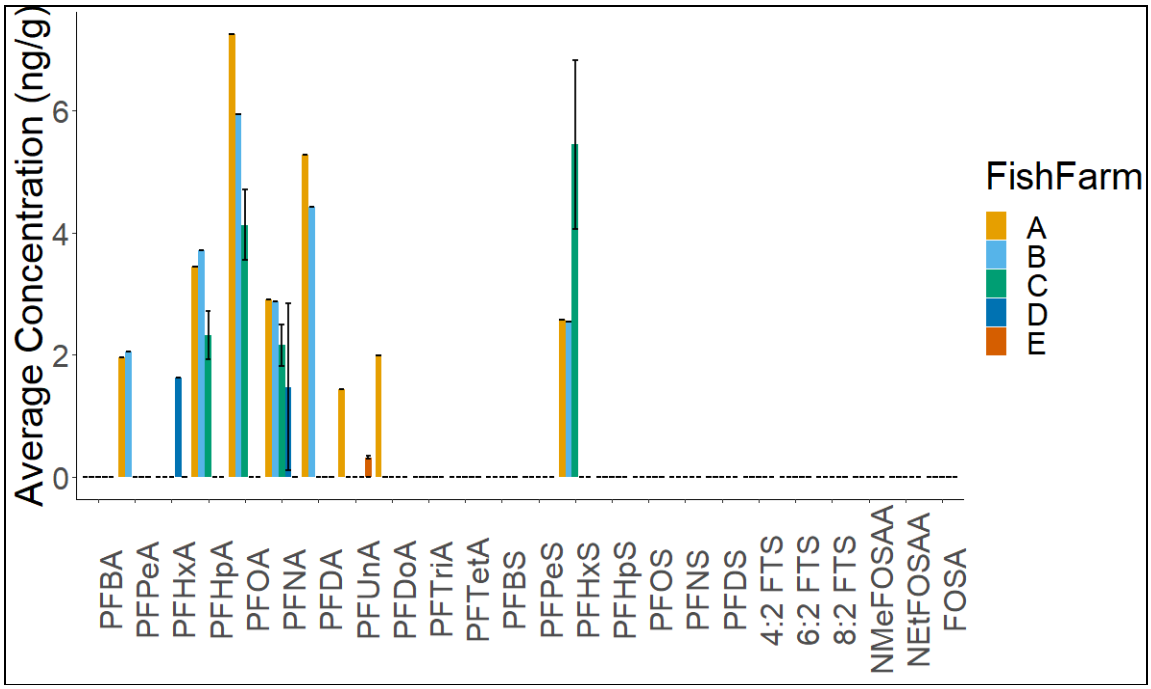

Figure 14. The PFAS concentration (ng/g) found in five aquaculture farm fathead minnow samples where the error bars represent a single standard deviation with PFOS removed.

While farms A, B, C, and D did not differentiate between sexes of the fish that

were provided, farm E provided and separated female and male *P. promelas*. As a result, PFAS contamination between sexes of *P. promelas* could be studied. However, given the overall low concentration of PFAS in minnows from farm E, few conclusions could be drawn. Female minnows from farm E were only contaminated with PFOS at a concentration of 4.14 ng/g, while male minnows from the same farm were only contaminated with PFUnA at a concentration of 0.32 ng/g. Given the small sample size (n = 8 per sex) and the overall low concentration, these results should be considered as preliminary with differentiation of PFAS contamination between sex of minnows requiring further study.

The total PFAS profile for each farm varies, however, there are some similarities between farms. For most farms, short and mid chain length carboxylates are present in addition to PFOS and PFHxS, in some cases (Figure 15). The total PFAS contamination in the *P. promelas* at each farm is as follows: 192.90 ng/g for farm A, 21.54 ng/g for farm B, 17.24 ng/g for farm C, 3.11 ng/g for farm D, and 4.46 ng/g for farm E. Guillette et al found a total concentration of PFAS between 9 and 13 ng/mL of serum in bass from an aquaculture facility.[81] This study supports the findings of PFAS contamination in farms B, C, D, and E, however, farm A lies considerably outside of this range. Interestingly, and perhaps surprisingly, farm D is the distributor and provides *P. promelas* to farms A, B, and C. Farms B and C have a similar total concentration, which is larger than the distributor (farm D), however, farm A has a much higher total concentration of PFAS which is mostly made of PFOS. Considering that farms A, B, and C source their minnows from the same distributor, the large PFAS contamination in minnows from farm A likely has another source.

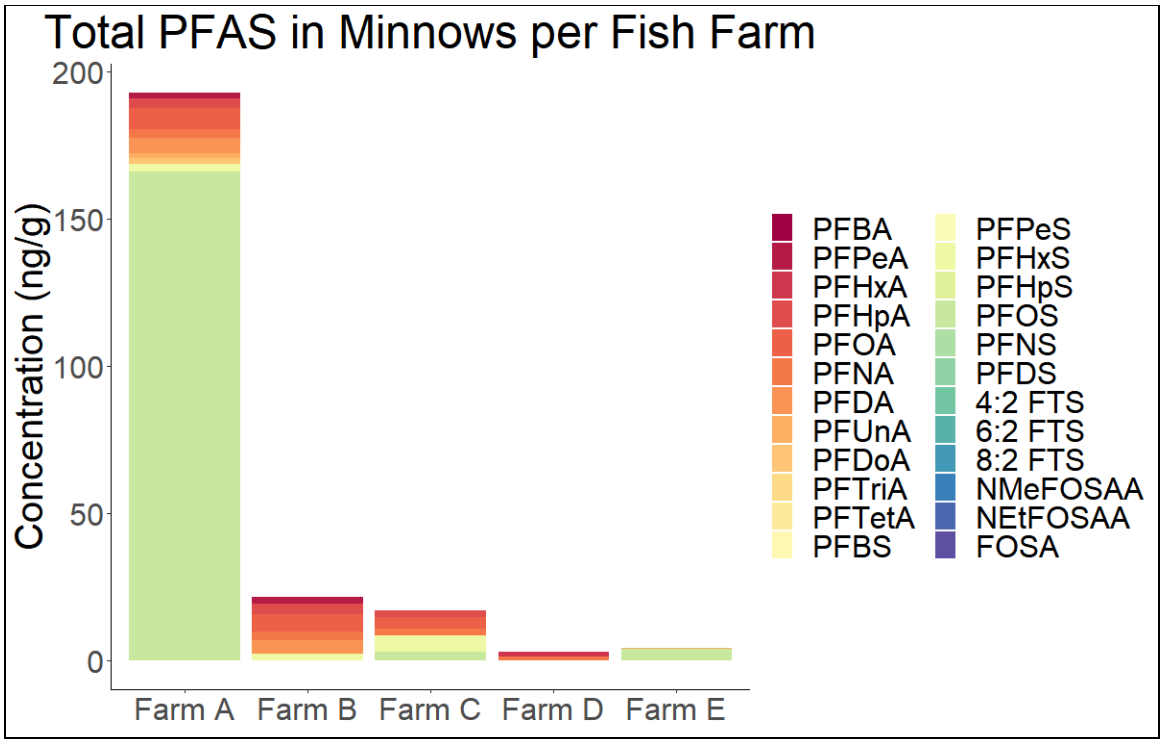

Figure 15. Total PFAS concentration (ng/g) and composition for each aquaculture farm sampled.

Given the findings that fish feed can be contaminated with a variety of PFAS (*Section 3.5.2*) the fish at aquaculture farms may be accumulating PFAS from contaminated feed at the farms. When provided the *P. promelas* from each aquaculture farm, samples of feed were also collected for analysis. Interestingly, the feed from farms A, C, D, and E had no PFAS above the detection limit and farm B had very low levels of contamination with a total PFAS concentration of 1.36 ng/g. Therefore, fish feed is thought to play a minimal role in PFAS bioaccumulation for the aquaculture farms sampled in this study. It is possible that this contamination comes from another source, such as the habitat materials or the aquaculture water.

The discrepancy between the high concentration of PFOS in *P. promelas* from farm A compared to PFOS present in *P. promelas* from farms C and E requires further examination. This large difference indicates that the *P. promelas* at aquaculture farm A are being exposed to additional PFAS compared to the *P. promelas* at farms C and E. Differences between aquaculture facilities could be in the water source that is used.

Depending on where the facility sources its water, or if there are PTFE pipes or other fluoropolymer applications, the water could be contaminated with PFAS. While water samples were not collected from any aquaculture farms, future research should investigate the PFAS contamination in the water to conclude where this additional PFAS contamination is coming from. Additionally, the isomer composition in the water may indicate the age of the contamination considering Schulz et al have shown that over time the linear PFOS that is in water will preferentially sorb to solids and be uptaken by organisms while the branched isomer becomes enriched in the water.[106] Overall, the source of the PFOS contamination in the *P. promelas* from farm A cannot be further speculated on at this time, however, there is likely a source coming from the habitat in the aquaculture facility.

Organisms from aquaculture facilities are no exception to PFAS contamination. Total PFAS concentrations range from 3.11 ng/g to 192.90 ng/g with PFOS making up a large portion of the total contamination of PFAS. While similarities exist between the PFAS contamination profile for *P. promelas* at different aquaculture facilities, the total concentration varies widely. Ultimately, the background PFAS contamination from aquaculture farms in stock organisms can greatly influence toxicity and bioaccumulation results from laboratories that source their organisms from these farms and, therefore, it needs to be considered when designing these studies.

2.5 Limitations

There are a number of limitations that should be weighed when considering this study. First, the habitat materials were chosen to provide estimates of PFAS contamination for a specific project and represent a small subset of possible aquatic tank accessories that can be used. Additionally, methanol rinsing materials may change the properties of the habitat materials with risk of possible degradation depending on the material type. This could potentially lead to chemical sorption in exposure studies, therefore, care should be taken when considering rinsing materials with methanol before use. Additionally, new habitat materials were extracted which may differ from weathered habitat materials. Depending on the type and age of the material, the PFAS that may be

released into the system may differ. Next, the feed samples used also represent a subset of all possible feeds available, therefore, it is paramount that initial feed stocks be tested for contaminants of concern, whether or not that contaminant is the one being investigated. Finally, the aquaculture farms chosen were ones considered for use for a specific bioaccumulation application and were chosen out of convenience rather than a specified sampling scheme. While these limitations exist for this study, the results should still be considered with respect to its impact.

2.6 Impact and Future Directions

Unknown PFAS present in laboratory materials can have a large impact on exposure experiments, specifically with respect to toxicity and bioaccumulation results. Habitat materials, such as aquatic tank parts and screens, should be rinsed with methanol when applicable. Future studies should also investigate weathered habitat materials to determine the PFAS load from new compared to used tank fittings and screens. Additionally, feed options should be tested before use to ensure low to no contamination of PFAS. Finally, stock organisms, such as *P. promelas*, should be tested for PFAS contamination before use in exposure experiments or as controls.

Future research should perform a more in depth analysis of PFAS present in all laboratory materials for a variety of applications, not simply where PFAS is being studied. Additionally, including more aquaculture farms could provide useful geographic data and allow scientists to potentially trace PFAS contamination in water back to a common source. Testing the water of aquaculture facilities would also be a useful addition to this study and the field. Furthermore, continued research should consider what other contaminants may be present in laboratory materials, feed, and stock organisms, especially chemicals that may leach from plastics into water, feed, or onto aquatic tank accessories and impact bioaccumulation or toxicity results.

3.1 Conclusion

This thesis reviewed the bioaccumulation parameters used in the field and suggested standardization of bioaccumulation factors given unknown environmental factors and potential diet contamination. There was PFAS contamination found in several aquatic tank accessories that could be removed using methanol. In fish feed, short chain acids and PFOS were the most common contaminants. Of the four types of feed analyzed, freeze dried bloodworms and frozen brine shrimp were the most contaminated. There can be a large difference in total PFAS contamination and profile based on the type and brand of fish feed used. Finally, aquaculture *P. promelas* can be highly contaminated with PFAS, although it varies significantly between farms. PFOS was the most common contaminant in the aquaculture *P. promelas*. Contributions from diet were minimal as the fish feed provided by each farm was minimally contaminated with PFAS, if at all.

Starting materials for any type of exposure experiment need to be tested for potential PFAS contamination. Otherwise, the bioaccumulation factors that are determined may be influenced by the contamination found in the laboratory materials and organisms. Ensuring PFAS free starting materials is a necessary first step in identifying and comparing bioaccumulation parameters. The calculation and measurement of these bioaccumulation parameters need to be standardized across the field to use the kinetically determined bioaccumulation model to make sure the results are not influenced by the potential lack of equilibrium or other external chemical factors (i.e. pH, salinity, suspended particles). PFAS contamination is ubiquitous and often overlooked, especially in laboratory settings.

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