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ROLE OF SALINITY IN PERFLUOROOCTANE SULFONATE (PFOS) BIOCONCENTRATION BETWEEN AQUATIC SPECIES AND THE DIFFERENTIAL REGULATION OF TRANSPORTERS IN *FUNDULUS HETEROCLITUS*

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Environmental Toxicology

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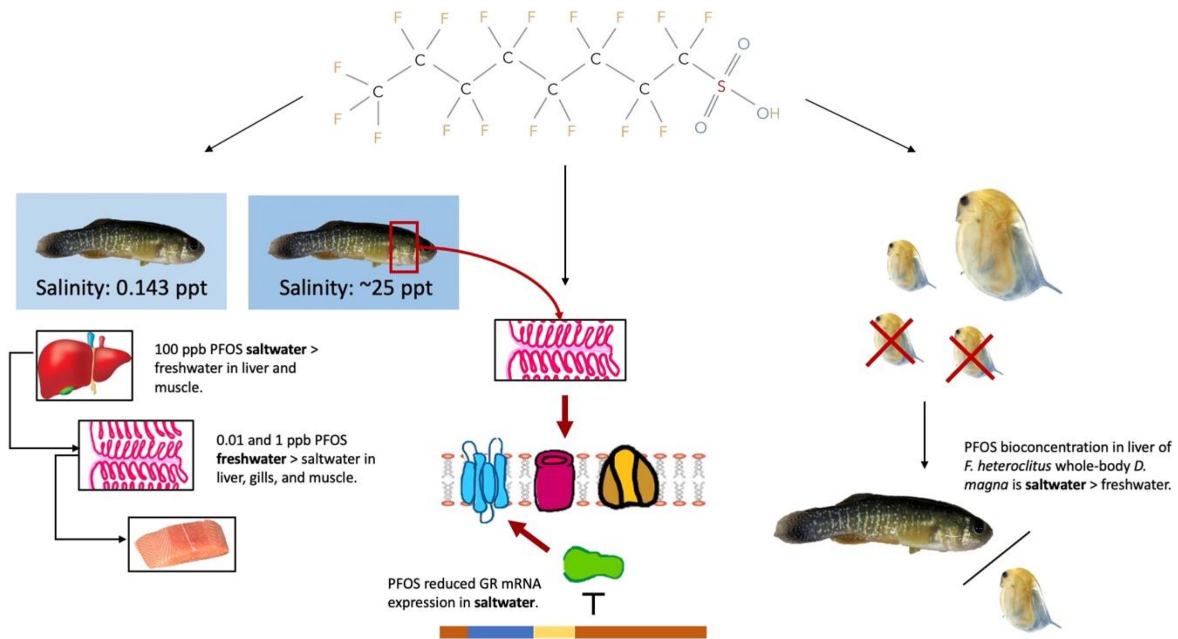
by
Tyler T. Davis
May 2023

Accepted by:
Dr. William S. Baldwin, Committee Chair
Dr. Peter van den Hurk
Dr. Charles D. Rice

ABSTRACT

Perfluorooctane sulfonate (PFOS) is a persistent organic pollutant that is known for its surfactant properties and its resistance to degradation. The persistence and lipophilicity of PFOS in surface waters have led to its bioaccumulation in fish, which in turn leads to its accumulation and toxic effects in humans that consume fish. PFOS is also hypothesized to perturb reproduction and survival of low and mid-trophic level zooplankton species such as *Daphnia magna* that are food for multiple fish species. In this study, a 21-day *D. magna* chronic toxicity test was performed using 1,10, 100, 1000, and 5000 ppb of PFOS to determine the concentration of PFOS in the whole body and its effects on fecundity, survival, and estimated population growth. PFOS showed concentration dependent decreases in survival and fecundity in *D. magna* and reduced reproductive success at concentrations above 10 ppb of PFOS. Salinity is a physiochemical property of water that can potentially enhance PFOS accumulation due to the presence of ions such as Ca^{+} and Mg^{2+} in the water; however, the difference in PFOS bioconcentration between freshwater and saltwater fish is not well studied. Estuarine fish species *Fundulus heteroclitus* (mummichog) is known for its adaptation to both hypertonic and hypotonic environments, which makes it an ideal organismal model to determine differences in the bioconcentration of PFOS in saltwater compared to freshwater in the same species. *F. heteroclitus* were adapted to both saltwater (23-26 ppt) and freshwater (moderately hard water; 0.143 ppt) conditions for 30 days before being treated with PFOS for 20 days to determine if bioconcentration of PFOS is higher in the liver, gills, and muscle of saltwater mummichogs compared to freshwater-acclimated mummichogs. At 100 ppb, PFOS bioconcentrations were higher in saltwater versus freshwater in the liver and muscle of *F. heteroclitus*. At 1 ppb PFOS and below, some of these effects were reserved but rarely statistically significant. The

physiological adaptability of *F. heteroclitus* in different environments is facilitated by changes in expression of ionic and xenobiotic transporters within the gills. The glucocorticoid receptor (*GR*) showed significant decreases in mRNA expression when exposed to PFOS in saltwater mummichogs which can lead to disruption in downstream signaling of the cystic fibrosis transmembrane receptor (*CFTR*). Bioconcentration factors for *F. heteroclitus* and *D. magna* were determined and concentrations in the liver and gills of both saltwater and freshwater-acclimated mummichogs were compared to *D. magna*. In general, PFOS accumulation was greater in *F. heteroclitus* than in *D. magna* in the liver but was greater in *D. magna* in the muscle. In conclusion, PFOS accumulation is exacerbated by salinity at high concentrations in the tissues of *F. heteroclitus* and PFOS hinders reproductive success in *D. magna*.



DEDICATION

I'd like to dedicate this thesis to both my parents. My dad has worked very hard his whole life and got his bachelors and master's degree while working and taking care of his family. He has taught me that I can achieve anything and that it is never too late to accomplish greatness. My mother has also been my rock and a beautiful example of what it is to walk by faith. I would not be the woman I am today without her wisdom and her love. I would also like to dedicate this thesis to the friends that I've met while in Clemson who have made this place a home away from home.

ACKNOWLEDGMENT

I would like to first acknowledge my Principal Investigator, Dr. Bill Baldwin for taking me into his lab and provided me with guidance. He was patient with me throughout this entire process and I appreciate his willingness to work with me through all the ups and downs. Without him, I wouldn't have been able to complete this project in the span of time that I was given. I would also like to acknowledge my co-advisor Dr. Peter van den Hurk for providing me with assistance and encouragement when I needed it. He gave me assurance of my ability to grow and learn in the lab which enhanced my research experience greatly. I would also like to thank Dr. Charlie Rice for his knowledge of *Fundulus heteroclitus* and for giving me the necessary tools to perform dissections and qPCR.

I must give a special acknowledgement to Bricen Ghent, one of the undergraduate students in the Baldwin lab, who was my greatest help throughout this research project. She aided me in performing fish dissections, water changes and daily feedings. She also helped me get through countless RNA extractions and qPCR analyses. Without her help, I would not have been able to process the numerous tissues that we dissected for this experiment. I would also like to thank my lab mates in both the Baldwin and van den Hurk labs for getting me through my time in graduate school. I would like to give special thanks to Lauren Stoczynski and Jazmine Eccles for both helping me during the 30-day freshwater portion of this study. Lauren was also my trusted confidant and helped me get back on track whenever I encountered roadblocks. My lab mates Jazmine Eccles and Drew Evans helped me tremendously by showing me the ropes of the Baldwin Lab and giving me good advice in improving lab techniques and efficiency. Lanie Williams was an amazing researcher who, as an undergraduate, I was able to learn so much from and, as a graduate student, I was able to receive help from in conducting solid phase extractions.

I would like to thank Dr. Angela Slitt and graduate student Juliana Aguedelo for allowing me to come into their lab at the University of Rhode Island and work with them over the summer of 2022. I especially would like to thank Juliana for showing me the basics of solid phase extractions, how to generate an efficient workflow and some of the more complicating aspects of working with an LC/MS-MS. I would also like to thank Dr. Nishanth Tharayil and Elizabeth Leonard for working with me during my last full semester at Clemson. Without the help of the Multi-User Analytical Lab, I would not have a good portion of my data. They were able to analyze many samples in a very short amount of time using LC/MS-MS and I could not thank them enough for all their hard work. Thank you to Mr. John Smink for his help in the set up and acclimation of the mummichogs to the aquatic animal research facility.

Lastly, a special thanks goes out to my parents and my sisters for being the most important part of my support system while at Clemson, and for my friends that I've made along the way who have become like family.

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Chapter I: Background

Per- and polyfluoroalkyl substances (PFAS) and Perfluorooctane sulfonate (PFOS)

Per- and polyfluoroalkyl substances (PFAS) are highly fluorinated, anthropogenic compounds that are characterized by their persistence, mobility, bioaccumulation potential, and toxicity (Lee et al. 2020). PFAS's widespread uses in industrial and commercial products can be attributed to its carbon-fluorine bonds that make them resistant to degradation (Lee et al. 2020). PFOS was used for its repellent properties in the production of plastics and textiles and has also been used in many industrial and commercial products, which include lubricants, fire retardants, pesticides, and surfactants (Khazaee and Ng 2018). The leaching of PFAS into sediment and waterways has resulted in PFAS continued longevity in terrestrial and aquatic ecosystems.

There are an estimated 4730 known PFAS in existence, with the one's of most concern being the long chain PFAS that have carbon fluorine bonds varying from 7-12 carbons in length (Baldwin et al. 2022). Long chain PFAS tend to persistence in the environment (water, soil, and food) and have long half-lives within the human body that range between 1-16 years.

A PFAS of particular concern that is one of the most prevalent persistent chemicals in the environment is perfluorooctane sulfonate (PFOS). Perfluorooctane sulfonate is a fluorosurfactant that is characterized as a long-chain PFAS that contains a sulfonic acid at the end of its 8-carbon chain. PFOS has a molecular weight of approximately 500 g/mol and has an octanol-water partitioning coefficient of 6.3, which adds to its lipophilicity. It was phased out of production in 2002 after its primary manufacturer 3M discontinued it voluntarily ("Drinking Water Health Advisory for Perfluorooctane sulfonate (PFOS)" 2016). It remains prevalent in surface waters, sediments, wastewater effluent, etc.

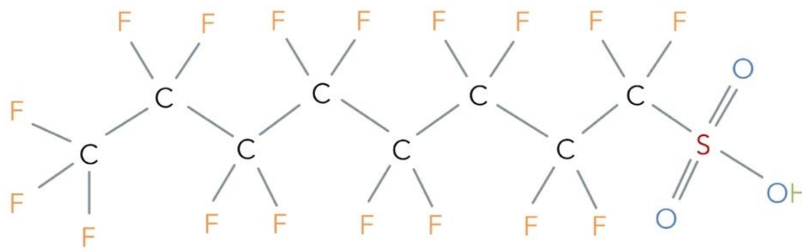

Figure 1. Chemical structure of perfluorooctane sulfonate (PFOS).

PFOS Exposure & Accumulation in Humans

PFOS has been known to bioaccumulate – increase in concentration within an organism over time, and bioconcentrate – have higher concentrations in the tissues of organisms as compared to their environment (typically water). Studies have been conducted to test for increases in concentration of PFAS via dietary uptake, and some have provided evidence of PFOS biomagnification – incremental increase in concentration due to trophic transfer of energy from producers to tertiary consumers (Goeritz et al. 2013; Ren et al. 2022). Perfluorooctanesulfonate (PFOS) is a perfluorinated compound (PFC) that has high energy carbon fluorine bonds that resist hydrolysis, biodegradation and metabolism (Ji et al. 2008).

PFOS is known to be a persistent organic pollutant that has made its way into surface and groundwater throughout the globe as a byproduct of the processes that are used to make the materials that are listed above. Although PFOS is mostly a lipophilic compound, its organic carbon partitioning coefficient (Log K_{oc} = 3) indicates that it is also mobile in soil. Not only does PFOS adsorb to sediments, soils, and organics, it also tends to leach into ground water and make its way into surface water. The leaching of PFOS into water systems not only impacts the aquatic organisms that inhabit those areas, but also humans that live near those sites. There have been studies conducted to determine the concentrations of PFCs in drinking water because it is

one of main routes that PFOS enters the human body. A study that analyzed PFAS concentrations in public water systems (PWS) took place in the state of New Jersey (2006/2009-2010) where the results detected that PFOS was found in 30% of the sites that were tested for PFCs (Post et al. 2013). Prolonged exposure to PFCs via drinking water has been known to substantially increase total human exposure (Post et al. 2013). Another study done in Pensacola; Florida did a survey on concentrations of perfluoroalkyl acids (PFAAs) in the surface waters near the various bays in the area because of run off from a military installation that used aqueous film forming foams (AFFF). It was found that PFOS had the highest concentration of 269 ng/L (da Silva et al. 2022). A population study that focused on residents exposed to PFASs in AFFF living near Peterson Air Force Base (AFB) in Colorado Springs, Colorado looked at the concentrations of perfluoro-carboxylic acids (PFCAs) and perfluoro-sulfonic acids (PFSAs) in drinking water and human serum. The study found that the recovery of L-PFOS(linear isomeric PFOS) in residents of Wakefield, Security, and Fountain averaged 103 ng/L and the average serum concentrations was 7.23 ng/mL in females (n = 132) and 10.09 ng/mL in males (n = 82) (McDonough et al. 2021).

In previous population studies done to examine the concentration of PFAS in human blood samples across the United States, the concentrations of PFOS have differed but have been nearly ubiquitous. In the U.S. National Health and Nutrition Examination Survey (NHANES) from 2003-2004, the geometric mean concentration of serum for the total population studied was 20.7 µg/L (Calafat et al. 2007). Serum concentrations of PFOS in a Michigan population showed median concentrations of 31.2 ng/mL (Kannan et al. 2004). Another study, known as the C8 Health Project, was conducted from 2005-2006 to assess the impact of PFOS as well as other PFCs in the Ohio River on blood serum concentrations in residents, students and workers in Ohio

and West Virginia. The cause for the study stemmed from the improper waste management by the DuPont Washington Works Plant that led to pollution of water and aquifer systems and contamination of well and drinking water in the area. The total population who participated (69,030) had serum PFOS concentrations ranging from 9.7-30.3 ng/mL with an average concentration of 23.3 ng/mL (Frisbee et al. 2009).

Uptake of PFOS can occur via food sources that are exposed to contaminated water or near PFAS production, the main food source being fish. Based on a previous study looking at the plasma concentrations of PFCs in bottlenose dolphins off the coast of Charleston, South Carolina, with the average concentration of PFOS being 1,420 ng/ml, it was speculated that the dolphin food web could result in exposure of humans to PFCs (Fair et al. 2013). In a population of people where seafood is a large part of their diet, the Gullah African Americans in coastal South Carolina had PFOS concentrations from ranging from 5–155 ng/mL with a mean of 53 ng/mL in 2003 (Gribble et al. 2015). After the abolishment of PFOS use in 2002, the reduction in PFOS concentration in the serum of coastal Gullah Geechee populations was approximately 9% annually from 2003-2013.

Although the main routes of exposure to PFAS chemicals such as PFOS is through drinking water and contaminated food sources, other potential pathways include direct skin contact and inhalation of aerosolized PFASs. In a research study looking at the serum concentrations of 149 firefighters who are occupationally exposed to AFFF, which contains various fluorinated surfactants in order to extinguish fires involving highly flammable liquids, the mean concentration of PFOS found in the serum averaged 74 ng/mL (Rotander et al. 2015). Levels of PFOS are reported to be higher in individuals that are occupationally exposed

compared to the general population and have been associated with high blood lipids such as total cholesterol and low density lipoproteins (LDL) (Nilsson et al. 2022).

PFOS Distribution, Bioaccumulation, and Elimination in Humans

PFOS accumulates because it is recalcitrant to degradation, metabolism, and elimination. Reasoning for this includes the number of carbon fluorine bonds and binding affinity to proteins and transporters, specifically the OAT and OATP transporters, in the blood and liver (Baldwin et al. 2022). A positive correlation between number of carbon fluorine bonds and bioaccumulation potential serves as evidence that long chain PFASs are cleared from the body less than short chain PFASs (Martin et al. 2003). In humans, the highest PFOS concentrations are found in the blood and liver, but can also accumulate in the kidneys and the lungs (Perez et al. 2013). In a study comparing pharmacokinetic modeling and measured experimental data showed that liver fatty acid binding proteins and serum albumin are good predictors for long chain PFAS distribution and concentration (Khazaee and Ng 2018). The estimated half-life of PFOS and PFOA in humans are 5.4 years 3.8 years respectively, and both PFASs are readily absorbed and poorly eliminated in both human and wildlife species (Takacs and Abbott 2007). Other PFAS chemicals have various elimination half-lives. Perfluorobutanoic acid (PFBA), perfluorobutane sulfonate (PFBS), and perfluorohexane sulfonate (PFHxS) have half-lives of 3.1 days, 25.8 days, and 5.3-15.5 years respectively (Pizzurro et al. 2019).

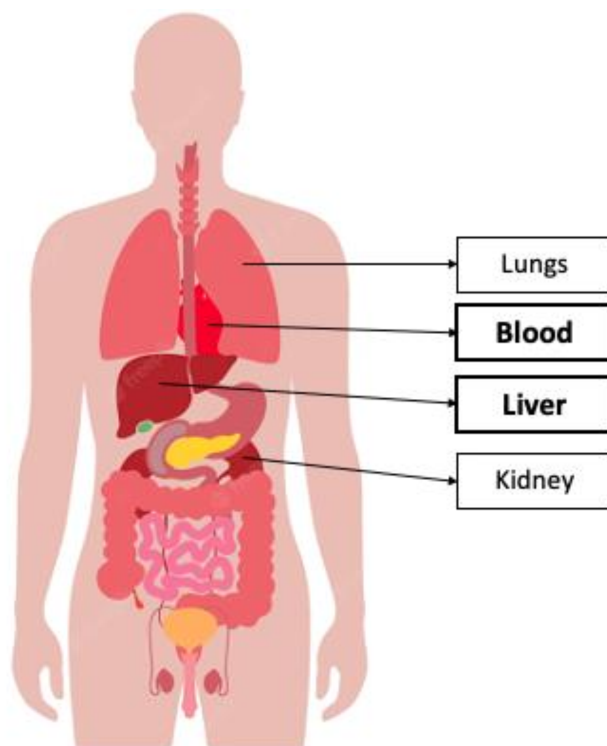

Figure 2. Distribution of PFOS in the human body (according to Perez et. al. 2013).

PFOS Toxicity in Humans

The U.S. Environmental Protection Agency (EPA) stated that the developing fetus and newborns are sensitive to PFOS induced toxicity and some human studies suggest an association with bladder, colon, and prostate cancer although the literature is inconsistent and some are confounded by failure to control certain environmental risk factors (Tsuda 2016). Other effects include non-alcoholic fatty liver disease and peroxisome proliferation (Di Nisio et al. 2020; Fei et al. 2009; Hamilton et al. 2021; Takacs and Abbott 2007). The primary effects of PFOS toxicity that has been the focus in many PFAS literature is association with non-alcoholic fatty liver disease and steatosis. PFOS has been known to suppress genes involved in lipid metabolism – such as cytochrome P450 2B6 (CYP2B6) and hepatocyte nuclear factor 4-alpha (HNF4α) – and upregulate genes involved in lipogenesis – such as peroxisome, proliferation activated receptor gamma (PPARγ) (Hamilton et al. 2021; Takacs and Abbott 2007). PFOS toxicity takes

different forms within each organ system, to include reproductive toxicity that consists of longer time-to-pregnancy (TTP) and late age of menarche (menstruation) (Fei et al. 2009). A study that observed alteration of semen morphology in rats showed that PFOS exposure was tied to lower levels of testosterone that resulted in the reduction in the maturation and number of Leydig cells (Mao et al. 2021). PFOS immunotoxicity is also evident through decreases in antibody concentrations and resistance to vaccines which can increase human susceptibility to diseases such as diphtheria and tetanus (Grandjean et al. 2012).

Exposure & Accumulation in Aquatic Ecosystems

In an aquatic ecosystem, the bioaccumulation of a chemical can occur by either ingestion of food or absorption through the gills during respiration. The bioconcentration of chemicals is the accumulation of the chemical into the organism from the water. In fish, the bioconcentration of PFOS occurs through respiration. The leaching of PFOS into major bodies of water results in the accumulation of PFOS in aquatic organisms. PFOS (as well as other PFCs) were found to accumulate in different animals within the marine food web and PFOS and other long chained PFCs were shown to accumulate and potentially biomagnify to high concentrations in organisms positioned higher on the trophic ladder (Ji et al. 2008). The bioconcentration factor (BCF) of a chemical is the difference in concentration between the environment and the organism. The concentration of PFOS in waters and coastal sediments is dependent on the sorption coefficient and site-specific physiochemical characteristics such as pH, salinity and temperature (Jarvis et al. 2021). Because PFOS sorption is affected by the water chemistry such as salinity, the bioconcentration of PFOS in aquatic species could also be affected by these same properties.

PFOS is known to accumulate in the tissues of fish, specifically the liver, blood, and the kidneys (Goeritz et al. 2013). The liver has been shown to be the best model organ to show correlation between PFAAs and protein interactions within organ tissue (Khazaee and Ng 2018). However, in a study on rainbow trout (*Oncorhynchus mykiss*), of all of the organ tissues that were sampled, total PFAS was the highest in the muscle tissue (41% of the whole body burden of PFOS) and the liver (12% of the whole body burden of PFOS) (Goeritz et al. 2013). By determining how much PFOS accumulates in the skeletal muscle of fish we will provide more information on its potential for biomagnification in humans that consume fish.

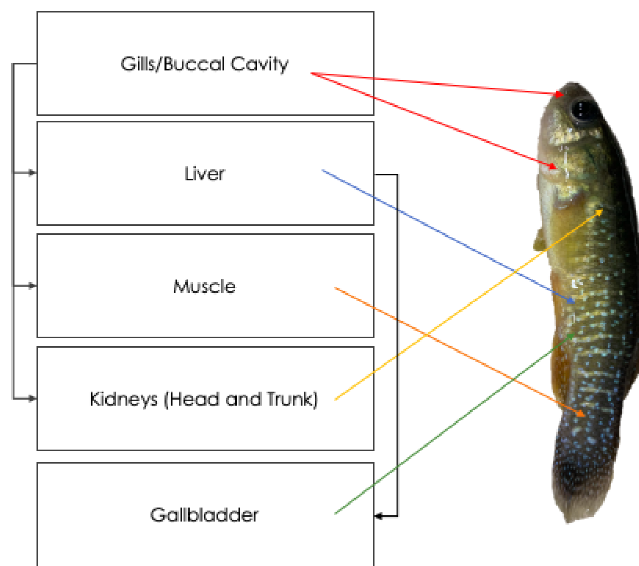

Figure 3. Major organs of distribution in estuarine fish (with *Fundulus heteroclitus* as a model).

Toxicology of PFOS in Fish

Toxicology tests have been conducted on freshwater fish to see how PFOS affects embryology, fecundity, and survival as well as its distribution to the tissues (Tu et al. 2019; Sharpe et al. 2010; Goeritz et al. 2013; Squadrone et al. 2015; Chen et al. 2012). The bioconcentration of PFOS in the gills has not been studied nearly as much as other organ tissues.

According to a study by Consoer et. al., PFOS is eliminated at a higher volume via expiration through the gills than in the urine (Consoer et al. 2016). However, in a study on rainbow trout (*Oncorhynchus mykiss*), the gills carry only 2% of the whole body burden of PFOS (Goeritz et al. 2013). With higher ventilation rates in smaller fish species, it can be expected that PFOS will be eliminated at higher rates across the gills (Consoer et al. 2016). Because the gills play a role in uptake and elimination of toxic chemicals, they also may have a potential role in the bioconcentration of PFOS in fish. Current data indicates that the gills are not crucial in uptake of PFOS but are in elimination. PFOS also causes upregulation in gene expression even after periods of exposure (Oh, Moon, and Choe 2013). Differentially expressed genes (DEGs) associated with development and metabolism are known to be affected by PFOS bioaccumulation in adult fish species and miRNA associated with perturbations in development, lipid metabolism and hormone secretion have been found in fish embryos (Zhang et al. 2011).

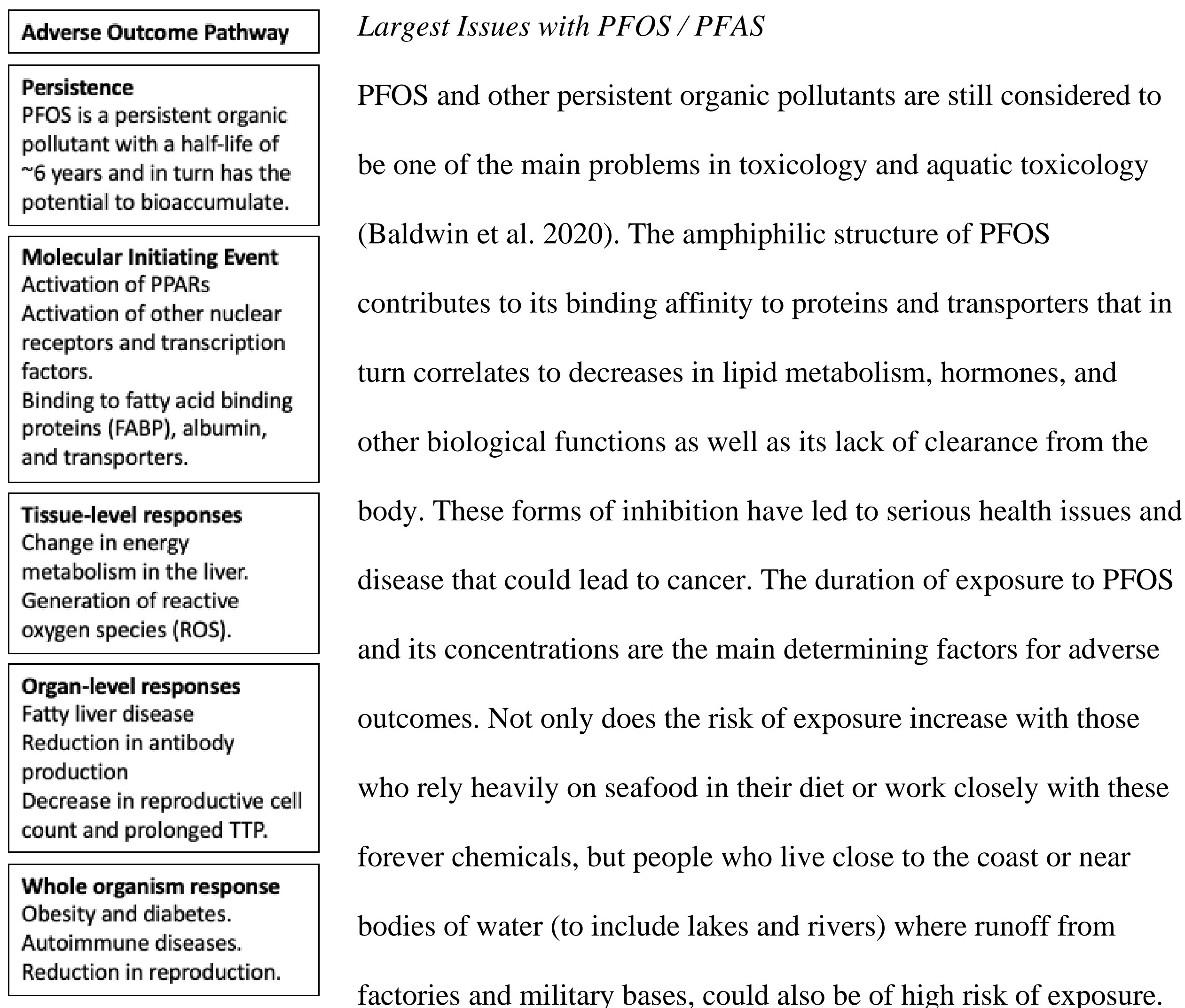

Figure 4. Adverse outcome pathway for PFOS. Modified from Baldwin et. al. 2022.

Largest Issues with PFOS / PFAS

PFOS and other persistent organic pollutants are still considered to be one of the main problems in toxicology and aquatic toxicology (Baldwin et al. 2020). The amphiphilic structure of PFOS contributes to its binding affinity to proteins and transporters that in turn correlates to decreases in lipid metabolism, hormones, and other biological functions as well as its lack of clearance from the body. These forms of inhibition have led to serious health issues and disease that could lead to cancer. The duration of exposure to PFOS and its concentrations are the main determining factors for adverse outcomes. Not only does the risk of exposure increase with those who rely heavily on seafood in their diet or work closely with these forever chemicals, but people who live close to the coast or near bodies of water (to include lakes and rivers) where runoff from factories and military bases, could also be of high risk of exposure.

Aquatic Model Organisms: Fundulus heteroclitus and Daphnia magna

Fundulus heteroclitus (killifish; mummichog) is an estuarine fish species that has physiological adaptations to changes in salinity, such as ion uptake and secretion, that allow it to inhabit both saltwater and freshwater environments (Scott, Schulte, and Wood 2006) and thus make it a near perfect test subject for this study. *F. heteroclitus* has been used in previous studies

that focused on superfund sites because they have a relatively small home range (Di Giulio and Clark 2015). In turn, they have been used as a bioindicator species at Superfund sites such as those contaminated with chromium, polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs) (Roling et al. 2007; Roling, Bain, and Baldwin 2004; Di Giulio and Clark 2015; Nacci et al. 2009). Multiple biomarkers of exposure have provided data on the health of estuarine environments (Roling, Bain, and Baldwin 2004). Furthermore, resistant *F. heteroclitus* populations have adapted and evolved to live in these polluted conditions (Meyer, Nacci, and Di Giulio 2002; Meyer et al. 2003).

Gills in *F. heteroclitus* function in immune support, osmoregulation, and ion/xenobiotic transport. *F. heteroclitus* adapt to salt imbalances with transporters in the gills that absorb and expel ions such as Na^+, K^+, and Cl^-. These transporters include Na^+- K^+- 2Cl (NKCC1) which takes up Cl- along the apical side of the cellular membrane, Sodium Potassium ATPase-1a1 (NKATPase1a1) which is upregulated in response to decreases in salinity that allows for NaCl secretion (McCormick et al. 2013), and the cystic fibrosis transmembrane receptor (CFTR) that secretes Cl^- along the basolateral side of the membrane (Shaw et al. 2007). CFTR is directly regulated by the glucocorticoid receptor (GR)(Shaw et al. 2007). The purpose of using *F. heteroclitus* in this study is to see if there are differences in accumulation in the organ tissue under two significantly different environmental conditions using the same species.

Daphnia magna is a zooplankton that belongs to the superorder Cladocera that are known for their short-life span, reproduction through parthenogenesis, and large clutch size. *D. magna* has been frequently used in toxicology studies due to its ease of laboratory culture (Kovisto 1995) and are considered to be effective bioindicators of PFOS contamination due to the effects

of PFOS on their survival and reproduction (Ji et al. 2008). The purpose for using *D. magna* for this study is to determine its toxicity and compare the PFOS body burden with mummichog liver.

Research Objectives and Specific Aims

The purpose of this research is to:

(1) determine the reproductive effects of PFOS on *Daphnia magna* and compare PFOS bioconcentration between *D. magna* and *F. heteroclitus.*

(2) Test whether there are significant differences in PFOS accumulation between saltwater and freshwater fish using saltwater and freshwater adapted *F. heteroclitus,* and

(3) investigate the distribution of PFOS in tissues with a close look at gills as they may be important for elimination.

Aim 1: Determine PFOS's bioconcentration factor and reproductive effects on *Daphnia magna.*

D. magna are commonly used to study aquatic freshwater toxicity because they are small, have short life-cycles and reproduce in large numbers via parthenogenesis (Kovisto 1995). The current literature indicates that *D. magna* are not as sensitive to PFOS as fish (Ji et al. 2008). According to previous studies, PFOS has been shown to impact reproductive success and survival in *D. magna* (Seyoum et al. 2020). For this experiment, we will determine the adverse reproductive effects and body burden of PFOS in the exposed daphnids. This will provide us a No Observable Effects Concentration (NOEC) of PFOS in *D. magna,* as well as body burdens that can be compared to other aquatic organisms. Most research has indicated that *D. magna* and other invertebrates are not as sensitive to PFOS toxicity compared to vertebrates most likely

because of a lack of toxicodynamic effects (lack of PPARs), but toxicokinetic differences have not been carefully evaluated. Comparison to fish bioconcentration factors will help confirm or deny this hypothesis. *D. magna* was be exposed to PFOS in concentrations of 0, 1, 10, 100, 1000, and 5000 ppb for 21 days to assess how the contaminant affects fecundity, brood number, and mortality. Tissue analysis was be done to investigate PFOS bioconcentration.

Aim 2: Compare the bioconcentrations of PFOS in estuarine and freshwater-adapted killifish and determine PFOS organ distribution.

PFOS has been known to accumulate in both freshwater and saltwater fish species (Baldwin et al. 2020). However, whether saltwater or freshwater species may be more likely to accumulate PFOS has not been adequately studied. Fish have lower uptake of water in order to maintain proper osmotic pressure in freshwater (Jeon et al. 2010) and therefore, we hypothesize that PFOS accumulation is more likely in fish that live in saltwater due to increased drinking and uptake of water. In addition, saltwater fish may accumulate more PFOS because metal ions in saltwater, such as magnesium [Mg^{2+}] and calcium [Ca^{2+}], enhance PFOS sorption (Chen et al. 2012).

In contrast, Mg^{2+} and Ca^{2+} also increase PFOS sorption to sediment and PFOS's sorption affinity to sediment is approximately 10 times higher in saltwater than in freshwater (Chen et al. 2012) causing an effective drop in saltwater PFOS concentrations. This is not viable in our study because no sediment is available. However, food or waste in the water may still affect bioavailability. The lack of sediments reduces the realism of our study but increases our understanding of direct PFOS bioconcentration and the role of the organism in PFOS bioconcentration due to reducing the non-organic variables. In summary, the hypothesis for this

study is that PFOS will have significantly greater bioconcentration factors (BCFs) in saltwater killifish than freshwater killifish because of greater retention in the saltwater due to ions and greater water uptake in the fish due to countercurrent exchange and drinking (Jeon et al. 2010). To test whether PFOS uptake in fish is affected by salinity, PFOS accumulation will be observed in the same fish species under freshwater and saltwater conditions following acclimation. A major plus of *F. heteroclitus* is their ability to acclimate to both freshwater and saltwater.

PFOS is primarily accumulated in the liver and blood of rodents and humans (Oh, Moon, and Choe 2013). Uptake and accumulation by the gills have not been extensively studied and given that the gills are a point of contact, point of excretion, and receive significant blood flow, they are also an organ of interest. Skeletal muscle will also be investigated because it is critical in trophic transfer to humans (Chen et al. 2021). Therefore, we will expose *F. heteroclitus* to PFOS at 0 ppb, 0.1 ppb, 1 ppb, 100 ppb for 0, 10, 20, and 30 days and monitor bioconcentration in muscle, gills, liver, and blood.

Aim 3: Test whether PFOS differentially perturbs gill transporter expression in salt- and freshwater acclimated mummichog.

It is expected that gills are important in the elimination of PFOS because of differences in ion transporter expression. The response of transporters in the gills of euryhaline fish to PFOS nor the potential role saltwater versus freshwater plays in the response has not been evaluated. According to previous research, we hypothesize that the BCF for PFOS will be higher in the gills of saltwater killifish compared to freshwater killifish. Higher concentrations of PFOS in the tissues can be attributed to increases in ion pairing of PFOS to inorganics such as Mg^{+} and Ca^{+}, and the role of the gills in transport and accumulation of xenobiotics (Kropf et al. 2020; Lu et al.

2021). We expect concentration dependent increases in PFOS in the gills, increased transporter expression due to saltwater, and in turn potential for greater transporter expression with the combination of saltwater and PFOS compared to freshwater and PFOS. This may help increase clearance of PFOS in saltwater acclimated fish. If there is significant difference between the concentrations of PFOS in the gills of killifish, then the transporters that contribute to the expression of PFOS excretion will be determined such as organic anion transporters (OAT), organic anion transporting proteins (OATP), and ATP binding cassettes (ABC) transporters using targeted qPCR. Significant differences in the expression of xenobiotic transport genes will be determined by two-way ANOVA.

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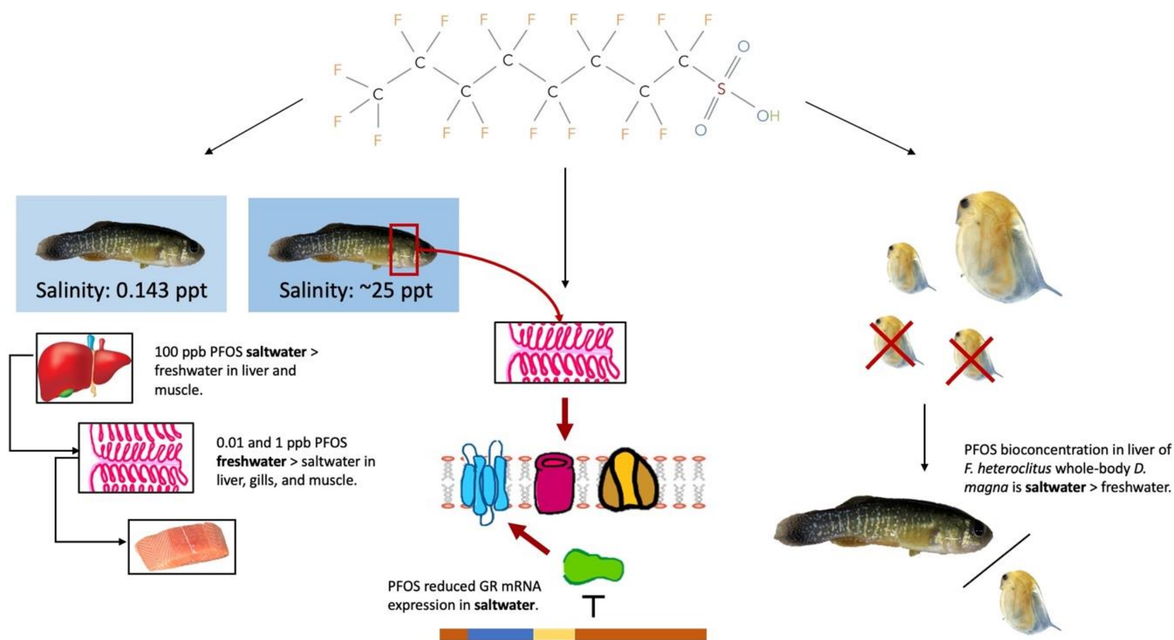
Chapter II: Salinity alters PFOS bioconcentration and distribution in *Fundulus heteroclitus* - Role of the gills and comparison to the invertebrate *Daphnia magna*

Abstract

Perfluorooctane sulfonate (PFOS) is a persistent organic pollutant that is known for its surfactant properties and resistance to degradation. PFOS's persistence in surface waters has led to its bioaccumulation in aquatic invertebrates and fish, which in turn leads to its accumulation and toxic effects in humans that consume fish. In this study, we compare the bioconcentration of PFOS between the the aquatic invertebrate, *Daphnia magna* and the estuarine fish species, Fundulus heteroclitus, and between saltwater and freshwater-acclimated *F. heteroclitus*. Differences in PFOS bioconcentration between freshwater and saltwater fish is not well studied and the estuarine fish species *Fundulus heteroclitus* (mummichog) known for its adaptation to both hypertonic and hypotonic environments makes an ideal organismal model to determine differences in the bioconcentration of PFOS in saltwater compared to freshwater because the experiments can be performed in the same species. *F. heteroclitus* were acclimated to both saltwater (23-26 ppt) and freshwater (moderately hard water; 0.143 ppt) conditions for 30 days before being treated with PFOS for 20 days to determine bioconcentration in the liver, gills, and muscle. At 100 ppb, PFOS bioconcentrations were higher in saltwater versus freshwater in the liver and muscle of *F. heteroclitus*. At 1 ppb PFOS and below, some of these effects were reversed but only statistically significant in gills. The physiological adaptability of *F. heteroclitus* in different environments is facilitated by changes in expression of ionic and xenobiotic transporters within the gills and these expression changes of glucocorticoid receptor (GR), cystic fibrosis transmembrane receptor (CFTR), and organic anion transporter 1 (OAT1) may play a role in the increased retention of PFOS in the gills of freshwater-acclimated

mummichog. In PFOS exposures with daphnids, PFOS decreased survival and fecundity in *D. magna* and reduced reproductive success at concentrations above 10 ppb of PFOS. Surprisingly, PFOS bioconcentration was almost as high in daphnids as mummichogs with the exception of the higher concentration exposures in liver. This indicates previous reports of decreased sensitivity of daphnids to PFOS is not due to lower bioconcentration. In conclusion, PFOS accumulation is exacerbated by salinity at high concentrations in the tissues of *F. heteroclitus* and PFOS hinders reproductive success in *D. magna*.



Introduction

Heptadecafluorooctane sulfonic acid potassium salt (PFOS) is a useful industrial chemical and surfactant that is used in stain repellents, pesticides, lubricants, and flame retardants (Khazaee and Ng 2018). However, PFOS's long chain of carbon-fluorine bonds make it resistant to degradation and therefore an environmental hazard that persists in sediments and waterways and bioaccumulates in both terrestrial and aquatic environments (Miranda et al. 2021; Lee et al. 2020). In turn, it impacts ecosystems primarily because of biomagnification in higher

trophic ladder organisms (Goeritz et al. 2013). For example, concentrations of linear and branched (L-PFOS and Br-PFOS) isomers of PFOS (0.49 and 0.09 ng/g whole body wet weight) in a system of bivalves, fish, and crustaceans resulted in a total biomagnification factor (TMF) of 1.53, which indicates bioaccumulation and biomagnification of PFOS across all trophic levels (Miranda et al. 2021).

PFOS leaches into drinking water and accumulates in seafood and other food sources and therefore poses a significant risk to human health ("Drinking Water Health Advisory for Perfluorooctane sulfonate (PFOS)" 2016; Gribble et al. 2015). PFOS has been found to accumulate in human serum at an average concentration of 23.3 ng/mL with a long half-life of 5.4 years (Takacs and Abbott 2007; Frisbee et al. 2009). The highest concentrations of PFOS are found in the liver, blood, and kidney of humans, rodents, and fish (Goeritz et al. 2013). However, significant concentrations of PFOS are measured in the muscle tissue and this can directly affect human bioaccumulation, especially in populations that consume fish regularly in their diet. The Gullah African-Americans in coastal South Carolina had PFOS concentrations ranging from 5–155 ng/mL with a mean of 53 ng/mL in 2003. After the abolishment of PFOS use in 2002, serum concentrations reduced about 9% annually from 2003-2013 (Gribble et al. 2015). The accumulation and distribution of PFOS in an aquatic organism are influenced by its uptake and elimination, and PFOS is predominantly eliminated via the urine and gills in fish. Therefore the gills are also key organs to study when investigating bioconcentration or biomagnification (Consoer et al. 2016).

PFOS and other perfluorinated compounds (PFCs) can disrupt ion homeostasis in fish and cause lesions within the gills of *Oryzias melastigma* (rainbow trout) with greater damage in freshwater fish than saltwater fish (Lu et al. 2021). In contrast, PFOS increases in concentration

relative to salinity in the liver and blood of Black Rockfish (Jeon et al. 2010). Considering the rate of uptake and elimination, the BCF of PFOS in Black Rockfish is about 10 - 35 practical salininty units [psu](Jeon et al. 2010). The chemical species that form between Mg^{2+}, Ca^{2+}, and PFOS increase its hydrophobicity, which may increase its uptake by transporters at higher salinities. PFOS bioaccumulation and Na^{+} and Mg^{+} ion concentrations in water (Chen et al. 2012) are associated, which suggests that tissue concentrations of PFOS would most likely be higher in the saltwater-acclimated fish compared to the freshwater-acclimated fish. In addition, marine fish species drink much more water than freshwater fish because they lose water through the gills due to osmosis; instead freshwater fish uptake greater amounts of water through the gills because of osmotic differences between the freshwater and tissues (Wood and Marshall 1994; Smith 1930). Therefore, PFOS exposure and ultimately organ bioconcentration may differ betweeen saltwater- and freshwater-acclimated species.

One of the major plusses of studying *F. heteroclitus* (mummichog; killifish) is their hardiness and ability to live in diverse saline environments (Burnett et al. 2007). In saltwater, *F. heteroclitus* take in more water to maintain osmotic pressure and actively excrete ions into the water to maintain ion balance within their cells. Euryhaline fish gill epithelial cells undergo morphological changes that either facilitate or prevent uptake of ions depending on the salinity which allows *F. heteroclitus* to thrive in hypersaline and hyposaline environments (Laurent, Chevalier, and Wood 2006). This allows us to study and compare bioconcentration between saltwater and freshwater-acclimated fish using the same species. In turn, increased bioconcentration in one of these groups or in specific tissues us understand PFOS bioconcentration and help inform human risk from consumption of freshwater or saltwater fish.

Key xenobiotic transporters function to eliminate chemicals includes ATP binding cassette (ABC) transporters such as the organic anion transporters (OATs), organic cation transporters (OCTs), multidrug resistant associated proteins (MRPs), and multidrug resistant proteins (MDRs). In mammals, PFOS is transported within the kidney and liver by organic anion transport polypeptides (OATPs); specifically, OATP1B1, OATP1B3, and OATP2B1 (Zhao et al. 2017). Of the homologous transporters with the potential to transport PFOS, *F. heteroclitus* express organic anion transporter 1 (*OAT1*) and P-glycoprotein (*P-gp*); both of which are inducible by several xenobiotic activated transcription factors such as the pregnane-X-receptor (PXR) in mummichogs (Grans et al. 2015). OAT1 has been shown to transport PFAS chemicals; however, P-gp does not (Weaver et al. 2010; Kim et al. 2016; Zhao et al. 2017). The expression of *P-gp* and *OAT1* in response to PFOS has yet to be determined.

Key transcription factors and ion transporters that regulate salt balance under osmotic stress within the gills include the glucocorticoid receptor (*GR*), cystic fibrosis transmembrane receptor (*CFTR*) and sodium potassium ATPase-1a1 (*NKATPase1a1*). The activation of cortisol by *GR* in fish has been known to increase the gene expression of *CFTR* that in turn increases Cl^- secretion as a physiological adaptation to seawater (Shaw et al. 2010; Shaw et al. 2007) and Cl- absorption as a physiological adaptation to freshwater (Marshall and Singer 2002). *CFTR* channels located on the apical side of the plasma membrane secretes Cl^- back into seawater as a measure to maintain NaCl homeostasis in environments of high salinity (Shaw et al. 2007). *NKATPase1a1* channels take up Na^+ into the basolateral side of the plasma membrane inside the gills (Scott et al. 2005); *NKATPase1a1* is highly expressed in freshwater compared to saltwater (McCormick et al. 2013).

In general, PFOS toxicity is higher in vertebrates that invertebrates (Ji et al. 2008). For example, the no observable effect concentration (NOEC) for reproduction of *D. magna* is 1.25 mg/L while hatchability for the Japanese medaka (*Oryzias latipes*) significantly decreases at concentrations as low as 0.01 mg/L (Ji et al. 2008). The primary hypothesis for this difference is the lack of peroxisome proliferator activated receptors (PPARs) in protostomes (Litoff et al. 2014; Capitao et al. 2020). However, there are other modes of action of PFOS such as inhibition of fatty acid oxidation and perturbation of triglyceride and cholesterol homeostasis (Fragki et al. 2021). Furthermore, the bioconcentration of PFOS in invertebrates has not been well studied and therefore it is possible that some of the differences in toxicity is due to reduced bioaccumulation. Therefore, in addition to evaluating bioconcentration between saltwater and freshwater acclimated *F. heteroclitus*, we will also measure and compare bioconcentration between *D. magna* and *F. heteroclitus*.

The purpose of this study was to determine if there are differences in BCF between *D. magna* and *F. heteroclitus*, and between *F. heteroclitus* adapted to saltwater or freshwater. We determined the chronic toxicity of PFOS in daphnids and measured PFOS bioaccumulation. Furthermore, we exposed mummichogs to PFOS for 20-days and measured bioconcentration at day 10 and 20 at three different PFOS concentrations in saltwater and freshwater-acclimated fish. Bioconcentration was compared between species and saline-acclimated fish. Differences in tissue bioconcentration was measured in liver, muscle, and gill of fish and compared between groups. Lastly, the expression of key transporters was measured before and after PFOS exposure to determine if transporter expression differs in saltwater versus freshwater-acclimated fish with and without exposure to PFOS.

Materials & Methods

D. magna Culture & Maintenance

A culture of *D. magna* has been maintained in the Department of Biological Sciences for greater than 25 years (Baldwin et al. 2001). The artificial media used to house *D. magna* was moderately hard water composed of 60 mg/L $MgSO_4$, 60 mg/L $CaSO_4$, 96 mg/L $NaHCO_3$, and 4 mg/L KCl in Millipore water kept at a pH between 8.2 and 8.4 (Ginjupalli, Gerard, and Baldwin 2015). The artificial media was aerated continuously before being used. *D. magna* were kept in a controlled environment that was maintained at 21.0°C with a 16:8-hour light:dark photoperiod. and fed a mix of fish food and *Pseudokirchneriella subcapitata.*

Pseudokirchneriella subcapitata Culture

P. subcapitata was cultured in medium containing 5 ml of each of the salt solutions #1-5. Salt solution #1 was made of 1.85 g $MgCl_2$, 2.2 g $CaCl_2 \bullet 2H_2O$, and 12.75 g $NaNO_3$ in 500 mL of deionized (DI) water. Salt solution #2 was made of 7.35 g $MgSO_4 \bullet 7H_2O$ in 500 ml of DI water. Salt solution #3 was made of 0.684 g $K_2HPO_4 \bullet 3H_2O$ in 500 ml of DI water. Salt solution #4 was made of 7.5 g $NaHCO_3$ in 500 ml of DI water. Salt solution #5 was made of 92.8 mg H_3BO_3, 2.08 mg $MgCl_2 \bullet 4\ H_2O$, 79.9 mg $FeCl_3 \bullet 6\ H_2O$ in 150 ml NaEPTA • $6H_2O$. NaEPTA • $6H_2O$ was made by combining 0.5 ml $ZnSO_4 \bullet 7H_2O$ (690 mg in 100 ml of DI water), 0.5 ml $CaCl_2 \bullet 2H_2O$ (60 mg in 580 ml of DI water), 0.5 ml $CaCl_2 \bullet 6H_2O$ (142.8 mg in 100 ml DI water), 0.5 ml $NaSeO_3 \bullet 5H_2O$ (333.0 mg in 100 ml of DI water), and 0.5 ml $Na_2MgO_3 \bullet 2H_2O$ (18.3 mg in 10 ml of DI water). *P. subcapitata* was cultured using a previous stock containing 3 x 10^7 cells/mL. *P. subcapitata* that was rapidly mixed with a stir bar in ~3.5 L of sterilized DI water under fluorescent light and aeration for 24 hours a day over 12 days. On the twelfth day,

the cultured algae were centrifuged out of the medium to be reconstituted in a 1 L autoclaved flask of Millipore water. All cultures were diluted to 30,000,000 cells / mL for feedings.

D. magna Chronic Toxicity Test with PFOS

A chronic toxicity test was performed using an adaptation of the standard methods for static renewal chronic toxicity tests *for D. magna* (Biesinger, Williams, and van der Schalie 1987). Twelve neonates (<24 hours old) were collected for each treatment group. Each daphnid was placed into separate 50 mL beakers containing 40 mL of culture media. PFOS was serial diluted to concentrations of 0, 1, 10, 100, 1000, and 5000 ppb (µg/L) from a stock solution dissolved in dimethyl sulfoxide (DMSO). All daphnid media solutions contained 0.1% DMSO.

D. magna were fed 3,000,000 cells per day (100 µL) of *P. subcapitata* for the first week of the experiment, 4,500,000 (150 µL) cells per day for the second week and 6,000,000 (200 µL) cells per day for the third week. Daphnid feedings were supplemented with 0.25 mg dry weight TetraFin Fish Flakes (Masterpet Corp., Australia) dissolved in a 50 µL aqueous suspension throughout the experiment. PFOS and control media were changed every other day at which time death, age at first brood, presence of a new brood, and number of neonates were monitored and quantified. The net reproductive rate for each exposure group at age 15- and 21-days was determined by multiplying the percent of survival by the number of offspring for each adult daphnid (Siegel, Swanson, and Shryock 2004). On the last day of the experiment, whole-body *D. magna* were weighed and stored at -80°C as groups of three for bioconcentration analysis. The BCF of PFOS in *D. magna* was determined by taking a ratio of the PFOS concentration in the whole daphnid and the PFOS concentration in the media.

F. heteroclitus Collection and Acclimation

F. heteroclitus were collected (wild-caught) from the brackish waters off the coast of South Carolina at the Baruch Institute of Marine & Coastal Science at the North Inlet Estuarine Research Reserve (NERR) at Georgetown, SC. Before experimentation, *F. heteroclitus* were divided into two large system tanks that were enough to house 64 fish in each tank. All fish were housed at 20 parts per thousand (ppt) in artificial saltwater (Instant Ocean Sea Salt, Spectrum Brands Pet LLC, Blacksburg, VA) until acclimation for the PFOS studies. The fish were fed a normal diet of TetraMin Tropical Fish Flakes (Masterpet Corp., Australia) daily.

F. heteroclitus Freshwater and Saltwater Acclimation

Freshly obtained mummichogs were separated into tanks for acclimation to saltwater or freshwater. Fish to be tested for PFOS bioconcentration in saltwater were acclimated for a week at a salinity of 23 ppt. The salinity of the water during the saltwater study was kept between 24-26 ppt throughout the 30-day testing period. For the freshwater study, the fish were acclimated over a four-week period in a step wise fashion in a slightly modified method described previously (Shaw et al. 2007). The fish were kept at 20 ppt during the first week and stepped down each week to 10, 3.5, and finally were housed at 0.143 ppt (moderately hard water). *F. heteroclitus* were further acclimated for 5 days in the moderately hard water before they were introduced to the testing environment for the freshwater studies. Moderately hard water was made in 45-gallon barrels of reverse osmosis (RO) water containing 10.2 g of magnesium sulfate ($MgSO_4$), 10.2 g of calcium sulfate ($CaSO_4$), 16.32 g of sodium bicarbonate ($NaHCO_3$), and 680 mg of potassium chloride (KCl).

F. heteroclitus Bioconcentration Testing with PFOS

Bioconcentration of PFOS in mummichogs was quantified for 30-days at several different concentrations of PFOS. Wild-caught *F. heteroclitus* [n=64] were separated into sixteen 5-gallon tanks each containing four fish (sixteen fish for each treatment group). The fish were treated with PFOS suspended in DMSO solvent to create water concentrations of 0 ppb, 0.01 ppb, 1 ppb, and 100 ppb. On days 2, 10, 20, and 30, one fish was taken from each tank for necropsies and dissection. Four fish from each treatment group were dissected on day 0 to determine baseline concentrations before PFOS exposure.

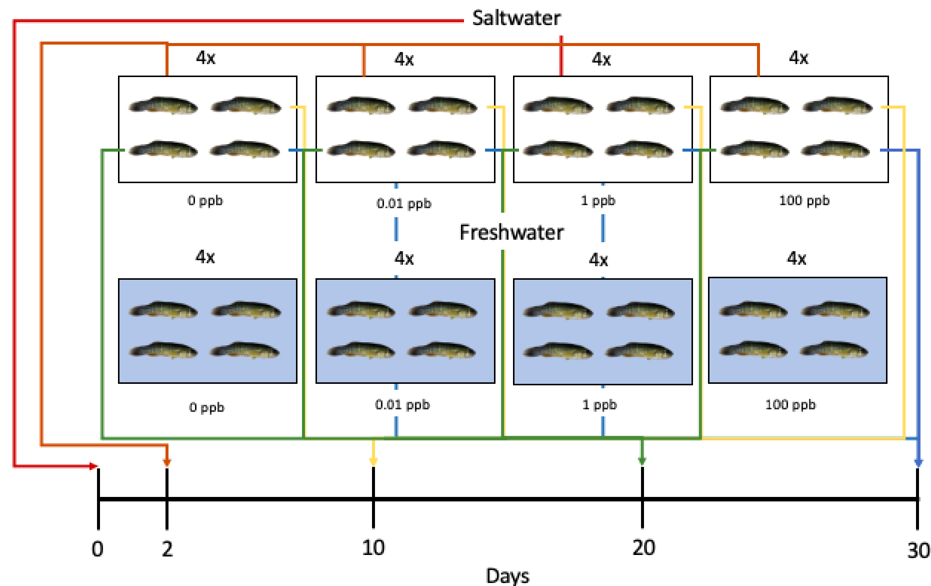


Figure 5. Diagram of 30-day bioconcentration testing of *F. heteroclitus* in saltwater and freshwater environment. On days 0/2, 10, 20, and 30, one fish was taken from each tank for dissection of the liver, muscle, and gills. The 30-day saltwater tests were performed before the 30-day freshwater tests. The dissection process and schedule were the same for both the saltwater and freshwater bioconcentration tests.

Fish were housed in saltwater/brackish (25 ppt) or freshwater (0.143 ppt). The fish were exposed to a 12:12-hour light:dark photoperiod and 50% water changes with fresh PFOS were administered three times a week. During testing, the fish were fed a normal diet of TetraMin

Tropical Fish Flakes (Masterpet Corp., Australia) daily and water quality testing was performed using the API Saltwater (or Freshwater) Testing Kit (Aquatic Pharmaceuticals, Inc., Chalfont, PA).

F. heteroclitus Necropsies

On days 0, 2, 10, 20, and 30, one fish from each treatment group were anesthetized in 1-1.5 g/L of tricane methanosulfonate (MS-222) solution buffered with calcium carbonate ($CaCO_3$). The total body weight of each *F. heteroclitus* was measured and then the fish were decapitated to confirm euthanasia prior to the necropsy. Liver, gills, and muscle tissue were dissected, weighed, placed in 2-mL vials, and snap frozen in liquid nitrogen until they were stored at -80°C.

PFOS Water Extractions

To extract PFOS from the water media, 160 ml of media for every four *Daphnia* and 160 ml of media for every fish (640 ml for each tank) in each PFOS treatment group was vacuumed through a 500 mg/6mL Bond Elut SPE column (Agilent Technologies, Santa Clara, CA[Part. No. 12255021]). The columns were conditioned with 15 mL of methanol and 18 mL of millipore water before running the sample water through a 12-position solid phase extraction (SPE) vacuum manifold at a flow rate of 1 drop/1-2 seconds. The columns were washed with 2 mL of millipore water for the *D. magna* media and 5 mL for the *F. heterocltus* media before drying the filter for 5 minutes at high vacuum. PFOS that remained in the filter was eluted with 8 mL of methanol. The methanol-PFOS was concentrated under a stream of nitrogen in a 40°C water bath to dryness using the OA-SYS heating system and N-EVAP 111 nitrogen evaporator

(Organomation Associates, Inc., Berlin, MA) and stored at -80°C in autosampler vials to be brought up in 200 µl of 50:50 acetonitrile:water before running the samples through LC-MS.

PFOS Tissue Extractions

Tissue samples were prepared using the QuEChERS Extraction Method for PFAS with modifications from other previously used methods (Chang et al. 2017; Marques et al. 2021) where respective amounts of tissue samples were spiked with 100 ng/mL of 13C internal standard (5,000 ng/mL PFOS [Lot # MPFOS0122] in acetonitrile) and homogenized in 2 mL OMNI vials containing ceramic beads in Millipore water with the Bead Ruptor Elite Bead Mill Homogenizer (OMNI, Bedford, NH, SKU 19-042E). Following homogenization, 100% LC/MS-grade acetonitrile was added to the vials and then vortexed for 30 seconds. The ratio of tissue:water:acetonitrile was 1 g: 10 mL: 10 mL. Once vortexed, the supernatant was then added to an RNase-free 2 mL tube containing a ratio of roQ Extraction Kit-Original Salts (4g $MgSO_4$ and 1g NaCl). The ratio of tissue sample : salts was 1 g : 5 g. The solution was then vortexed again for 20-30 seconds and centrifuged for 20 mins at 5000 rpm at 23°C. 150 µL of the resulting top layer of PFAS supernatant in acetonitrile was transferred into 650 µl RNase-free tubes containing 30 mg of roQ dSPE powder (150mg $MgSO_4$ and 50mg PSA, KSO-8920), vortex for 30 seconds, and then centrifuged for 20 minutes at 5000 rpm at 25°C. 100 µL of clean acetonitrile was pipetted out into another 2 mL tube for storage at -20°C. The extracts were eluted through a 200 mg/3 mL Strata SBD-L 100 µm Styrene-divinylbenzene SPE column (Phenomenex, Torrance, CA) and washed with methanol. The methanol-PFOS was concentrated under a stream of nitrogen in a 40°C water bath to dryness using the OA-SYS heating system

and N-EVAP 111 nitrogen evaporator (Organomation Associates, Inc., Berlin, MA and reconstituted to its original extract volume prior to evaporation.

RNA Extraction and cDNA Synthesis

RNA was isolated from 50-100 mg of gill tissue using TRIzol Reagent according to the manufacturer's instructions (Invitrogen, Waltham, MA). RNA was quantified with a NanoDrop™ One^{C} Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific, Waltham, MA, Cat. No. ND-ONE-W) before storage at -80°C. RNA quantity was confirmed with a Qubit 4 Fluorometer (Invitrogen, Waltham, MA) using the RNA Broad Range assay (Invitrogen, Waltham, MA). RNA (2 µg) was synthesized into cDNA by reverse transcription using Moloney Murine Leukemia Virus-Reverse Transcriptase (MMLV-RT), dNTPs, 5xRT Buffer and random hexamers. Samples were then incubated at 37°C for 1 hour and stored at -20°C.

Quantitative Polymerase Chain Reaction (qPCR)

Quantification of transporter expression was determined by real-time quantitative polymerase chain reaction (qPCR). qPCR was performed using primers specific for glucocorticoid receptor (*GR*), cystic fibrosis transmembrane receptor (*CFTR*), organic anion transporter-1 (*OAT1*), Sodium potassium ATPase-1a1 (*NK-ATPase1a1*), P-glycoprotein (*P-gp*), *18S* and *β-Actin*. Master mix was generated using 1 µL of diluted cDNA (1:10 cDNA/Millipore water) mixed with 1 µL each of forward and reverse primers, 9.5 µL of Millipore water, and 12.5 µL of RT^2 SYBR Green (Qiagen, Frederick, MD) to get a total volume of 25 µL in each well. Primers were denatured at 95°C for 30 seconds, lowered to their respective annealing

temperatures (Table 1) for 30 seconds, and extended at 72°C for 30-45 seconds for 50 cycles to determine threshold cycle (C_t). For the standard curve, serial dilutions of 1:4 (from 1:1-1:1024) was used for all primers including the housekeeping genes. Samples were diluted 1:10 prior to qPCR. The target genes were normalized to β-Actin as previously described using the inverted Muller's equation (Muller et al. 2002; Roling, Bain, and Baldwin 2004).

Table 1. qPCR Primer Information for Selected Transporters & Receptors.

Primer	Forward Sequence	Reverse Sequence	Annealing Temperatures (°C)
*β-actin	5'-CAG GGC TGT GAT CTC CTT C-3'	5'-TCC ACG AGA CCA CCT ACA AC-3'	59
*18S	5'-TTT CTC GAT TCT GTG GGT GGT GGT-3'	5'-TAG TTA GCA TGC CGG AGT CTC GTT-3'	57
Glucocorticoid Receptor (GR)	5'-GTA CCA AAA GAA GGC CTG AAG TG-3'	5'-CCT TGA TGT AAG TCA TCC TGA TCT CA-3'	53
Cystic Fibrosis Transmembrane Receptor (CFTR)	5'-AAT CGA GCA GTT CCC AGA CAA G-3'	5'-AGC TGT TTG TGC CCA TTG C-3'	54
Na/K/ATPase1a1	5'-AAG ATC ATG GAG TCC TTT AAG AAT CTG-3'	5'-CAC CTC CTC TGC ATT GAT GCT-3'	57
P-glycoprotein (P-gp)	5'-GGC TTC ACC TTC TCC TTC TC-3'	5'-ATA CTG CTT CCA CAT CCA TCC-3'	54
Organic Anion Transporter 1 (OAT1)	5'-TCG CTG AGG AGG AAC TTA GA-3'	5'-TGC AGC CCA GCT ACA TAA TC-3'	56

Liquid Chromatography/Mass Spectrometry

The liver, muscle and right gills were run on the SCIEX Triple Quadrupole 4500 Liquid Chromatography Tandem Mass Spectrometer (LC-MS/MS) System (SCIEX, Framingham, MA)

using a modification of methods described previously (Marques et al. 2020). The solvent used for the samples was 50% acetonitrile and 50% Millipore water. Dilutions were made for samples that were above the upper limit of quantification (ULOQ). The diluted samples were produced and transferred into autosampler vials for analysis. The buffers used to run the samples through the LC-MS was 1% ammonium hydroxide in methanol and 1% ammonium hydroxide in water. The Waters X-Bridge C18 (100 mm x 4.6 mm, 5µm) column was used to separate solutes in the mobile phase. A calibration standard composed of acetonitrile and internal standard (IS) with concentrations ranging from 500 ng/mL to 1 ng/mL of PFOS were used to verify PFOS retention time (approximately 4.5 seconds). The product ion was optimized by injecting 10 µL of each calibration standard. Using Analyst, a LC-MS data system software, a linear regression calibration curve was constructed based off 6 calibration standards (500, 250, 100, 15, 5, and 1 ng/mL) with an r^2 =0.99. 10 µL of each environmental sample was injected into the mass spectrometer. The solvent gradient started at 40% of 0.1% ammonium hydroxide in methanol from 0.01-0.1 mins, up to 80% at 0.6 mins, up to 100% at 2 mins and remained at 100% until 3.5 mins, dropped back down to 40% at 3.51 mins and remained at 40% until the controller stopped the program at 6.5 mins. The MRM ion pair used to quantify PFOS was 498.9/79.8.

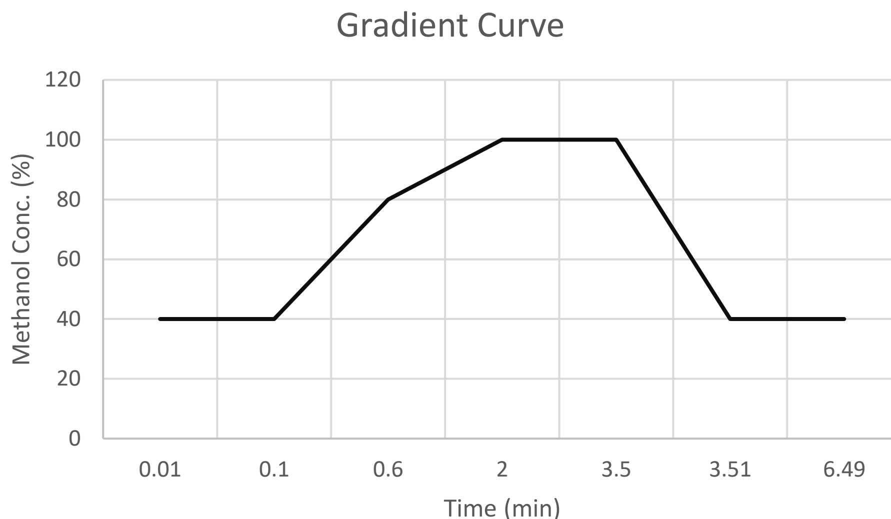


Figure 6. Gradient curve for running solvents (0.1% ammonium hydroxide in methanol & water).

Results

PFOS reduces fecundity in Daphnia magna

A 21-day chronic toxicity test was performed to determine the reproductive effects of PFOS. PFOS decreased daphnid survival in a concentration-dependent manner with significant decreases at 1000 and 5000 ppb (**Fig. 7**). None of the 5000 ppb-exposed daphnids survived past day 3.

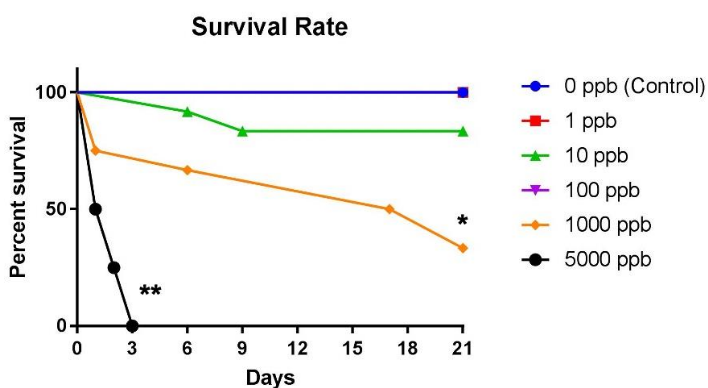


Figure 7. Effects of PFOS on mortality. Data were determined by chi-squared analysis and are expressed as the total number of deaths from an original population of 12. Statistical differences between the survival curves were assessed using Mantel-Cox test, Logrank test for trend, and Gehan-Breslow-Wilcoxson test. ** denotes significant difference with p-value<0.0001, * denotes significant difference with p-value<0.05.

Fecundity was also reduced in a concentration-dependent manner in daphnids exposed to higher PFOS concentrations by day 13 (**Fig. 8a**) in the 100 ppb (29.7%) and 1000 ppb (33.9%)

exposure groups. By day 21 the 100 ppb and 1000 ppb groups showed 16.9% and 39.2% reduction of fecundity compared to the control, respectively. This is consistent with previous results with *D. magna* that demonstrated concentration-dependent reductions in fecundity starting at about 40 ppb (Lu et al. 2015). From survival and reproduction, the net reproductive rate (R_0) was determined (**Fig. 8b**). R_0 is a ratio of the number of offspring produced over an organism's entire lifespan that also takes survival into consideration. Because it combines the effects of survival and fecundity, it is a more sensitive marker of the adverse effects of a chemical that estimates the effects of PFOS on total population growth (Lu et al. 2015). Net reproductive success (R_0) was reduced by PFOS in *D. magna* in a concentration-dependent manner starting at 10 ppb (**Fig. 9**).

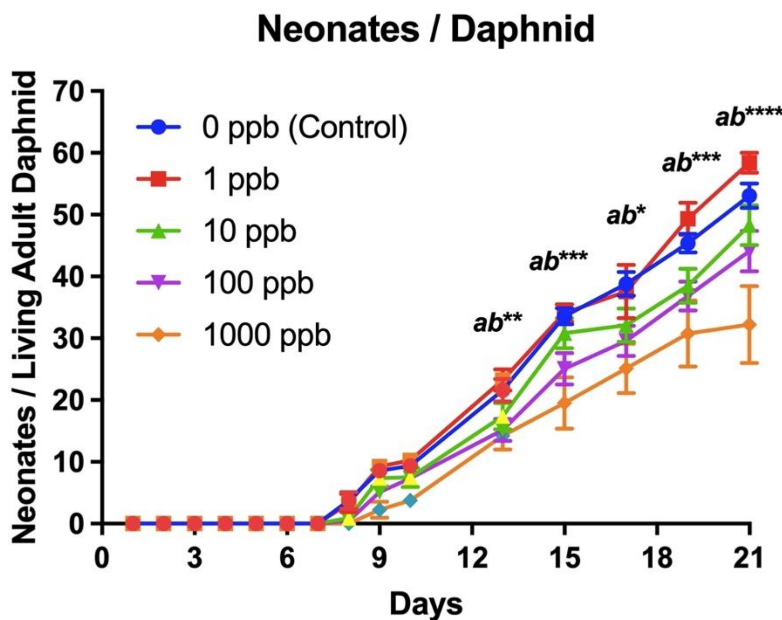


Figure 8. PFOS decreases fecundity and in a concentration-dependent manner. (a) Fecundity was determined as described in the Materials & Methods. Differences were determined by ANOVA followed by Fisher LSD and are expressed as mean ± SEM (n = 12). Asterisks denote an overall significant difference from the control. The treatment groups that are statistically different are noted with an 'a' (100 ppb) or 'b' (1000 ppb),and **p-value <0.01, ***p-value<0.005, and ****p-value<0.0001.

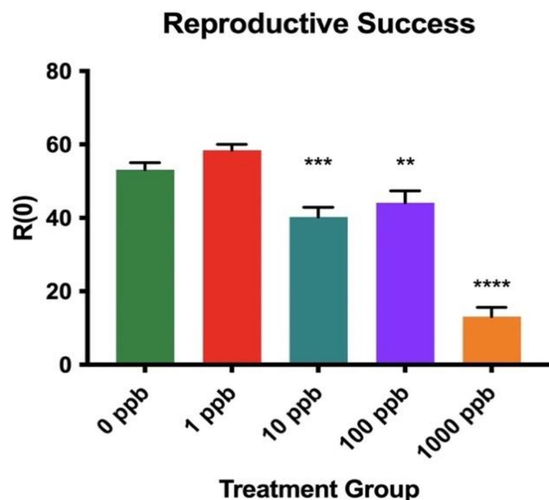


Figure 9. PFOS reduces reproductive success and population size in *D. magna*. Over one lifespan, reproductive success declined with higher concentrations of PFOS; determined as described in the Materials and Methods. Differences were determined by one-way ANOVA followed by Fisher's LSD and are expressed as mean ± SEM. Significant differences are denoted by ** ($p<0.01$), *** ($p<0.005$), and **** ($p<0.0001$).

Parameters that may affect fecundity such as age at first brood, number of broods, and neonates/brood were determined. The most sensitive parameter was neonates / brood, which was reduced in *D. magna* treated with 100 and 1000 ppb PFOS (**Fig. 10a**). The number of broods produced per daphnid (**Fig. 10b**) was not significantly altered and the age at first brood was only weakly increased in the 100 ppb group (**Fig. 10c**). Overall, The R_0 indicates reproductive success is 35.77% and 86.50 % lower in the 10 ppb and 1000 ppb PFOS-exposed groups, respectively. The decline in reproductive success, in combination with high mortality rate, will reduce population size. Most of this drop is due to mortality in the 1000 ppb group making adult survival the key factor in reproductive success. Of the parameters that measure reproduction, the primary driver of reduced reproduction is a change in the number of neonates produced in each brood. Other studies have also shown reduced survival due to similar concentrations of PFOS although not as powerfully as our data and the key driver of reproductive success was neonates per brood. (Ji et al. 2008; Lu et al. 2015).

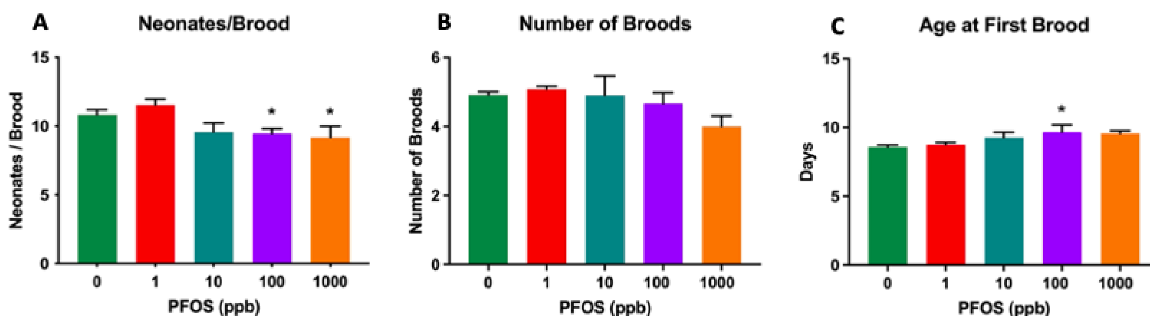


Figure 10. PFOS primarily decreases fecundity by reducing the number of neonates per brood in a concentration-dependent manner. (a) Neonates produced per brood. (b) Number of broods produced per adult daphnid. (c) Age of the average daphnid when they produced their first brood. Data were determined by ANOVA followed by Fisher's LSD and are expressed as mean ± SEM (n = 12). * denote significant difference from the control (p-value < 0.05).

<u>Bioconcentration of PFOS in D. magna</u>

PFOS concentrations were measured from pooled samples of 3 whole daphnids in each treatment group of 12 (n = 4). PFOS concentrations increased in a concentration-dependent manner (**Table 2**). However, concentrations at the 10, 100, and 1000 ppb concentrations were above our standard curve limitations and therefore are in part estimated. These concentrations were compared to measured PFOS concentrations in the media (**Table 3**) to determine bioconcentration factors (BCFs) [BCF = $Concentration_{water}$ / $Concentration_{dapnid}$]. BCFs were determined at each concentration over 21-days and found to decrease with increasing concentrations. Lower BCFs at higher concentrations are due to higher amounts of PFOS accumulating in the tissues over time compared to the steady concentration in the water. Few studies have performed BCFs in invertebrates (Xia et al. 2015) and none have evaluated bioconcentration in *D. magna* at different concentrations to our knowledge. The bioconcentration factors for *D. magna* are 0.372 ± 0.139 (mean ± SEM) for 0 ppb, 0.659 ± 0.331 for 1 ppb, 0.267

± 0.001 for 10 ppb, and 0.399 ± 0.132 for 100 ppb. There was no significant difference found in the BCFs of *D. magna* at any treatment of PFOS.

Table 2. Measured concentrations of PFOS in whole body of *D. magna*.

Concentration (ppb)	Measured (ppb)
0	0.192 ± 0.109
1	1.076 ± 0.280
10	3.556 ± 0.435#
100	25.80 ± 3.630#

Data expressed as mean ± SEM

# indicates data that is above the upper limits of detection.

Table 3. Nominal and measured concentrations of PFOS in the *D. magna* media.

Concentration (ppb)	Measured (ppb)
0	0.558 ± 0.193
1	3.231 ± 1.921
10	22.82 ± 9.540
100	64.65 ± 10.33#
1000	1244 ± 631.7#

Data expressed as mean ± SEM

# indicates that some samples are above the quantitative upper limits of detection using the standard curve and are therefore estimated.

$ Efficiency of water extractions were not determined and based on liver we might expect them to be about 70%.

Bioconcentration of PFOS in F. heteroclitus

PFOS concentrations in the saltwater and freshwater media were measured by LC-MS (**Table 4-5**). Concentrations were higher than expected in the controls of both concentrations, especially the saltwater acclimated water suggesting that there may be PFOS contamination trapped within the seawater salts. The bioconcentration of PFOS in *F. heteroclitus* was determined in liver, gill, and deskinned muscle from fish tested at 0, 10, and 20-days post initial laboratory exposures from saltwater and freshwater acclimated fish trapped from the NERR site in Georgetown, SC. The purpose of this experiment was to compare bioconcentration between freshwater and saltwater fish and use *F. heteroclitus* as a model because of its ability to adapt to either saltwater or freshwater conditions (Shaw et al. 2007).

Table 4. Nominal and measured concentrations of PFOS in *F. heteroclitus* saltwater.

Concentration (ppb)	Measured (ppb)
0	1.899 ± 1.399
0.01	0.358 ± 0.079
1.00	22.000 ± 1.446

Data expressed as mean ± SEM

Table 5. Nominal and measured concentrations of PFOS in *F. heteroclitus* freshwater.

Concentration (ppb)	Measured (ppb)
0	0.015 ± 0.005
0.01	0.071 ± 0.006

1.00	24.476 ± 4.040

Data expressed as mean ± SEM

PFOS concentrations were the highest in the liver tissue out of the tissues that were analyzed (**Table 6-8**). Liver PFOS concentrations increased over time and exposure concentration with only the 100 ppb concentration showing statistically significant increases by two-way ANOVA probably because of high background concentrations in the wild captured fish (**Table 6; Fig. 11**). The concentration of PFOS in the liver tissue was higher in saltwater acclimated mummichogs than freshwater acclimated mummichogs in the 100 ppb treatment group (**Table 6; Fig. 12**). PFOS concentrations were also significantly increased in the 1 ppb relative to control and group if the analysis was performed by one-way ANOVA (p-value < 0.001). Previous studies have shown increases in PFOS at higher salinities in the liver of other euryhaline fish species (Jeon et al. 2010).

Table 6. PFOS Concentrations in the liver of freshwater and saltwater acclimated fish.

	Freshwater		Saltwater	
Concentration (ppb)	Day 10	Day 20	Day 10	Day 20
0	0.119 + 0.076		0.579 ± 0.121	
0.01	1.092 ± 0.289	2.429 ± 1.134	0.075 ± 0.024 ^{b***}	0.225 ± 0.064 ^{b**}
1.0	1.932 ± 0.612	2.379 ± 0.656	0.527 ± 0.073 ^{b***}	7.086 ± 1.337 ^{b***}
100	7.755 ± 1.731^{a*}	11.78 ± 3.733^{a**b**c*}	32.10 ± 15.77 ^{a}	61.21 ± 26.23 ^{a***c*}

Data presented as mean ± standard error mean. Statistical significance was determined by 2-way ANOVA followed by Uncorrected Fisher's LSD and ordinary one-way ANOVA.

'a' indicates difference from day 0.

'b' indicates difference over time in corresponding treatment.

'c' indicates difference between salinity.

Letter with no asterisk indicates p-value <0.05.

* indicates p-value < 0.01.

** indicates p-value < 0.001.

*** indicates p-value < 0.0001.

PFOS concentrations in the gills were lower than the liver but higher than the muscle tissue in *F. heteroclitus* (**Table 6-8**). The concentration of PFOS in the gill tissue differs from that of the liver tissue in that the freshwater, not the saltwater-acclimated mummichogs, showed the greatest increase in comparison to day 0. There were no significant differences in gill concentrations caused by salinity (**Table 7**; **Fig. 11**). However, gill PFOS concentrations were only measured in the 0, 0.01, and 1 ppb groups; not the 100 ppb group because of loss of individual samples (death and small gill size in which extractions were not possible). Liver concentrations were also often greater in the freshwater fish at lower concentrations (day 10 – 0.01 and 1.0 ppb) similar to gills (**Table 7,8**; **Fig. 12**).

Table 7. PFOS concentrations in the gill of freshwater and saltwater acclimated fish.

	Freshwater		Saltwater	
Concentration (ppb)	Day 10	Day 20	Day 10	Day 20
0	0.391 ± 0.185		0.179 ± 0.094	
0.01	2.415 ± 0.742[a**c**]	0.980 ± 0.251[b]	0.447 ± 0.318[c**]	0.278 ± 0.141
1.0	1.523 ± 0.281[a]	1.672 ± 0.320[ac]	0.766 ± 0.228	0.594 ± 0.337[c]
100				

Data presented as mean ± standard error mean. Statistical significance was determined by 2-way ANOVA followed by Uncorrected Fisher's LSD and ordinary one-way ANOVA.

'a' indicates difference from day 0.

'b' indicates difference over time in corresponding treatment.

'c' indicates difference between salinity.

Letter with no asterisk indicates p-value <0.05.

* indicates p-value < 0.01.

** indicates p-value < 0.001.

*** indicates p-value < 0.0001.

The concentrations of PFOS in the muscle tissue are lower than the other tissues measured (**Table 6-8**). Once again, saltwater tissue concentrations were higher than the freshwater muscle concentrations at the highest concentration of 100 ppb (**Table 8**; **Fig. 12**).

Table 8. PFOS concentrations in the muscle of freshwater and saltwater acclimated fish.

	Freshwater		Saltwater	
Concentration (ppb)	Day 10	Day 20	Day 10	Day 20
0	0.108 ± 0.043		0.926 ± 0.415	
0.01	0.626 ± 0.465	0.276 ± 0.086	0.609 ± 0.308	0.196 ± 0.116
1	0.542 ± 0.232	0.553 ± 0.208	0.223 ± 0.070	0.221 ± 0.063
100	1.494 ± 0.329[c**]	1.902 ± 0.616	6.242 ± 4.359[a***bc**]	3.849 ± 1.136[a*b]

Data presented as mean ± standard error mean. Statistical significance was determined by 2-way ANOVA followed by Uncorrected Fisher's LSD and ordinary one-way ANOVA.

'a' indicates differences from day 0.

'b' indicates difference over time in corresponding treatment.

'c' indicates difference between salinity.

Letter with no asterisk indicates p-value <0.05.

* indicates p-value < 0.01.

** indicates p-value < 0.001.

*** indicates p-value < 0.0001.

The BCF was calculated by comparing the dry weight (ng/mg) of tissue to the concentration of PFOS in the media (ng/mL). The BCF of *F. heteroclitus* gills, liver, and muscle significantly decreased from the lowest treatment group in freshwater while the BCF of the corresponding tissues in saltwater show no significant difference (**Table 9**).

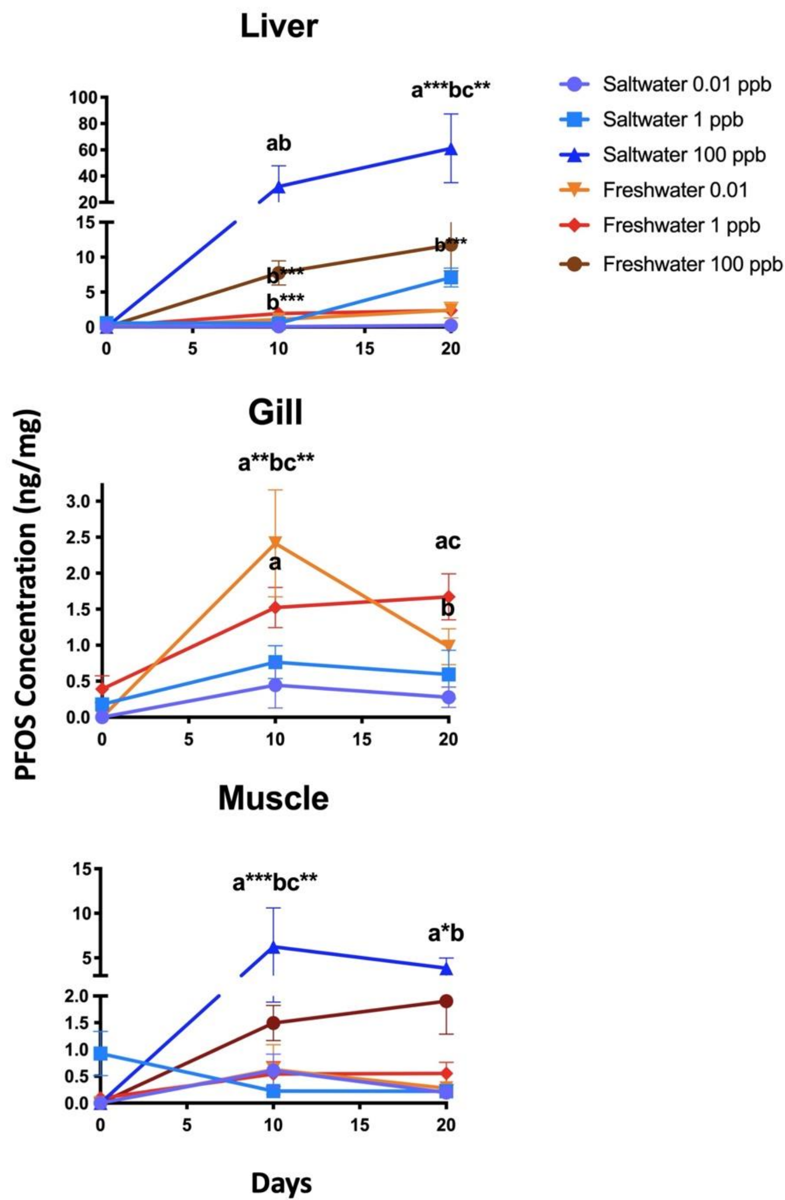


Figure 11. PFOS concentration in tissues of *F. heteroclitus* are affected by salinity. Concentrations of PFOS in the liver, gills, and muscle in both freshwater and saltwater over 20 day tests show that PFOS concentrations differ between saltwater and freshwater among PFOS treated groups. Significant differences determined by 2-way ANOVA followed by Uncorrected Fisher's LSD. An [a] indicates difference from day 0. An [b] indicates difference in time. An [c] indicates difference in salinity. Letter with no asterisk indicates p-value <0.05. * indicates p-value < 0.01. ** indicates p-value < 0.001. *** indicates p-value < 0.0001.

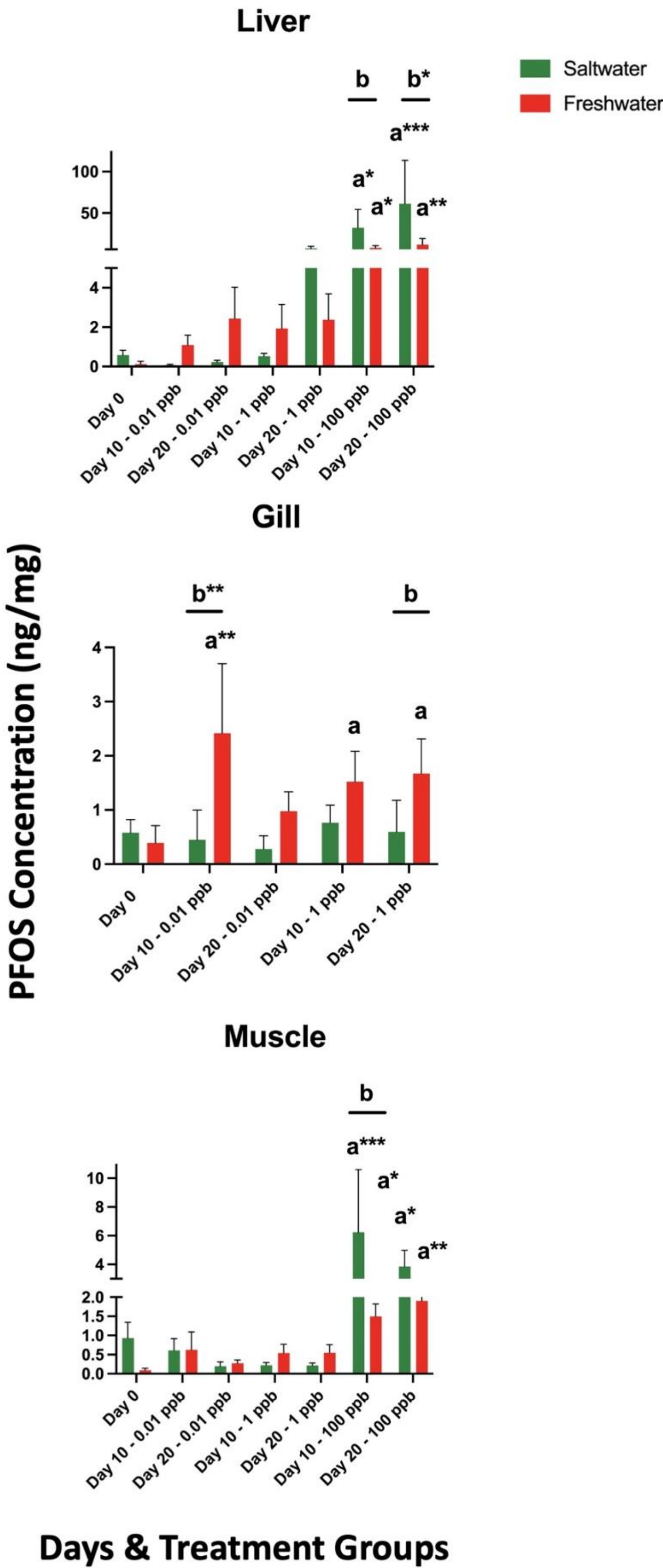


Figure 12. PFOS concentration in tissues of *F. heteroclitus* differ between saltwater and freshwater. Concentrations of PFOS in the liver, gills, and muscle in both freshwater and saltwater over 20 days show that PFOS concentrations differ between saltwater and freshwater between PFOS treated groups. Significant differences determined by 2-way ANOVA followed by Uncorrected Fisher's LSD. An [a] indicates difference from day 0 of either saltwater or freshwater. An [b] indicates difference in salinity among the treatment group. Letter with no asterisk indicates p-value <0.05. * indicates p-value < 0.01. ** indicates p-value < 0.001. *** indicates p-value $<$ 0.0001.

Table 9. PFOS Bioconcentration Factor (BCF) in *F. heteroclitus*.

	Freshwater			Saltwater		
Concentration (ppb)	Gill (ppb)	Liver (ppb)	Muscle (ppb)	Gill (ppb)	Liver (ppb)	Muscle (ppb)
0.01	14.839 ± 6.196	34.568 ± 19.207	4.092 ± 1.582	1.155 ± 0.741[a*]	5.038 ± 1.671[a**]	0.746 ± 0.460[a*]
1.00	0.078 ± 0.022[a**]	0.108 ± 0.029[a***]	0.029 ± 0.017[a*]	0.031 ± 0.020[a**]	0.333 ± 0.079[a***]	0.010 ± 0.003[a*]
100		0.118 ± 0.037[a***]	0.019 ± 0.006[a*]		0.612 ± 0.262[a***]	0.038 ± 0.011[a**]

Data expressed as mean ± SEM

Statistical differences determined by two-way ANOVA followed by Uncorrected Fisher's LSD.

[a] indicates differences from 0.01 ppb freshwater.

* indicates p-value < 0.01.

** indicates p-value < 0.001.

*** indicates p-value <0.0001.

Comparison of Bioconcentration between D. magna and F. heteroclitus

The concentration of PFOS in the liver and muscle tissue of *F. heteroclitus* at 1 ppb and 100 ppb PFOS was compared to the whole-body burden of PFOS in *D. magna* at the same corresponding concentrations and times (20/21-days). Invertebrates are often thought to be much less sensitive to PFOS and lower bioconcentration may play a factor in this reduced toxicity (Ji et al. 2008). This also allowed us to compare the bioconcentration of PFOS between two organisms at different trophic levels. The comparison of whole-body *D. magna* to both *F. heteroclitus* liver and muscle provides ratios to high and low concentration tissues in the fish, respectively. Between *F. heteroclitus* and *D. magna*, the accumulation of PFOS was greater in *D. magna* (zooplankton) than in the muscle tissue compared to freshwater and saltwater-acclimated *F. heteroclitus*. PFOS accumulation in *D. magna* was less than that of *F. heteroclitus* liver in both

concentrations of saltwater-acclimated fish and the 1 ppb treatment group of the freshwater-acclimated mummichogs. Only the 100 ppb PFOS liver group in freshwater mummichog showed lower bioconcentration in mummichog liver than *D. magna* (**Table 10**).

Table 10. Ratios of 20-day *F. heteroclitus* muscle and liver - to - *D. magna* tissue concentration.

	Freshwater		Saltwater	
Concentration (ppb)	Muscle	Liver	Muscle	Liver
1	0.514	2.211	0.205	6.586
100	0.074	0.457	0.149	2.372

Data presented as mean

Expression of ionic and xenobiotic transporters in response to PFOS

qPCR was performed on *GR*, *CFTR*, *OAT1*, *NKATPase1a1*, and *P-gp* to determine if changes and salinity or exposure to PFOS alter ionic and xenobiotic transporter gene expression as this may potentially change PFOS bioaccumulation. All the transporters and GR showed significant differences in expression between fish gills exposed to saltwater or freshwater. *NK-ATPase1a1* is a key biomarker of changes in salinity, and it is expected that freshwater will enhance expression (McCormick et al. 2013), which was the case at day 2 or 20. PFOS. The baseline expression of *NKATPase1a1*, *OAT1* and *P-gp* are significantly upregulated in *F.*

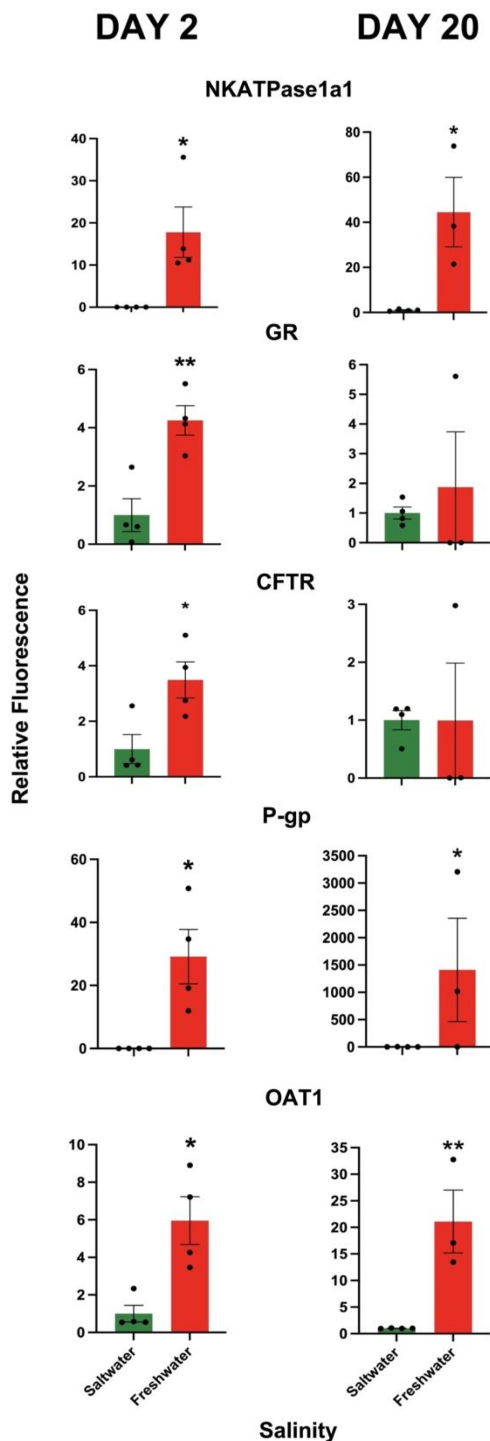


Figure 13. In freshwater conditions without PFOS treatment, gene expression of all transporters *F. heteroclitus* are upregulated. At 0.143 ppt of salinity at day 2, all transporters were upregulated. By day 20, all but GR and CFTR either increased in differential expression. GR and CFTR expression in freshwater were reduced by day 20. Differences were determined by unpaired t-test and are expressed as mean ± SEM (n=3-4). * denotes p-value < 0.05 and ** denotes p-value < 0.01**.**

heteroclitus at day 2 and 20. The increase in expression of ion channels such as *NKATPase* and *CFTR* is supported by transcriptomic analysis that showed freshwater gills of euryhaline fish show a higher percentage of gene homologs for ion channels, G-couple protein receptors, and transmembrane receptors than saltwater gills (Lam et al. 2014). In previous literature, the expression of *NKATPase1a* increases with decreases in salinity, vice versa to *NKATPase1b* (McCormick et al. 2013). OAT1 has been previously shown to eliminate PFOS, so this result may provide a mechanism by which freshwater fish could eliminate PFOS faster than saltwater-adapted fish. *GR* and *CFTR* showed significant differences in expression at day 2 of the experiment, but not day 20. This might be caused by further acclimation to the freshwater and lesser need for the transcriptional changes induced by GR that help the acclimation to freshwater (Shaw et al. 2007). There is some uncertainty as to whether GR and CFTR are good markers of changes in salinity over a long period of time (**Fig. 13**)

because mRNA expression is not reflective of the number of transporter proteins present (Scott et al. 2004).

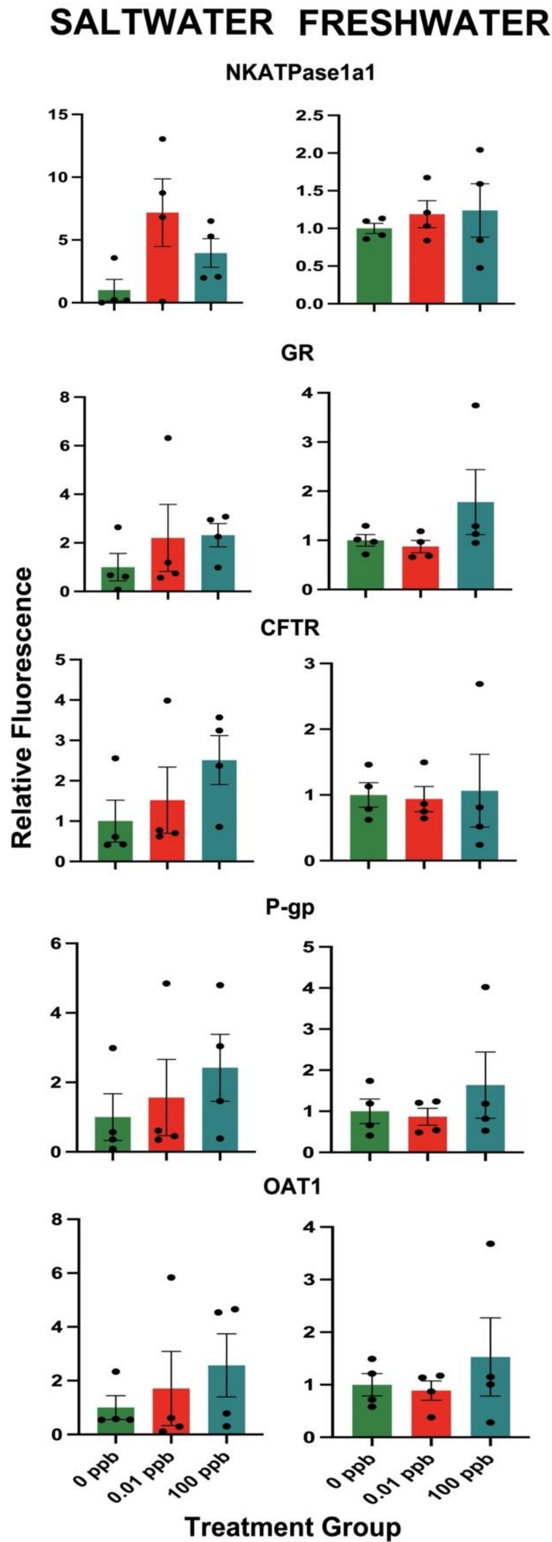


Figure 14. PFOS has no effect on transporter expression at day 2. In both saltwater and freshwater, there were no significant differences in gene expression of transporters in the gills of *F. heteroclitus*. Statistical analysis was performed using one-way ANOVA and data is presenting as mean ± SEM (n=3-4).

At day 2, there were no observed changes in gene expression in response to PFOS exposure (**Fig. 14**). There were similar patterns of increased expression in *GR*, *CFTR*, *P-gp*, and *OAT1* in the saltwater killifish gills, especially at day 2, but no individual gene showed significance. However, after 20-days PFOS significantly reduced the expression of *GR* in saltwater-adapted *F. heteroclitus* (**Fig. 15**). *GR* and *CFTR* in freshwater-adapted *F. heteroclitus* show upregulation in a concentration dependent

manner, however, there are not any statistically significant changes. No other significant alterations were found in the expression of the five genes involved in xenobiotic or ion transport. To our knowledge, no research has been conducted on the effects of PFOS on GR in *F. heteroclitus* gills. In rodents, PFOS inhibits the release of corticosteroids in the hypothalamic-pituitary-adrenal axis (Salgado-Freiria, Lopez-Doval, and Lafuente 2018). Because GR functions to increase mRNA expression of CFTR in response to being activated by cortisol and aids in acclimation to changes in salinity, the downregulation of GR in freshwater-acclimated killifish at day 20 could be the result of cortisol inhibition (Shaw et al. 2007). Furthermore, this data suggests that PFOS may reduce normal environmental plasticity and in turn hinder the fish from responding to normal salinity changes in an estuarine environment.

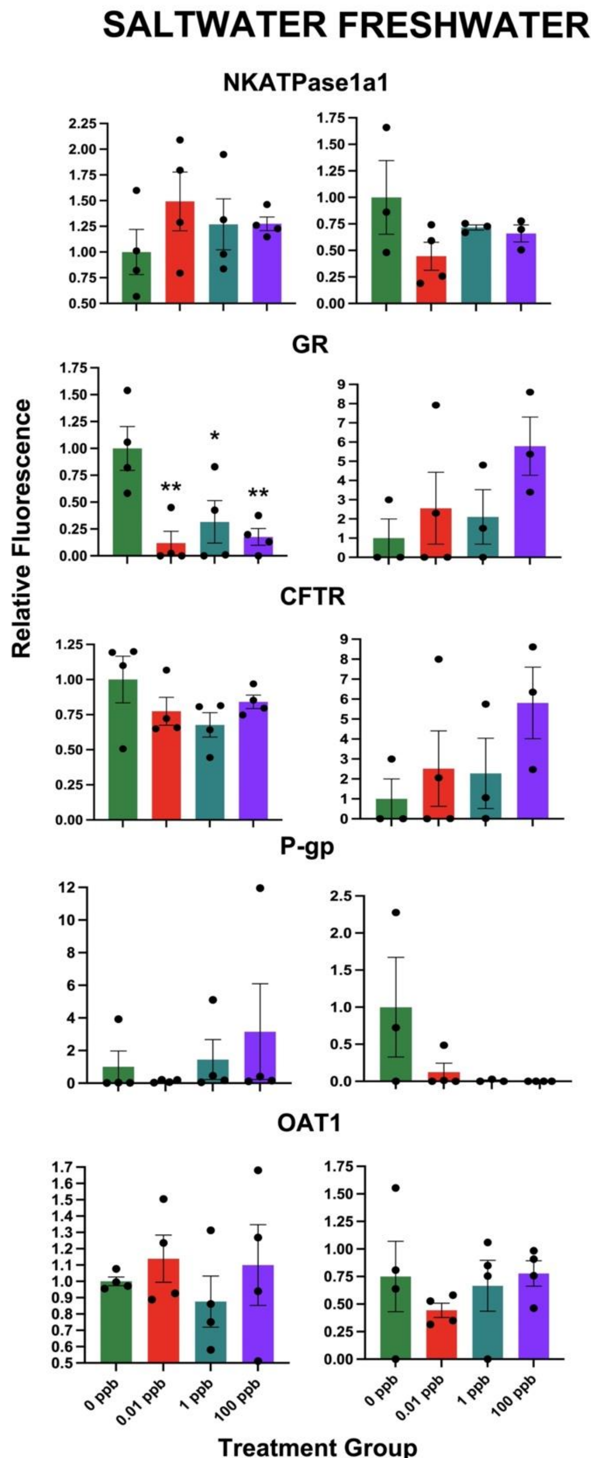


Figure 15. PFOS alters the expression of the glucocorticoid receptor (GR) under saltwater conditions in *F. heteroclitus*. PFOS showed significant reduction in the expression of GR in the 0.01, 1, and 100 ppb treatment groups in *F. heteroclitus* in 23-26 ppt salinity (saltwater). No statistical significance was found in any other transporter under either salinity conditions in response to PFOS exposure. Differences were determined by one-way ANOVA and are expressed as mean ± SEM (n=3-4). * denotes p-value < 0.05 and ** denotes p-value < 0.01.

Discussion

PFOS concentrations in the liver and the muscle tissues at 100 ppb were significantly higher in the saltwater-acclimated mummichogs than the freshwater-acclimated mummichogs, indicating greater bioconcentration in the saltwater-acclimated fish. The ratio of liver and muscle concentrations in the saltwater-acclimated mummichogs compared to the freshwater-acclimated mummichogs were 5.2:1 and 2.02:1 at 100 ppb, respectively. This trend was not as strong and in fact slightly reversed at some of the lower concentrations. The ratio of liver, gill, and muscle concentrations in the saltwater-acclimated mummichogs compared to freshwater-acclimated mummichogs were 2.98:1 , 1: 2.81, and 1: 2.5 at 1 ppb and 1: 10.80, 1: 3.5, and 1: 3.19 at 0.01 ppb, respectively.

In other studies, salinity has been shown to act as a protective mechanisms against xenobiotic uptake due to molecular competition with other ions such as Ca^{+} and Mg^{2+} (Loro et al. 2012). Marine fish species drink significant amounts of water because of osmotic water loss through the gills. Freshwater fish species drink markedly less water as they deal with increased osmotic water uptake of water through the gills in these hypoosmotic condition (Wood and Marshall 1994; Smith 1930). Therefore, PFOS exposure and ultimately bioconcentration may be greater in saltwater-acclimated species through the intestine from drinking water with a first-pass through the liver (Vidal et al. 2019). However, freshwater-acclimated species may be exposed more through the gills without first pass through the liver. Therefore, liver and bioconcentration would be expected to be greater in saltwater-acclimated species through greater influx volume from the gut, and gill bioconcentration greater in freshwater species by uptake through the gill filaments. Muscle tissue levels may be greatly influenced by the greatest influx volume into the fish and therefore may not be altered as much as gill or liver by the route of exposure as by the

amount of water entering the fish. This is what we observed with some caveats. In general, freshwater-acclimated mummichogs showed greater bioconcentration at 0.01 ppb in all tissues and saltwater-acclimated mummichogs showed greater bioconcentration at 100 ppb in all tissues. However, at 1 ppb, freshwater-acclimated mummichogs showed greater bioconcentration in gills and saltwater-acclimated mummichogs showed greater bioconcentration in liver (Tables 6, 7; Figures 11, 12). This is likely caused by the different modes of exposures; gills via freshwater and drinking via saltwater (Wood and Marshall 1994).

PFOS accumulation in the tissues of *F. heteroclitus* by organ are similar to that of other studies that observed PFOS distribution that ranged from highest to lowest from the liver, to the gill, and then lastly to the muscle (Chen et al. 2021). The magnitude of distribution, specifically from muscle-to-liver is greater in the saltwater than in the freshwater-acclimated mummichogs. Although a previous study concluded that 41% of PFOS is proportionally distributed in the muscle tissue of rainbow trout, PFOS concentrations in the muscle tissue were relatively low compared to the gill and the liver (Goeritz et al. 2013). The comparison between PFOS concentration in the liver, gill and muscle tissue are reflected in the results of this study.

The mechanisms by which PFOS transports into or is eliminated out of the gills differs from that of the liver or the muscle to where PFOS's bioaccumulative potential isn't hindered by other molecules in the water. In a study observing the branchial clearance and uptake of PFOS in rainbow trout, the branchial clearance rate is reportedly higher than the renal clearance rate (Consoer et al. 2016). Higher bioconcentrations of PFOS in freshwater tissues at lower levels compared to saltwater could result from the abundance of ion channels and receptors in freshwater-acclimated gills of teleost fish that can accumulate more ions (Lam et al. 2014).

Organic anion transporters (OATs) and their corresponding peptides (OATPs) contribute to the elimination of PFAS through the kidneys in fish species, the upregulation of OAT1 in the gills of freshwater acclimated mummichogs might provide insight into why concentrations of PFOS are lower in freshwater than saltwater acclimated mummichogs. According to another study that conducted toxicokinetic modeling of PFOS in rainbow trout, reportedly >99.99% of ionic PFOS accumulates in the gills (Vidal et al. 2019). Not only are the gills a potentially important route of uptake in fish, low concentrations of PFOS can be absorbed into the gills, which can further explain why PFOS concentrations were quantitatively higher in freshwater than in saltwater at low concentrations. The uptake and elimination of PFOS in the gills can only be speculated by the results of this study. In addition, variations in individual weight must be taken into account because higher ventilation rates in small fish could result in greater rates of PFOS elimination and therefore less bioconcentration (Consoer et al. 2016).

In the *F. heteroclitus* gills, the upregulation of CFTR in this experiment contrasts several studies that indicate higher expression of CFTR in saltwater in the apical membrane of *F. heteroclitus* (Marshall, Lynch, and Cozzi 2002; Scott et al. 2005). However, through immunostaining of mitochondrial rich cells, a potential isoform of CFTR is highly expressed on the basolateral membrane and in the cytosol of *F. heteroclitus* gills adapted to freshwater (Marshall, Lynch, and Cozzi 2002; Scott et al. 2004). Over time, the mRNA expression of both *GR* and *CFTR* decline after 20 days post acclimation (Scott et al. 2004). Similar results were found after 20 days in freshwater-acclimated killifish gills (**Fig. 11**). No known research has been conducted on the expression of *P-gp* and the teleost ortholog to mammalian *OAT1* in the gills of *F. heteroclitus*. Although both *P-gp* and *OAT1* are known to be found in the kidney of

most fish, *P-gp* has been identified in cultured epidermal cells of teleost fish *Oncorhynchus mykiss* (Shuilleabhain et al. 2005).

The ratio of liver: gill: muscle concentrations at day 20 is 32.1: 2.7: 1 in the 1 ppb group in the saltwater-acclimated mummichogs and 4.3: 3: 1 in freshwater-acclimated mummichogs. The ratio of liver: gill: muscle concentrations at day 20 is 1.1: 1.4: 1 in the 0.01 ppb group in the saltwater-acclimated mummichogs and 3.9: 1.6: 1 in freshwater acclimated mummichogs. The ratio of liver: muscle concentrations at day 20 is 15.9:1 in the 100 ppb group in saltwater-acclimated mummichogs and 6.2:1 in freshwater acclimated mummichogs. The general trend at 0.01 ppb and 1 ppb of PFOS treatment, concentrations were higher in the freshwater than in the saltwater for muscle and gill and less so with liver.

PFOS accumulation in liver of *F. heteroclitus* was greater than that of *D. magna* in both concentrations of saltwater-acclimated fish and the 1 ppb treatment group of the freshwater-acclimated mummichogs. Only the 100 ppb PFOS liver group in freshwater showed lower bioconcentration in *F. heteroclitus* liver compared to *D. magna* (**Table 10**). *F. heteroclitus* have a greater propensity to accumulate PFOS than *D. magna* because the comparison is between the liver of *F. heteroclitus*, tissue with a great propensity for retention, compared to whole *D.magna* that includes tissues with less propensity for retention such as muscle. It is possible that *D. magna* eggs increased this retention, but they were not measured separately. Previous research validates the increase in PFOS concentration from species in lower trophic levels to higher, factors such as animal length and size should also be taken into account determine a tighter correlation behind the increased contamination load on more complex organisms (Miranda et al. 2021). In an aquatic food web, zooplankton were reported to have the lowest concentrations of PFOS accumulation while prey fish that mainly consumed zooplankton had the lowest

concentrations of PFOS compared to other fish with the same fatty acid content, but consumed both zooplankton and other small prey fish(Ren et al. 2022). Positive correlation between proteins, fatty acids, and PFOS accumulation, biomagnification of PFOS from *D. magna* to prey fish and then to predatory fish is possible in a food web where prey fish predominately consume *D. magna* (Ren et al. 2022).

D. magna and invertebrates in general show less sensitivity to PFAS chemicals including PFOS with a high LC50 value of 22.43 mg/L. ("Draft Aqautic Life Ambient Water Quality Criteria for Perfluorooctane Sulfonate (PFOS)" 2022). It should be noted that D. magna are less sensitive to PFOS toxicity than most invertebrate species ("Draft Aqautic Life Ambient Water Quality Criteria for Perfluorooctane Sulfonate (PFOS)" 2022). We hypothesized that this could be due to either significantly lower bioaccumulation or the lack of a target such as PPARs. Because our data indicates high bioconcentration in *D. magna*, the lack of PPARs or another unknown target is more likely the reason for less sensitivity in invertebrate species such as *D. magna*. In general, the BCFs in *D. magna* were similar to or lower than BCFs of algae, amphipods and zebra mussel that were determined by the wet weight concentration compared to the media (Kannan et al. 2005). However, the dry weight comparison to these low trophic organisms cannot be concluded.

PFOS perturbation of fecundity, specifically the number of neonates produced per daphnid as well as neonates per brood, was similar to that found in other studies with *D. magna* (Lu et al. 2015). The reproductive success rate (R_0) at the end of the 21-day toxicity test indicated that population size would be significantly reduced by PFOS exposure and can therefore be a good indicator of PFOS toxicity within an ecosystem that includes *D. magna* as one of its key primary consumers.

Further research is needed to determine the pathways of PFOS transport and distribution in *F. heteroclitus* as well as its potential mechanisms of toxicity that can lead to reduction in activity and reproduction or an increase in mortality. The same is also true for *D. magna* as it appears that *D. magna* lack some mechanisms of PFOS clearance that may occur in many fish species based on its high whole body BCF. The downregulation of mRNA expression in GR needs to be further investigated to find how PFOS disrupts the CFTR signaling pathway. The increase in expression of *P-gp* and *OAT1* in untreated *F. heteroclitus* in freshwater, as well as other teleost fish, needs further investigation since the expression of xenobiotic transporters in the gills is not well known.

In conclusion, at the highest levels of PFOS in the water, the concentrations of PFOS found in the tissues of *F. heteroclitus* were higher in saltwater-acclimated mummichogs than in freshwater-acclimated mummichogs. At lower levels of PFOS, concentrations were higher in the freshwater-acclimated mummichog compared to the saltwater-acclimated mummichog. The downregulation of *GR* in the gills of *F. heteroclitus* might be an indicator of PFOS's effect on the *CFTR* signaling pathway. Lastly, PFOS accumulates more in the liver of *F. heteroclitus* than in the whole body of *D. magna* while PFOS accumulates less in the muscle of *F. heteroclitus* than in *D. magna*.

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The views expressed in this thesis are those of the author and do not reflect the official policy or position of the United States, Air Force, Department of Defense, or the U.S. Government.

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Chapter III: Summary and Conclusions

Although the use of PFOS in manufacturing processes was phased out in the early 2000s ("Drinking Water Health Advisory for Perfluorooctane sulfonate (PFOS)" 2016), the longevity of PFOS in the environment and its ability to bioaccumulate in aquatic organisms, such as zooplankton and fish, makes it a continued risk to human populations that consume seafood as a regular staple in their diet (Gribble et al. 2015). It is important to determine how physiochemical properties in water affect its accumulation in aquatic organisms, as concentrations of PFOS in fish tissues have been reported to be affected by salinity (Jeon et al. 2010). High salinity environments may protect against toxicity of some environmental contaminants (Loro et al. 2012), however, other studies have reported that PFOS sorption to surfaces and accumulation in the tissues increases with the concentration of Ca^{+} and Mg^{2+} in the water (Chen et al. 2012; Jeon et al. 2010). Understanding how salinity affects PFOS bioconcentration from the water into other organisms is relevant to determining how PFOS will accumulate in humans.

Daphnia magna serve as an effective biomarker for PFOS toxicity due to its lack of PPARs which are key receptors and targets of PFOS toxicity (Litoff et al. 2014). In *Daphnia magna*, PFOS reduces survival, the number of neonates produced per daphnid and reproductive success in a concentration dependent manner with the greatest perturbations in reproduction found at 100 and 1000 ppb. These findings verify previously identified indicators of PFOS toxicity in *Daphnia magna* while the mortality rate experiences in this study is greater than other chronic toxicity tests performed in the past (Lu et al. 2015).

The accumulation of PFOS solely from the media has not been the focus of other studies on PFOS bioconcentration, mainly because PFOS's main route of accumulation is through food (Goeritz et al. 2013). However, the impact of PFOS in the water should be taken into account

since its accumulation in water and ability to be taken up through drinking, contributes to the total body burden of PFOS in aquatic organisms (Wood and Marshall 1994). The focus for this study was to determine the bioconcentration of PFOS from the water in the tissues of *D. magna* and is the first study, to our knowledge, to determine the BCF of PFOS in *Daphnia magna*. The BCF found for *D. magna* was lower than other reported BCFs determined in other invertebrate species, however, this study aimed to determine the dry mass of PFOS in the tissue and eliminate the additional burden of PFOS in water contained within the body cavity.

The overarching effect of salinity on PFOS accumulation is that bioconcentration of PFOS in the tissues of teleost fish are greater in a hypersaline environment compared to hyposaline environment (Jeon et al. 2010). PFOS concentrations in *F. heteroclitus* were markedly higher in the liver and muscle of saltwater-acclimated fish than in the same organs in the freshwater-acclimated fish when present at higher levels in the water. But the contrasting results found at 0.01 and 1 ppb of PFOS treatment in all tissues must be further explained. Because the presence of ions in the water can affect the activity of larger molecules, water partitioning and sorption models might be useful in determining how the uptake of PFOS into the gills of *F. heteroclitus* is either inhibited or facilitated, depending on the concentration in the water.

The importance of comparing the bioconcentration of two species of different trophic levels is to determine if complexity, size, and ability to metabolize and eliminate PFOS plays a role in its accumulation (Miranda et al. 2021). This study is the first to compare bioconcentrations of PFOS between a vertebrate and invertebrate species. The accumulation of PFOS in the liver and muscle of *F. heteroclitus* was compared to the whole-body burden of *D. magna*. The observed ratios between the tissues and species showed greater accumulation in *F.*

heteroclitus liver at higher concentrations while *D. magna* had greater accumulation of PFOS compared to *F. heteroclitus* muscle. To get a better representation for the total accumulation of PFOS between species, the body burden of PFOS in *F. heteroclitus* should be measured. Overall, PFOS accumulation in the aquatic environment is dependent on its concentration, the physiochemical properties of the water system, and the organisms that are found within the affected ecosystem.

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