Effects of Environmentally Relevant PFOS Concentrations on Unionid Bioaccumulation, Benthic-pelagic Coupling, and Gene Expression

by

Amanda Jo Strozier

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 7, 2021

Keywords: Unionid, PFOS, bioaccumulation, benthic-pelagic coupling, gene expression

Approved by

Jim Stoeckel, Chair, Associate Professor of Fisheries, Aquaculture, and Aquatic Sciences
Elise Irwin, Associate Professor of Fisheries, Aquaculture, and Aquatic Sciences
Dean Schwartz, Associate Professor of Physiology

Abstract

Per- and polyfluoroalkyl substances (PFAS) are persistent organic chemicals that have high energy carbon and fluorine bonds that make them resistant to metabolic breakdown and degradation. These substances are emerging containments of interest that are very widespread in the environment today. Perfluorooctanesulfonic acid (PFOS) is a type of PFAS that was created in the 1950s and can be found in fire prevention agents, weatherproof clothing, and other everyday household items. Alabama waterways host the highest diversity of unionid mussels (Unionidae) in the world but the majority of the species in the state are threatened and/or declining. PFOS has been detected in many of Alabama waterways and poses a potential threat to unionids. In the present study, we examined the effects of feeding on unionid bioaccumulation of PFOS, the effects of unionid size on tissue and sediment PFOS concentration, whether the presence of unionids resulted in the enrichment of PFOS in surrounding sediments, and if environmentally relevant concentrations of PFOS caused a gene expression change in the gills and mantle tissue of male and gravid female unionids. We found that bioconcentration of PFOS by fasted unionids was greater than bioaccumulation by fed unionids, but no evidence that PFOS body burden was affected by size of adult unionids. Sediment PFOS concentrations increased significantly in the presence of unionids suggesting that bivalves may affect non-trophic pathways of PFOS bioaccumulation in benthic invertebrates via benthic-pelagic coupling. We found no significant response in Bag 4, HSP 70 or Krüppel 5 gene expression in either the male and female gills or in female mantle tissue. However, there was a 2.37-fold change in the Krüppel 5 gene expression in the male mantle tissue suggesting that environmentally relevant PFOS concentration may influence pathways regulated by the Krüppel 5 gene.

Acknowledgments

First, I would like to thank Dr. Jim Stoeckel for his support and guidance during my time at Auburn University. Thank you for taking a chance on me allowing me the opportunity to explore my interests (sharks and rays) and teaching me about freshwater benthic organisms. I would also like to thank you Dr. Elise Irwin and Dr. Dean Schwartz for being great committee members and amazing teachers. Dr. Elise Irwin thank you for making me an adopted member of your lab and being there for me when I needed to talk to someone. Dr. Dean Schwartz, thank you so much for your support and patience on the gene expression analysis (who knew we would have a global pandemic right in the middle of everything). I would like to thank Dr. Hisham Abdelrahman, who provided massive statistical support and Dr. Vanisree Mulabagal for analyzing all my samples. A big thank you to Kaelyn Fogelman, who helped during my time at Auburn with preparing and formatting my presentations. I would like to recognize and say thank you to Christine Camp for being a great mentor and friend. I could not have gotten through this journey without you!

Last but not least, I would like to thank my best friend, Michael Freeman, for all of his encouragement, patience, and support as I pursued my master's degree. Thank you so much for believing in me.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

PFAS	Per- and polyfluoroalkyl substances
PFOS	Perfluorooctanesulfonic Acid
PFOA	Perfluorooctanic acid
SAFRS	South Auburn Fisheries Research Station
SAFW	Soft artificial freshwater
DI	Deionized water
HDPE	High-density polyethylene
RO/DI	Reverse osmosis deionized water
MPFOS	Sodium perfluoro-1-[1,2,3,4-13C4] octanesulfonate
SPE	Solid phase extraction
LC	Liquid chromatography
rpm	Revolutions per minute
UHPLC-MS/MS	Ultra-high performance liquid chromatography tandem mass spectrometry
IDL	Instrument Detection Limits
DNA	Deoxyribonucleic acid
HSP	Heat-shock protein
TNF	Tumor necrosis factor
KLF5	Krüppel-like factor 5
RT-PCR	Real-time Polymerase Chain Reaction
RNA	Ribonucleic acid
cDNA	Complementary DNA

Chapter 1: PFOS, Body Burden and Benthic-Pelagic Coupling

1.1 Introduction

Per- and polyfluoroalkyl substances (PFAS) are persistent organic chemicals that have high energy carbon and fluorine bonds that makes them resistant to metabolic breakdown and degradation (Bossi et al. 2005). The fluorocarbon chain has hydrophobic and hydrophilic properties which make it repel water and oil (Rotander et al. 2015). These substances are emerging contaminants of interest that are very widespread in the environment today (Li 2009).

Perfluorooctanesulfonic acid (PFOS) is a type of PFAS was created and used in the 1950s for industrial material and can be found in fire prevention agents, weatherproof clothing and other everyday household items (Li 2009, Amraoui et al. 2018). PFOS has been described as a non-volatile chemical and is the final degradation product of many other perfluorinated substances (Giesy et al. 2010, Amraoui et al. 2018). PFOS has been shown to adsorb to several different soil and sediment types through chemisorption (Giesy et al. 2010).

Along with perfluorooctanic acid (PFOA), PFOS is one of the most frequently detected PFAS compounds in the environment (Li 2009, Giesy et al. 2010). High levels of PFOS have been found in water and biological samples downstream from airports that conduct firefighting training, and facilities that manufacture or use PFAS (Rotander et al. 2015, Newton et al. 2017).

PFOS is found in many Alabama waterways including the Tennessee River downstream of Decatur, Alabama and in the Tensaw River (Newton et al. 2017). Concentrations of 220 ng/L of PFOS have been recorded in the water of the Tennessee River near Decatur, Alabama. In this same area, 62-87 % of the total PFAS found in the river sediments was comprised of PFOS (Newton et al. 2017).

Alabama waterways host the highest diversity of unionids in the world, but the majority of the species in the state are threatened and/or declining. A potential contributor to these

declines is water pollution. Through the filter-feeding process unionids are exposed to contaminants present in the water column, suspended sediments, and food. Because of their high filtration rate (up to 38 L/day), exposure to PFOS can be substantial (Machtinger 2007).

Bivalves, including unionids, perform important ecosystem functions such as transferring materials from the water column to the sediments via filtration and biodeposition. Biodeposits come in the form of feces (nutrients that have been ingested and digested), and pseudofeces, particles that are not digested (Navarro and Thompson 1997). Nutrients and organic material transferred to the sediments may benefit benthic invertebrates by enhancing food quantity and quality (Vaughn and Spooner 2006). However, bivalves may also increase exposure of benthic invertebrates to waterborne contaminants via this same process. For example, PFOS that has entered the water column has the potential to be absorbed and/or filtered out by unionids and deposited into the sediment in feces or pseudofeces. This could have negative ecological impacts via increased exposure to contaminated sediments or via contaminated feces and pseudofeces that are a potential food source for other benthic organisms (Navarro and Thompson 1997, Vaughn and Spooner 2006). The more food or algae that is available to the unionid the more feces and psudofeces the unionid should produce (Vaughn et al. 2004).

In this study, we addressed the following objectives:

1. Determine if PFOS bioaccumulation in unionid tissues is higher when they are fed, as opposed to when they are fasted.
2. Determine the effects of unionid size and feeding on PFOS concentration in unionid tissue.
3. Determine if sediment PFOS concentrations are higher when unionids are present.

4. Determine the relative importance of feeding and unionid size on PFOS concentrations in sediment.

1.2 Methods

1.2.1. Experimental protocol

To minimize prior exposure and PFOS accumulation in experimental animals, we used *Ligumia subrostrata* that had been raised in an earthen pond for several generations at the Auburn University South Auburn Fisheries Research Station (SAFRS). In February 2020, we collected 21 adult male unionids from the pond, tagged each individual with a FPN 8 mm x 4 mm glue-on shellfish Hallprint tag (Hindmarsh Valley, South Australia 5211, Australia) and randomly assigned them to one of two upweller systems (Haney et al. 2020) at 12 ℃ - the same temperature as the pondwater at time of collection. Temperature was subsequently raised by 1 ℃ per day until reaching the experimental temperature of 25 ℃. Animals were then acclimated to laboratory conditions for an additional 14 days before initiating experiments.

Each upweller contained 70 L of soft, artificial freshwater (SAFW) using a recipe modified from (Smith et al. 1997): 0.0048 g/L $NaHCO_3$, 0.025 g/L $CaSO_4$-H_2O, 0.025 g/L CaC12, 0.015 g/L $MgSO_4$, and 0.002 g/L KCl in reverse osmosis de-ionized (RO/DI) water, yielding a final alkalinity of 120 mg/L and final hardness of 50 mg/L. Water quality was monitored to help ensure the survival of the unionids during the acclimation period. Photoperiod was maintained at 12:12 L:D. Unionids were fed 6.2 ml of a 2:1 mixture of Shellfish diet 1800® and Nanno 3600 (Reed Mariculture, 900 E Hamilton Ave, Suite 100 Campbell, CA 95008) with a GHL Doser 2.1 automatic feeder (GHL USA LLC, 5212 Carolina Beach Road, Wilmington, NC 28412) every 2 hours.

Just prior to each experimental trial, five unionids were randomly selected from the holding tanks and sacrificed via opening the valves and cutting through the adductor muscles. All soft tissue was removed from the shell and frozen in 25 ml plastic bags (Zipper Poly Bags, Auburn University Scientific Supply Store, 36849) at -20 ℃ for future analysis of initial PFOS body burden.

Each experimental system consisted of a 70 L cooler, which served as a common sump, with ten plastic 0.95 L containers suspended above it. SAFW was pumped from the sump to a manifold system which distributed water to individual containers, hereafter cups, which held individual unionids. Water flowed through each cup, draining back into the sump at a rate of 14.4 L/h (Fig. 1.1). Water temperature was maintained at 25 ± 2 ℃. Each cup contained 226.8 g of sand (Quickrete Premium Play Sand) that had been rinsed clear of fine particles with tap water, sterilized with a 10% solution of bleach for >3 h and then rinsed again with tap water, followed by rinsing with SAFW. Prior to placing sand in cups, four 100 g samples of sand were removed from the sand source, placed in high-density polyethylene (HDPE) containers, and stored at -20 ℃ for later PFOS analysis.

During each experimental trial, we used two experimental systems with 10 cups each. Both systems received PFOS but only one system received food. A volume of 70 L SAFW was maintained in each sump via calibrated marks on the inner walls. Water was circulated through each system for 24 hours prior to initiation of a trial. Ten unionids were then randomly chosen from the holding tanks and five individuals assigned to each experimental system such that alternating cups contained either unionid-plus-sand or sand only. Shell length of each unionid was measured to the nearest mm and whole wet mass measured to the nearest gram (Table 1.1). Unionids in the “fed” system received 6.2 ml of a 2:1 mix of Shellfish diet 1800® and Nanno

3600 added to the sump each day, for a nominal concentration of 300,000 cells / ml. The other system did not receive any algae.

A stock solution was made by adding 0.05 g of PFOS (Synquest Laboratories,CAS Number 1763-23-1) to 1 liter of DI/RO water in a HDPE bottle which was covered with a plastic lid and placed on a stir plate. After stirring for >12 hours 250 ml of the resultant solution was diluted with 750 ml DI/RO water. This was repeated three more times from the original solution, resulting in four, 1 L stock solutions at a nominal concentration of 12.5 mg/L of PFOS each. One stock solution was refrigerated and used for experiments within 30 days while the other 3 stock solutions were stored at -20 ℃ for use within 6 months.

After the unionids had acclimated to the experimental system for 24 hrs, 1.12 ml of stock solution was pipetted into the fed and unfed systems for an initial nominal concentration of 200 ng PFOS/L. A preliminary trial in our experimental system was used to estimate how much PFOS was typically lost from the water column within 24 hrs (Fig. 1.2). During experiments, an additional 0.56 ml of stock solution was added to each experimental system every 24 hrs to bring the nominal concentration in the water column back up to 200 ng/L. In order to determine the true concentrations of PFOS in the water column during each trial, we collected replicate 100 ml samples from each system one hour (daily maximum), and 24 hrs (daily minimum) after each PFOS addition. Samples were stored in HDPE 125mL bottles (414004-112, VWR) at -20 ℃ for future analysis. After each sample collection, an equivalent amount of fresh SAFW was added back to the sump to maintain a constant water volume. If the water level was observed to be below the 70 L mark between sampling events, the loss was assumed due to evaporation and the sump brought back up to the 70 L mark with RO/DI water.

We conducted two trials, with each trial lasting for 8 days (one day acclimation to system without PFOS followed by 7 days of PFOS exposure). In the second trial, experimental systems were switched between the fed and unfed treatments such that a given system was not assigned to the same treatment twice. At the end of day 8, the pumps in both systems were turned off and chambers allowed to sit for 1 hour to allow any solids to settle to the bottom. Water was then carefully pipetted out of each chamber. Sand from each chamber was removed and stored in an individual HDPE 500 mL wide mouth jar (89094-102, VWR) at -20 ℃. Experimental unionids were sacrificed and soft tissue removed and stored following the same procedures described previously for the initial unionid samples (Table 1.1).

1.2.2 Sample analysis

Water samples: water samples were thawed to room temperature before analysis. Duplicate water samples from each time interval were combined, spiked with an internal standard (MPFOS, 1 ng/mL) and subjected to cleanup using solid phase extraction (SPE) approach. Prior to loading the sample, SPE cartridges were preconditioned with 0.1% ammonium hydroxide in methanol (4 mL) followed by methanol (4 mL) and the portions were discarded. The cartridges were then rinsed with liquid chromatography (LC) grade water (4 mL) and sample were loaded and eluted by adjusting the flow to drop per second. After sample loading step was complete, SPE cartridges were rinsed with water to eliminate salts stayed on the column. Also, SPE cartridges were washed with 25mM ammonium acetate in water (4 mL) to adjust the pH to 4.0 and the fraction was discarded. Finally, cartridges were dried under vacuum and then eluted with extraction solvents methanol (2 mL) followed by 0.1% ammonium

hydroxide in methanol (3 mL). Both the solvent fractions were combined and filtered through Agilent nylon glass-fiber syringe filters (0.2 µm).

Sediment and unionid tissue samples: Sediment samples were removed from the -20 ℃ storage and freeze-dried for 72 hrs in individual HDPE 500 mL wide mouth jars (89094-102, VWR). Tissue samples were removed from the -20 ℃ storage, and freeze-dried for 96 hrs in individual plastic bags. Tissues were then ground into a fine powder using a mortar and pestle. Ground tissue was transferred to transparent, 50 ml autoclavable polycarbonate centrifuge tubes (21009-342, VWR), and returned to the -20 ℃ freezer. Individual sediment and tissue samples were subsequently thawed to room temperature, spiked with an internal standard (MPFOS, 1 ng/mL) and extracted with 30 ml extraction solvent (methanol/water, 70:30, v/v). Samples were placed on an orbital shaker at 250 revolutions per minute (rpm) for 3 hrs. Each sample was then allowed to stand for 2 minutes, sonicated at 60 Hz for 2 hrs, and centrifuged at 13,000rpm, 4 ℃ for 10 minutes. Sample supernatant was transferred into a HDPE bottle (414004-112, VWR) and diluted to 500 mL with DI water. The diluted extracts were purified using the SPE method described above and eluted with extraction solvents methanol (2 mL) followed by 0.1% ammonium hydroxide in methanol (2 mL), and then filtered through Agilent nylon glass-fiber syringe filters (0.2 µm).

Ultra-high performance liquid chromatography-triple quadrupole mass spectrometry (UHPLC-MS/MS) Analysis: Prior to analysis, all the extracts were spiked with internal standard (MPFOS) to adjust the final concentration to 2 ng/mL and analyzed using UHPLC-MS/MS according to published method (Mulabagal et al. 2018). Samples were quantified with a PFOS calibration curve developed using standard concentrations ranging from 0.05 to 50 ng/mL in 80% methanol in water.

Statistical Analysis: The statistical analysis was carried out using SAS 9.4. All data were tested for normality. An ANOSIM was used to compare the treatment water data from each tank. ANOSIM is an analogue of one-way ANOVA that is used to test for dissimilarities among groups. The effects of unionid dry weight and the presence of food on unionid tissue PFOS concentration was assessed by using an ANCOVA. The effect of feeding and unionid presence on sediment PFOS was assessed using a two-way ANOVA. A multiple linear regression model using backward selection method was used to assess the effect of feeding, unionid mass, and unionid PFOS body burden on sediment PFOS.

1.3 **Results**

The Instrument Detection Limits (IDLs) for PFOS for the Ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry and JetStream ion source (UHPLC-QqQ-MS). Initial unionid tissue samples and initial sediment samples did not show any peak corresponding to PFOS. During trials, there was no significant difference in mean PFOS concentration (ng/L) of the sump water among the treatments and trials (ANOSIM: R=-0.012, p=0.5904) (Figure 1.3) suggesting all unionid and sediments had a similar exposure history. PFOS concentrations were nonlethal as evidenced by a lack of unionid mortality during trials.

PFOS accumulation in sediments

There was no evidence of a significant relationship between mean water PFOS concentration and final sediment PFOS concentrations (ANCOVA: $F_{1,536} = 0.01$, $p = 0.9986$). After removing water PFOS concentration as a non-significant co-variate, we found no effect of food (present/absent) on sediment PFOS (Two-way ANOVA: $F_{1,36} = 0.98$, $p = 0.3298$)

(Fig.1.4A) However, sediment PFOS concentrations were significantly higher when unionids were present (Two-way ANOVA: $F_{1,36} = 7.56$, $p = 0.0093$) (Fig. 1.4B).

Because sediment PFOS concentrations could have been affected by additional variables, we used a multilinear approach to develop a predictive equation for sediment PFOS concentrations after 7 days of exposure when unionids were present. After removing water PFOS concentration as a non-significant co-variate, multilinear regression using the backward selection method showed no evidence of the effect of food ($t_1 = 3.65$, $p = 0.0741$) on final sediment PFOS concentrations when unionids were present. Sediment PFOS concentrations were negatively affected by unionid PFOS body burden (MLR: $t_1 = 3.84$, $p = 0.0013$, partial $R^2 = 0.28$), and unionid soft tissue dry weight dry weight (MLR: $t_1 = 5.59$, $p < 0.0001$, partial $R^2 = 0.40$). The final predictive model (Table 1.2) was determined as:

Sediment PFOS concentration (ng/g sediment dry mass) = $2.5929 – 0.4390 \cdot DW – 0.1409\ PFOS_m$

(adjusted $R^2 = 0.64$, $F_{2,19} = 17.93$, $p < 0.0001$)

Where

$PFOS_m$ = unionid PFOS body burden (ng / g dry tissue mass); range = 2.13- 11.33

DW = unionid tissue dry weight (g); range = 0.5919- 4.1999

2.5929 = the intercept

PFOS accumulation in unionid tissue

There was no evidence for the effect of unionid tissue dry weight as a covariate on unionid tissue PFOS concentration (ANCOVA: $F_{1, 17} = 2.81$, $p = 0.1122$) (Fig. 1.5A). Therefore, it was removed from the model. Feeding had a significant effect on unionid tissue PFOS

(ANCOVA: $F_{1, 16} = 5.02$, $p = 0.0397$), with fed unionids having lower PFOS concentrations than fasted unionids (t-test: $t_{18} = 2.30$, $p = 0.0338$) (Fig. 1.5B).

1.4 Discussion

Benthic ecosystems are sensitive to chemical pollution caused by anthropogenic pressures. PFOS is an emerging contaminant that has been found in almost all ecosystems around the world (Wang et al. 2017). Because PFOS is resistant to metabolic breakdown, degradation, and is bioaccumulative, it is globally an environmental concern (Giesy et al. 2010). Research is now being performed to better understand the effects of PFOS on different types of organisms and ecosystems(Simpson et al. 2021). Aquatic organisms can be exposed to PFOS through food, sediment and water combined (bioaccumulation) or through the water column only (bioconcentration) (Barron 1995). In the present study, we examined whether presence of food affects unionid bioaccumulation of PFOS, and whether the presence of unionids resulted in the enrichment of PFOS in surrounding sediments.

There was no mortality observed in the unionids in the present study. Other studies have shown a 96 h median lethal concentration (LC50) of 69.5 mg PFOS/L for the unionid mussel *Unio ravoisieri*, 3.6 mg PFOS/L for the saltwater shrimp *Mysidopsis bahia,* 10 mg PFOS/L for the freshwater shrimp *Neocardina denticulate*, 9.1 mg PFOS/L for fathead minnows (*Pimephales promelas*), and 7.8 mg/L PFOS for rainbow trout (*Oncorhynchus mykiss*) exposure (Oakes et al. 2005, Qi et al. 2011, Hazelton et al. 2012, Amraoui et al. 2018). All of these lethal thresholds were much higher than environmentally relevant concentrations, suggesting PFOS concentrations reported from the Tennessee River (Newton et al. 2017) are not lethal to a range of aquatic organisms, including unionid mussels. However, it is unknown whether toxicity of

PFOS changes when mixed with additional PFAS compounds that have also been reported from the Tennessee River.

Our hypothesis that fed unionids will accumulate more PFOS than fasted unionids due to exposure from food in addition to ambient water was not supported by the present study. Bioconcentration (exposure from water only) of PFOS by fasted unionids was greater than the bioaccumulation (exposure from water and food) by fed unionids. This indicated that PFOS adsorbed to or absorbed by food over a short period of time was not an important route of exposure to unionids. In a study using *Corbicula fluminea*, (Asiatic clam) it was reported that the presence of food in the water column affected the ventilation rate of these bivalves. Lower algal density in the water column correlated to a higher ventilation rate (Fournier et al. 2006). The ventilation rate of bivalves is defined as the amount of water that flows over the gills over a given time (Winter 1978). A high ventilation rate would mean that the fasted unionids would be subjected to increased exposure to PFOS due to a greater volume of PFOS contaminated water passing over the gills. This may explain the greater bioconcentration of PFOS in fasted unionids compared to the bioaccumulation of the fed unionids. In the wild, unionids are faced with many different stressors (Tuffnail et al. 2009). If one of those stressors caused reduced algae density in PFOS contaminated water, resulting in an increase ventilation rate, exposure to PFOS and subsequent accumulation in unionid tissues may also increase. Studies have shown that PFOS can affect the cellular membrane of the green algae *S. obliquus*, which can cause the cell to absorb other chemicals that may be in the water (Liu et al. 2008). When the green algae had a co-exposure to PFOS (10-40 mg/L) and pentachlorophenol, growth was inhibited (Liu et al. 2009).

We found no evidence that PFOS body burden was affected by size of adult unionids. This is supported by the findings of Robinson et al. (2005), using the oyster, *Saccostrea*

glomerata, and trace metal contaminants. However, in contaminated natural systems, because larger (older) individuals have presumably been exposed to PFOS contamination for a longer time than smaller (younger) individuals, body burden may differ with unionid size. Relationship between PFOS body burden and unionid size in natural unionid beds deserves further study.

Bivalves, such as unionids, play in an important role in benthic- pelagic coupling. Griffiths et al. (2017) defines benthic- pelagic coupling as, "the processes which connects the bottom substrate and the water column habitats through the exchange of mass, energy, and nutrients." Unionids are vital to the health of the benthic ecosystems because they provide organic matter to the benthos through filter-feeding and deposition of nutrient rich feces and pseudofeces in the sediment, cause sediment bioturbation through movement, and provide a food source to predators (Vaughn et al. 2004, Vaughn and Spooner 2006).

Our hypothesis that fed unionids will deposit more PFOS into the sediment than fasted unionids due to increased production of biodeposits was not supported in the present study. However, unionid presence, regardless of feeding, did have a significant, positive effect on sediment PFOS concentration. This finding, coupled with reduced bioaccumulation of PFOS by fed unionids, suggests that feces and pseudofeces may not be highly contaminated by PFOS. Sediment enrichment may occur by alternate routes such as bioturbation during burrowing which increases the sediment-water content (Vaughn and Hakenkamp 2001, Vaughn and Spooner 2006) and/or through the excretion of urine. Studies have shown that urine is a principal depuration route for PFOS compared to feces (Cui et al. 2010, Zhang et al. 2015). Regardless of the specific mechanisms the presence of unionids is likely to significantly increase the amount of PFOS in the sediment, increasing the exposure of PFOS to the benthic organisms that burrow into the sediment. Additionally, it may increase exposure of juvenile unionids. Juveniles tend to

burrow more extensively than adults and be exposed to PFOS by ingesting contaminated sediment via pedal-feeding and/or through direct contact (Yeager et al. 1994, Vaughn and Hakenkamp 2001). The long-term effects on constant exposure to PFOS on juvenile unionids is still unknown (Hazelton et al. 2012).

Our present study provides strong evidence that unionids may affect non-trophic pathways of PFOS bioaccumulation in aquatic ecosystems via benthic-pelagic coupling. Similar effects have been shown for marine mussels exposed to polychlorinated biphenyl (PCB), although in this case, biodeposits were implicated as a primary mechanism (Prince et al. 2021). Lasier et al. (2011) showed that there was a positive relationship with sediment PFOS concentration and macroinvertebrate tissue PFOS concentration. Since PFOS does not biodegrade (Giesy et al. 2010), it could be transferred and biomagnified in other organisms through the food chain (Giesy and Kannan 2001, Kannan et al. 2005) or could be dispersed back through the water column through bioturbation (Vaughn and Spooner 2006).

Although unionids provide important ecological functions to river ecosystems, they may also serve as a vessel for increased PFOS exposure for other organisms, as well as increasing exposure of their own juvenile stages. Our study had a time and financial limitation that allowed us to expose the *Ligumia subrostata* to environmentally relevant PFOS concentration for 7 days. Subjecting unionids to longer exposure times could be beneficial in determining the long-term fate and effects of PFOS in freshwater ecosystems that host large bivalve populations.

1.5 References

Amraoui, I., N. Khalloufi, and S. Touaylia. 2018. Effects to perfluorooctane sulfonate (PFOS) on the mollusk Unio ravoisieri under laboratory exposure. Chemistry and Ecology **34**:324-339.

Barron, M. G. 1995. Bioaccumulation and bioconcentration in aquatic organisms. Handbook of ecotoxicology:652-662.

Bossi, R., F. F. Riget, R. Dietz, C. Sonne, P. Fauser, M. Dam, and K. Vorkamp. 2005. Preliminary screening of perfluorooctane sulfonate (PFOS) and other fluorochemicals in fish, birds and marine mammals from Greenland and the Faroe Islands. Environmental Pollution **136**:323-329.

Cui, L., C.-y. Liao, Q.-f. Zhou, T.-m. Xia, Z.-j. Yun, and G.-b. Jiang. 2010. Excretion of PFOA and PFOS in Male Rats During a Subchronic Exposure. Archives of Environmental Contamination and Toxicology **58**:205-213.

Fournier, E., C. Adam, J.-C. Massabuau, and J. Garnier-Laplace. 2006. Selenium bioaccumulation in Chlamydomonas reinhardtii and Subsequent transfer to Corbicula fluminea: Role of selenium speciation and bivalve ventilation. Environmental Toxicology and Chemistry **25**:2692-2699.

Giesy, J. P., and K. Kannan. 2001. Global Distribution of Perfluorooctane Sulfonate in Wildlife. Environmental Science & Technology **35**:1339-1342.

Giesy, J. P., J. E. Naile, J. S. Khim, P. D. Jones, and J. L. Newsted. 2010. Aquatic Toxicology of Perfluorinated Chemicals. Pages 1-52 Reviews of Environmental Contamination and Toxicology. Springer New York, New York, NY.

Griffiths, J. R., M. Kadin, F. J. A. Nascimento, T. Tamelander, A. Törnroos, S. Bonaglia, E. Bonsdorff, V. Brüchert, A. Gårdmark, M. Järnström, J. Kotta, M. Lindegren, M. C. Nordström, A. Norkko, J. Olsson, B. Weigel, R. Žydelis, T. Blenckner, S. Niiranen, and M. Winder. 2017. The importance of benthic–pelagic coupling for marine ecosystem functioning in a changing world. Global Change Biology **23**:2179-2196.

Haney, A., H. Abdelrahman, and J. A. Stoeckel. 2020. Effects of thermal and hypoxic stress on respiratory patterns of three unionid species: implications for management and conservation. Hydrobiologia **847**:787-802.

Hazelton, P. D., W. G. Cope, T. J. Pandolfo, S. Mosher, M. J. Strynar, M. C. Barnhart, and R. B. Bringolf. 2012. Partial life-cycle and acute toxicity of perfluoroalkyl acids to freshwater mussels. Environmental Toxicology and Chemistry **31**:1611-1620.

Kannan, K., L. Tao, E. Sinclair, S. D. Pastva, D. J. Jude, and J. P. Giesy. 2005. Perfluorinated Compounds in Aquatic Organisms at Various Trophic Levels in a Great Lakes Food Chain. Archives of Environmental Contamination and Toxicology **48**:559-566.

Lasier, P. J., J. W. Washington, S. M. Hassan, and T. M. Jenkins. 2011. Perfluorinated chemicals in surface waters and sediments from northwest Georgia, USA, and their bioaccumulation in Lumbriculus variegatus. Environmental Toxicology and Chemistry **30**:2194-2201.

Li, M.-H. 2009. Toxicity of perfluorooctane sulfonate and perfluorooctanoic acid to plants and aquatic invertebrates. Environmental Toxicology **24**:95-101.

Liu, W., S. Chen, X. Quan, and Y.-H. Jin. 2008. Toxic effect of serial perfluorosulfonic and perfluorocarboxylic acids on the membrane system of a freshwater alga measured by flow cytometry. Environmental Toxicology and Chemistry **27**:1597-1604.

Liu, W., Y.-B. Zhang, X. Quan, Y.-H. Jin, and S. Chen. 2009. Effect of perfluorooctane sulfonate on toxicity and cell uptake of other compounds with different hydrophobicity in green alga. Chemosphere **75**:405-409.

Machtinger, E. T. 2007. Native freshwater mussels. Natural Resources Conservation Service.

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

Navarro, J. M., and R. J. Thompson. 1997. Biodeposition by the horse mussel Modiolus modiolus (Dillwyn) during the spring diatom bloom. Journal of Experimental Marine Biology and Ecology **209**:1-13.

Newton, S., R. McMahen, J. A. Stoeckel, M. Chislock, A. Lindstrom, and M. Strynar. 2017. Novel Polyfluorinated Compounds Identified Using High Resolution Mass Spectrometry Downstream of Manufacturing Facilities near Decatur, Alabama. Environmental Science & Technology **51**:1544-1552.

Oakes, K. D., P. K. Sibley, J. W. Martin, D. D. Maclean, K. R. Solomon, S. A. Mabury, and G. J. Van Der Kraak. 2005. Short-term exposures of fish to perfluorooctane sulfonate: Acute effects on fatty acyl-CoA oxidase activity, oxidative stress, and circulating sex steroids. Environmental Toxicology and Chemistry **24**:1172-1181.

Prince, K. D., S. M. Crotty, A. Cetta, J. J. Delfino, T. M. Palmer, N. D. Denslow, and C. Angelini. 2021. Mussels drive polychlorinated biphenyl (PCB) biomagnification in a coastal food web. Scientific reports **11**:1-11.

Qi, P., Y. Wang, J. Mu, and J. Wang. 2011. Aquatic predicted no-effect-concentration derivation for perfluorooctane sulfonic acid. Environmental Toxicology and Chemistry **30**:836-842.

Robinson, W. A., W. A. Maher, F. Krikowa, J. A. Nell, and R. Hand. 2005. The use of the oyster Saccostrea glomerata as a biomonitor of trace metal contamination: intra-sample, local scale and temporal variability and its implications for biomonitoring. Journal of Environmental Monitoring **7**:208-223.

Rotander, A., L.-M. L. Toms, L. Aylward, M. Kay, and J. F. Mueller. 2015. Elevated levels of PFOS and PFHxS in firefighters exposed to aqueous film forming foam (AFFF). Environment International **82**:28-34.

Simpson, S. L., Y. Liu, D. A. Spadaro, X. Wang, R. S. Kookana, and G. E. Batley. 2021. Chronic effects and thresholds for estuarine and marine benthic organism exposure to perfluorooctane sulfonic acid (PFOS)-contaminated sediments: Influence of organic carbon and exposure routes. Science of The Total Environment **776**:146008.

Smith, M. E., J. M. Lazorchak, L. E. Herrin, S. Brewer-Swartz, and W. T. Thoeny. 1997. A reformulated, reconstituted water for testing the freshwater amphipod, Hyalella azteca. Environmental Toxicology and Chemistry: An International Journal **16**:1229-1233.

Tuffnail, W., G. A. Mills, P. Cary, and R. Greenwood. 2009. An environmental 1H NMR metabolomic study of the exposure of the marine mussel Mytilus edulis to atrazine, lindane, hypoxia and starvation. Metabolomics **5**:33-43.

Vaughn, C. C., K. B. Gido, and D. E. Spooner. 2004. Ecosystem Processes Performed by Unionid Mussels in Stream Mesocosms: Species Roles and Effects of Abundance. Hydrobiologia **527**:35-47.

Vaughn, C. C., and C. C. Hakenkamp. 2001. The functional role of burrowing bivalves in freshwater ecosystems. Freshwater Biology **46**:1431-1446.

Vaughn, C. C., and D. E. Spooner. 2006. Unionid mussels influence macroinvertebrate assemblage structure in streams. Journal of the North American Benthological Society **25**:691-700.

Wang, Z., J. C. DeWitt, C. P. Higgins, and I. T. Cousins. 2017. A never-ending story of per-and polyfluoroalkyl substances (PFASs)? ACS Publications.

Winter, J. E. 1978. A review on the knowledge of suspension-feeding in lamellibranchiate bivalves, with special reference to artificial aquaculture systems. Aquaculture **13**:1-33.

Yeager, M., D. Cherry, and R. Neves. 1994. Feeding and burrowing behaviors of juvenile rainbow mussels, Villosa iris (Bivalvia: Unionidae). Journal of the North American Benthological Society **13**:217-222.

Zhang, T., H. Sun, X. Qin, Z. Gan, and K. Kannan. 2015. PFOS and PFOA in paired urine and blood from general adults and pregnant women: assessment of urinary elimination. Environmental Science and Pollution Research **22**:5572-5579.

Tables

Table 1.1: The length and weight measurements for the male *Ligumia subrostrata* in trial 1 and 2. The wet weight is the visceral mass tissue at the time of dissection and dry weight is after the visceral mass tissue had been freeze-dried.

Trial 1						
Tag Number	Length (mm)	Fed/ Fasted	Before Total Body Weight (g)	End Total Body Weight (g)	Wet Weight (g)	Dry Weight (g)
Y659	86.86	NA	55.6	55.6	23.6	4.19535
Y660	81.6	NA	43.3	43.3	15.8	2.9746
Y661	73.84	Fed	37	36.2	12.3	2.1263
Y662	74.41	Fed	35.6	40.2	11.6	1.9892
Y663	80.8	Fed	45.4	43.8	15.6	2.81
Y664	92.46	Fasted	63.4	67.9	22.2	4.1999
Y665	59.92	Fasted	20.3	20.7	6.5	0.8629
Y666	77.26	NA	36.4	36.4	14.9	2.6156
Y667	74.58	Fasted	37.6	35.5	12.5	2.2114
Y668	83.64	NA	51	51	18.3	3.5871
Y669	77.1	Fed	36.5	36.9	13.3	2.29
Y670	87.79	Fed	47.7	46.4	17.3	2.9648
Y671	86.69	Fasted	58.6	58.8	18.8	3.5274
Y672	70.15	NA	29.6	29.6	11.4	2.1012
Y673	76.14	Fasted	38	35.6	11.1	1.8096

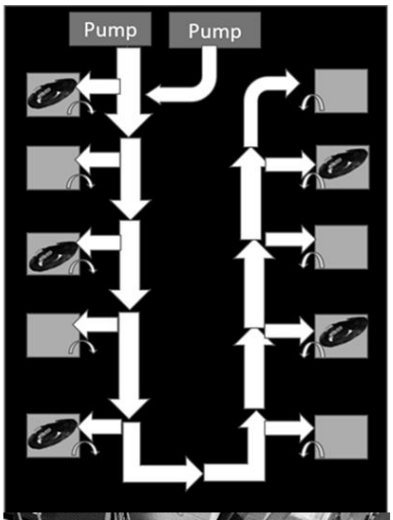
Trial 2						
Tag Number	Length (mm)	Fed/ Fasted	Before Total Body Weight (g)	End Total Body Weight (g)	Wet Weight (g)	Dry Weight (g)
Y675	88.36	NA	44.3	44.3	12.9	2.0495
Y676	68.32	Fasted	24.6	25.2	5.4	0.608
Y677	77.25	Fasted	40.3	40.9	8.7	1.2844
Y678	64.33	Fed	26.4	25.5	5	0.5919
Y681	65.39	Fed	24.3	24	5.3	0.6271
Y682	64.2	Fasted	23.6	22.3	5.5	0.7199
Y683	76.96	NA	37.1	37.1	11.2	1.7578
Y684	64.07	NA	22.5	22.5	6.2	1.0254
Y685	77.22	Fed	39.3	40.3	11.5	1.7671
Y686	69.26	Fed	28.7	28.3	8.3	1.3637
Y687	76.73	NA	32.1	32.1	6.7	0.8197
Y688	75.96	Fed	36.8	36.3	8.8	1.102
Y689	73.12	Fasted	29	28.5	7.7	1.1427
Y691	69.34	NA	26.7	26.7	7.2	0.9065
Y694	76.42	Fasted	37.1	37	10.4	1.5896

Table 1.2. Significant variables and associated statistics for final model predicting PFOS concentrations in sediments when unionids were present. Presence of food was not a significant variable.

Variable	DF	Estimate	SE	t Value	Pr > |t|	Partial R^2	95%	CI
Intercept	1	2.593	0.296	8.75	<0.0001		1.968	3.218
Unionid dry wt (g)	1	-0.439	0.078	-5.59	<0.0001	0.400	-0.605	-0.273
Unionid PFOS body burden (ng/g dry)	1	-0.141	0.037	-3.84	0.0013	0.279	-0.218	-0.063

Figures

A)



B)

Figure 1.1. A) A depiction of the manifold system that was built for the experiment. The white arrows indicate water flow. Every cup (the grey boxes) had 226.8 grams of wet sediment and one male unionid was placed in alternating cups. The water flowed from a sump, into each cup, and slowly poured out of each cup at a flow rate of 14.4L/hr. B) A photograph of the experimental setup showing the sediment and alternating cups with unionids.

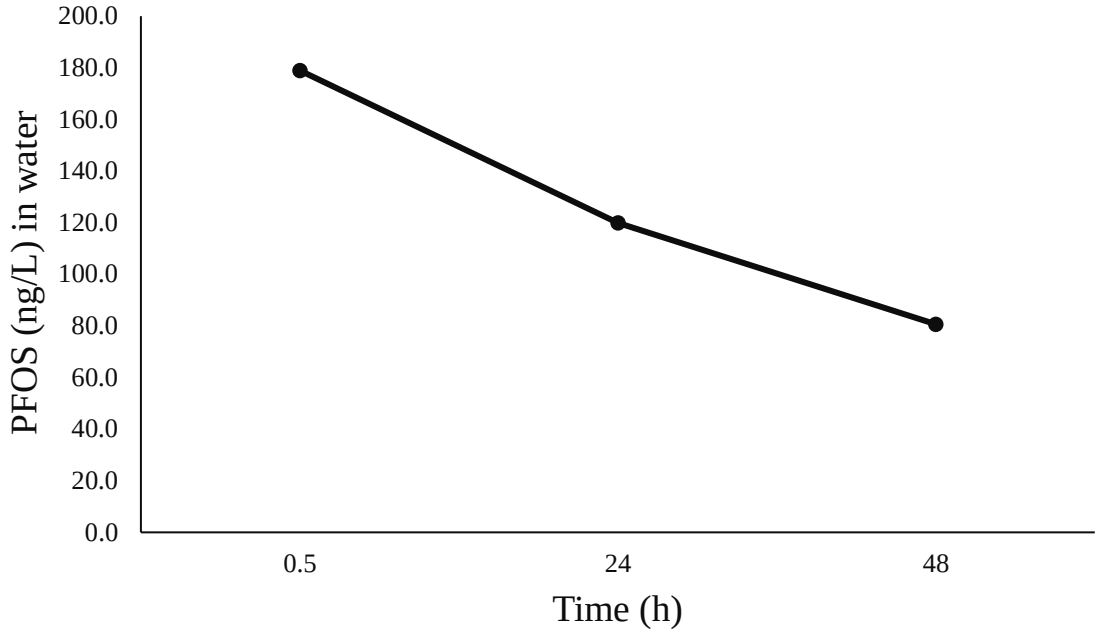


Figure 1.2. Declining PFOS concentration in experimental system water over time. PFOS was added to water at hour 0. Cups in experimental system contained sediment but no unionids.

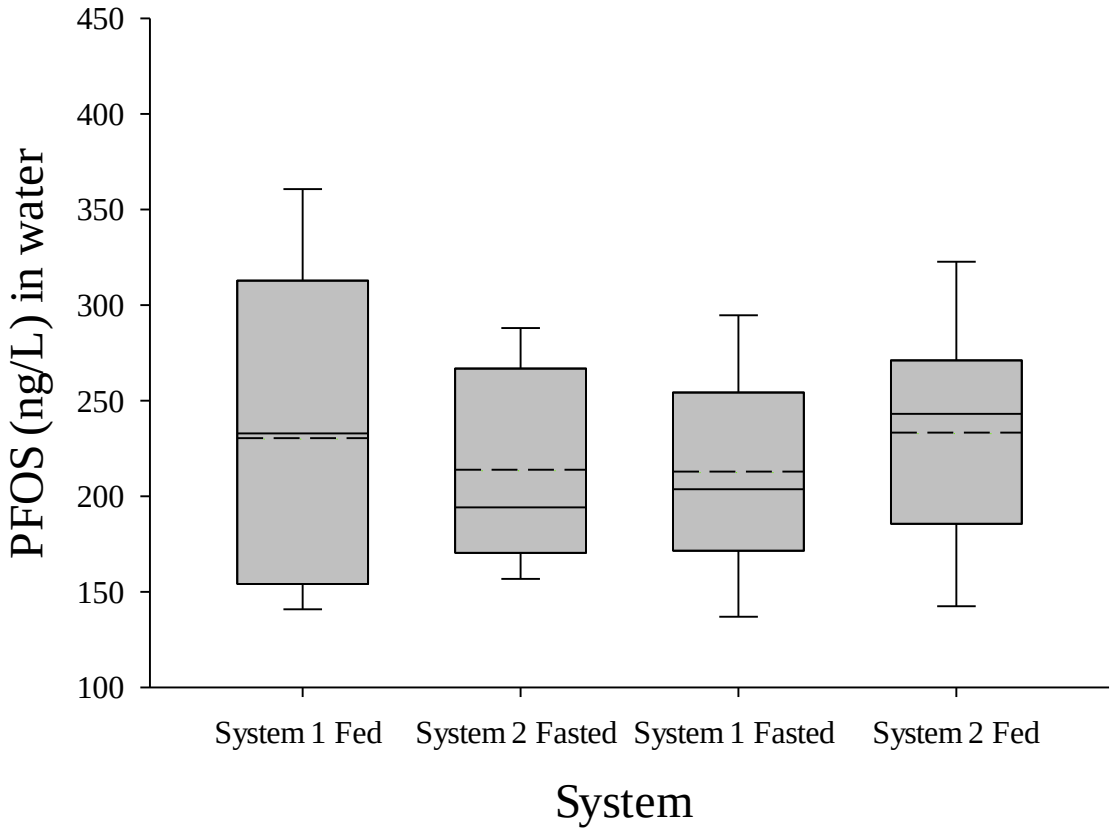


Figure 1.3. Box plot of mean PFOS (ng/L) concentration in the water column of each experimental system over 168 hours. No significant differences were found between trials. Boxes represent the upper and lower quartiles of data. Solid line inside each box represents the median value and the dotted line represents the mean.

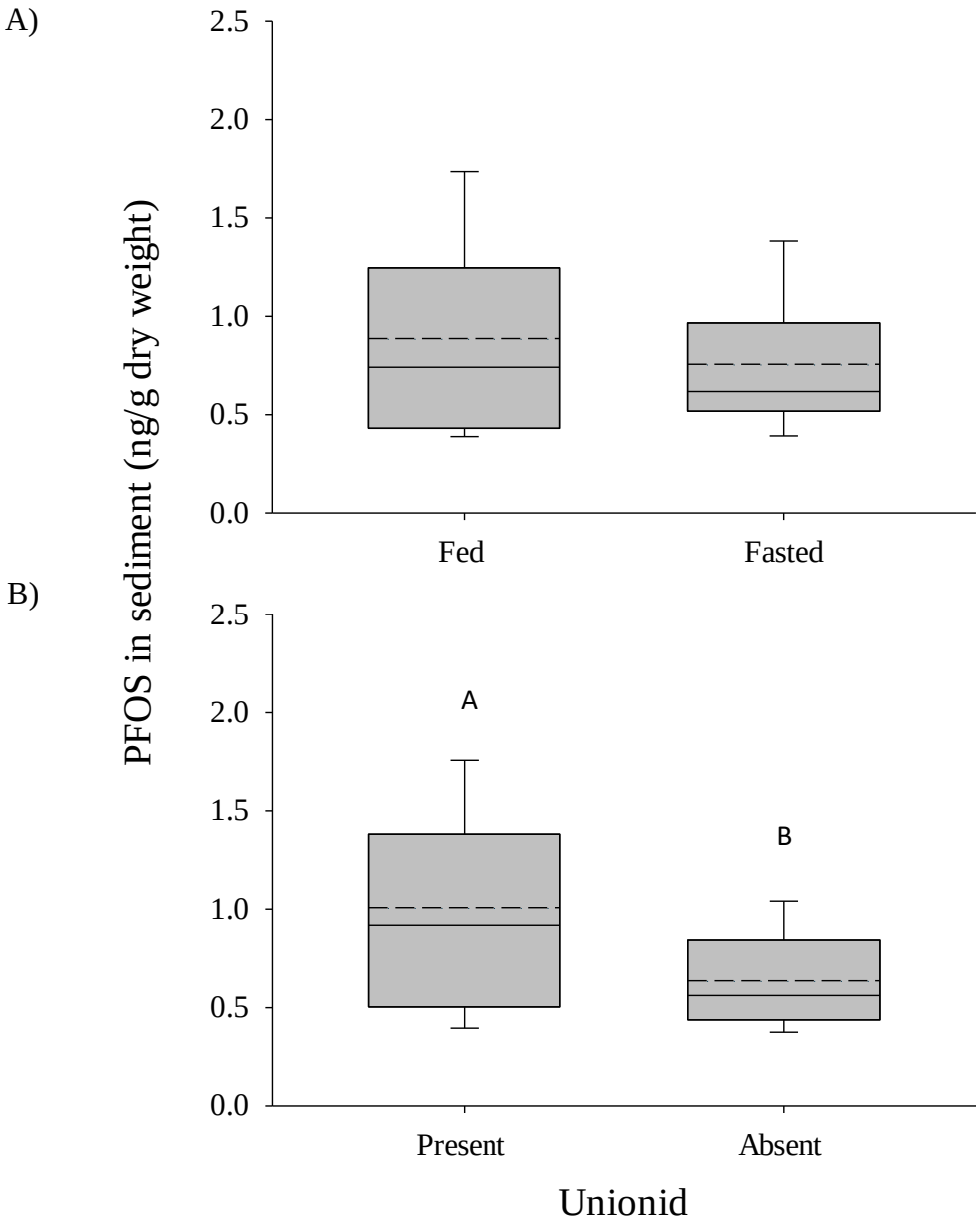


Figure 1.4 A) Box plot of mean PFOS concentration in the sediment with food present or absent regardless of unionid presence/absence. B) Concentration of PFOS in sediment when a unionid was present or absent, regardless of food treatment. Letters above each box represent a significant difference between treatments. Letters are absent when no significant difference was found. Boxes represent the upper and lower quartiles of data. Solid line inside each box represents the median value and the dotted line represents the mean.

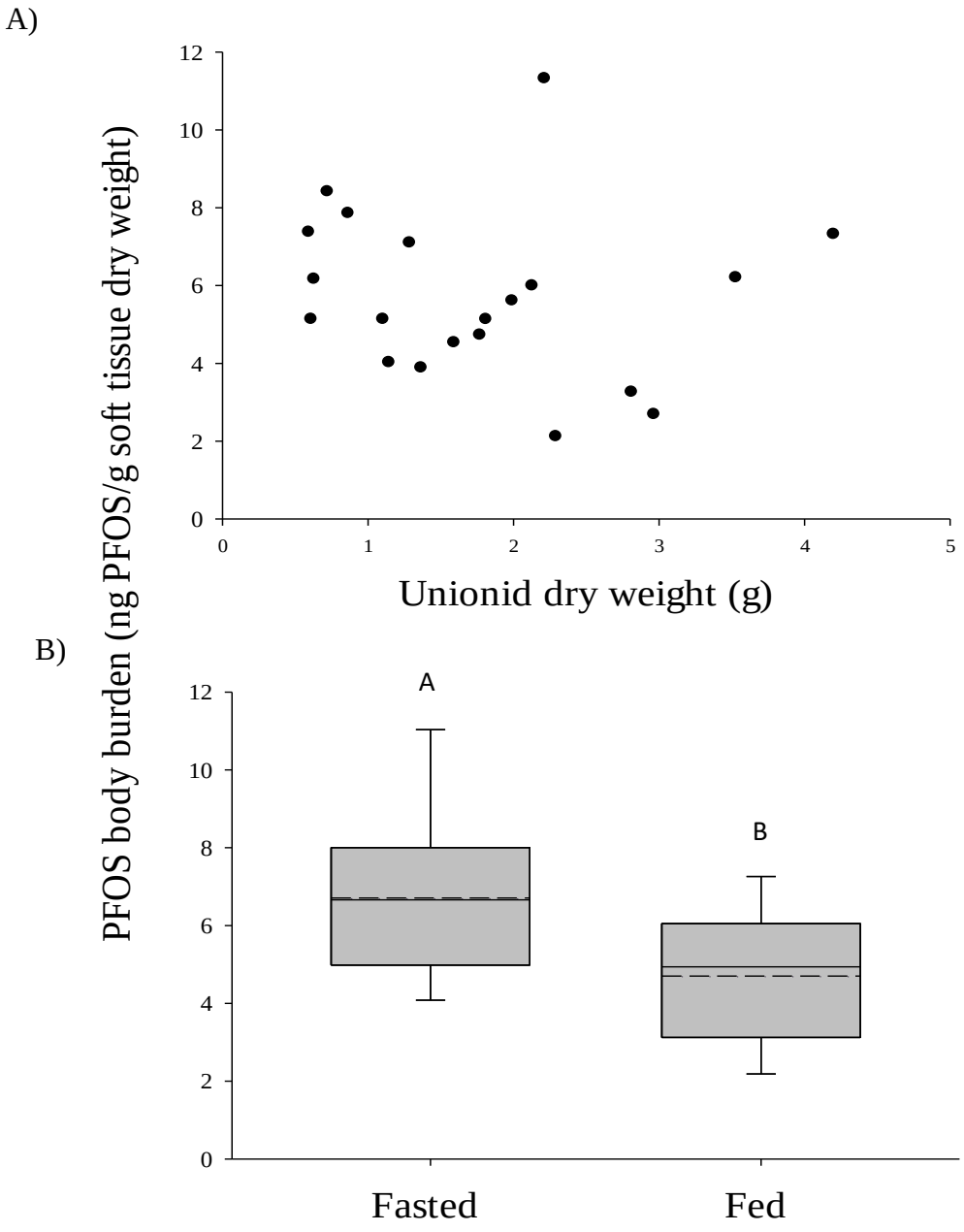


Figure 1.5 A) The relationship between unionid PFOS body burden and unionid size. No evidence was found for a significant effect of unionid size on PFOS body burden B) Box plot of PFOS body burden for fed and fasted unionids. Letters above each box represent a significant difference. Boxes represent the upper and lower quartiles of data. Solid line inside each box represents the median value and the dotted line represents the mean.

Chapter 2: PFOS Effect on Gene Expression on Freshwater Unionids

2.1 Introduction

Perfluorooctanesulfonic acid (PFOS) was created for multiple uses including fire retardants, lubricants, and stain resistant treatments for fabrics (Giesy et al. 2010). It is a major concern as an environmental pollutant because under normal conditions it does not biodegrade, break down in water, or photolyze (Krøvel et al. 2008). Even though the major manufacturers of PFOS, most notably 3M, have stopped production of PFOS, it is still persistently found in the environment (Hagenaars et al. 2008). Bioaccumulation of PFOS has been reported in the aquatic food chain and has also been found at high concentrations in livers of fish and fish-eating predators (Krøvel et al. 2008, Shi et al. 2008).

Freshwater mussels (Family Unionidae) can be used for assessment of pollution and threats to freshwater ecosystems (Amraoui et al. 2018, Modesto et al. 2018). Chemicals in the water can disrupt cellular function of unionids by accumulating in the gill and mantle tissue (Gómez-Mendikute et al. 2005). When unionids bioaccumulate contaminants, the gills may accumulate more than the mantle tissue (Sohail et al. 2016). Gravid females could be more sensitive to accumulated or waterborne chemicals because they carry and nourish their larvae, or glochidia, inside the gills (Cope et al. 2008). When exposed to an array of different PFOS concentrations, unionids have experienced inducement of antioxidant enzymes in the gills, and decreased glochidia viability (Hazelton et al. 2012, Amraoui et al. 2018).

When an organism is exposed to stress in its environment, reactive changes in gene expression can occur (Hagenaars et al. 2008). According to Sørensen et al. (2003), stress is defined as a condition that decreases an organism's fitness or interrupts the organism's natural biological functions. Oxidative stress can cause DNA damage and mutations of tumor suppressor

genes (Kang 2002). Mutation can cause a gene's function to be inhibited and mutation of a tumor suppressor gene could cause the formation of a tumor (Hinds and Weinberg 1994). Unionids have developed tumors in different types of tissues such as the foot and mantle tissue (Carella et al. 2016). Exposing organisms to chemicals can cause a stress response which could cause changes of expressions of certain genes. Genes that have an influence on apoptosis are of interest because apoptosis is a sign of severe stress in a cell (Kültz 2005). In the present study, we studied three stress related genes, Bag 4, Heat shock protein 70 and Krüppel 5, to determine if there was a change of expression by our study unionid, *Ligumia subrostrata*, in relation to exposure to environmentally relevant PFOS concentrations.

Bag genes are a group of proteins found in plants and animals that function in processes such as apoptosis and tumorigenesis (Kabbage and Dickman 2008). The Bag 4 gene, also known as the silencer of death, has been found to reduce tumor necrosis factor (TNF) — induced apoptosis when gene expression is upregulated (Gehrmann et al. 2005). Studies have also shown that upregulation of the Bag 4 gene is associated with many different types of cancers in humans (Yang et al. 2020). To our knowledge, there has not been a study to examine the effects on the expression of the Bag 4 gene when exposed to PFOS.

Heat shock proteins (HSP), especially HSP 70, are found in organisms from simple, one celled organisms to eukaryotes (Beere and Green 2001). These proteins are broken down into groups called families that are based on their molecular weight (Pockley 2003). The primary role of HSP 70 is to correct the folding of new proteins by preventing protein build-up and starting the breakdown of denatured and misfolded proteins (Mayer and Bukau 2005). PFOS could cause an over expression of HSP 70 by interacting with different proteins and change their functional structure, or by causing oxidative stress in the cell (San-Segundo et al. 2016). For example, in

Atlantic salmon hepatocytes, HSP 70 was significantly up regulated when exposed to 15.1 mg/L and 25 mg/L of PFOS (Krøvel et al. 2008).

Krüppel -like transcription factors that regulate transcription and cell proliferation have been identified in a number of organisms such as a nematodes, zebrafish, and humans (Kaczynski et al. 2003). Krüppel-like factor 5 (KLF5) binds to C-G rich segments of DNA and is identified as the intestinal rich Krüppel-like factor but is expressed in many different types of tissues (Zheng et al. 2009). KLF5 is implicated in apoptosis, tumorigenesis and cell proliferation (McConnell et al. 2007, Zheng et al. 2009). Upregulation of KLF5 has been shown to act as an oncogene in certain tissues and can act as a tumor suppressor in other tissues (Diakiw et al. 2013). Di Nisio et al. (2020) reported KLF5 was significantly upregulated in human endometrial cells when exposed to PFAS, particularly PFOA.

A house-keeping gene is generally used in real-time polymerase chain reaction (RT-PCR) as a reference gene for data normalization because the gene is expressed in all cells and should not vary under experimental conditions (Vandesompele et al. 2002). The purpose of a house-keeping gene for data normalization is to control for error by removing artificial variation among samples and a quality insurance of RNA that is below quantification levels (Dheda et al. 2004, Mane et al. 2008). The house-keeping genes that are typically used are Beta-actin and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Thellin et al. 1999). In the present study, Beta-actin was used as the reference gene.

In this study, *Ligumia subrostrata* was used to address the following objectives:

1. Determine if the expression of three-stress related genes are affected in response to exposure to environmentally relevant concentrations of PFOS.
2. Obtain evidence as to whether gravid females are affected differently than males.

3. Obtain evidence as to whether gill tissue is more strongly affected than mantle tissue.

2.2 Methods

2.2.1 Exposing the Unionids to PFOS

A stock solution of PFOS was made by adding 0.05 g of PFOS (Synquest Laboratories, CAS Number 1763-23-1) to 1 liter of DI/RO water in a high-density polyethylene (HDPE) bottle; the bottle was then covered with a plastic lid and placed on a stir plate. After stirring for >12 hours, 250 ml of the resultant solution was diluted with 750 ml reverse osmosis de-ionized (DI/RO) water. The dilution step was repeated three more times from the original solution, resulting in four, 1 L stock solutions at a nominal concentration of 12.5 mg/L of PFOS each. One stock solution was refrigerated and used for experiments within 30 days and the other 3 stock solutions were stored at -20 ℃ for use within 6 months.

In November 2019, 21 adult males and 23 adult females, *Ligumia subrostrata*, were collected from a farmed population at the Auburn University South Auburn Fisheries Research Station (SAFRS). Each individual was tagged with a FPN 8 mm x 4 mm glue-on shellfish Hallprint tag (Hindmarsh Valley, South Australia 5211, Australia) and randomly assigned them to one of three upweller systems (Haney et al. 2020). Temperature of the water in the upwellers was set to the temperature of the pond and was raised by 1 ℃ per day until reaching a final experimental temperature of 25 ℃. Animals were acclimated to laboratory conditions for an additional 14 days before initiating experiments. Each upweller system contained 70 L of soft, artificial freshwater (SAFW) using a recipe modified from Smith et al. (1997): 0.0048 g/L $NaHCO_3$, 0.025 g/L $CaSO_4$-H_2O, 0.025 g/L $CaC1_2$, 0.015 g/L $MgSO_4$, and 0.002 g/L KCl were added to RO/DI water, yielding a final alkalinity of 120 mg/L and final hardness of 50 mg/L. Water quality was measured over the acclimation period to help ensure the survival of the

unionids. Photoperiod was maintained at 12:12 L:D. Unionids were fed every 2 hours 6.2ml of a 2:1 mixture of Shellfish diet 1800® and Nanno 3600 (Reed Mariculture, 900 E Hamilton Ave, Suite 100 Campbell, CA 95008) with a GHL Doser 2.1 automatic feeder (GHL USA LLC, 5212 Carolina Beach Road, Wilmington, NC 28412) to distribute a total concentration of 300,000 cells/L in a 24-hour period.

The experimental systems consisted of a 70 L cooler, which served as a common sump, with 10 plastic 0.95 L containers suspended above it. SAFW was pumped from the sump to a manifold system which distributed water to each individual container. Water flowed through each container, draining back into the sump at a rate of 12 L/h (Fig. 2.1). Water temperature was maintained at 25 ± 2 ℃. Each cup contained 226.8 g of sand (Quickrete Premium Play Sand) that had been rinsed clear of fine particles with tap water, sterilized with a 10% solution of bleach for >3 h and then rinsed again with tap water followed by SAFW. Prior to placing in cups, four 100 g samples of sand were placed in HDPE containers and stored at -20 ℃ for later PFOS analysis.

We used two experimental systems with 10 cups each. One system received PFOS while the other system did not. Food was not added to either system. A volume of 70 L SAFW was maintained in each sump via calibrated marks on the inner walls of the sumps and water was circulated through each system for 24 hrs prior to the start of the experiment. Five female unionids and five male unionids were then randomly selected from the holding tanks and sacrificed via opening the valves and cutting through the adductor muscles. All soft tissue was removed from the shell and frozen in 25 ml plastic bags (Zipper Poly Bags, Auburn University Scientific Supply Store, 36849) at -20 ℃ for future analysis of initial PFOS body burden. Ten additional male and ten additional female unionids were then randomly chosen from the upweller

systems and assigned to each experimental system such that each cup alternated between male and female unionids (Fig 2.1). Shell length of each unionid was measured to the nearest mm and whole wet mass measured to the nearest gram prior to placing the unionids in the experimental system (Table 2.1).

After the unionids acclimated to the experimental system for 24 hrs, 1.12 ml of PFOS stock solution was pipetted into one of the experimental tanks for an initial nominal concentration of 200 ng PFOS / L. The other tank did not receive any PFOS and served as a control. A previous pilot run in our experimental system indicated approximately how much PFOS was lost from the water column within a 24-hour period (Fig 1.2). Assuming this depletion rate remained constant during the experimental period, 0.56 ml of the PFOS stock solution was added to the experimental system after the first set of samples were taken to bring PFOS concentration back up to approximately 200 µg/L. In order to monitor the true concentrations of PFOS in the water column during each trial, we collected replicate 100 ml samples from the system one hour (daily maximum), and 24 hrs (daily minimum) after each PFOS addition. Samples were stored in HDPE 125 mL bottles (414004-112, VWR) at -20 ℃ for future analysis. After each sample collection, an equivalent amount of fresh SAFW was added back to the sump to maintain a constant water volume. If the water level was observed to be below the 70 L mark between sampling events, the loss was assumed due to evaporation and the sump brought back up to the 70 L mark with RO/DI water

At the conclusion of the experimental trial, each unionid was sacrificed, mantle tissue and gills were dissected out and stored in 10 µl RNA*Later* per 1 mg tissue. The glochidia were flushed out using phosphate buffered saline (PBS) in a syringe. The remaining visceral mass

tissue was then cut into smaller pieces and stored in a small 25 ml Ziplock bag in a -20 ℃ until it was possible to freeze-dry the samples.

The visceral mass samples were freeze-dried for a total of 96 hours, crushed in a fine powder, collected in a transparent autoclavable polycarbonate tube, and stored in a -20 ℃ until analysis.

2.2.2 RNA Isolation

Total ribonucleic acid (RNA) was isolated from mantle and gill tissue from adult unionids, using the Qiagen RNeasy Plus mini kit (www.qiagen.com) according to the manufacturer's instructions. First the tissue was allowed to thaw in an ice bucket for ten minutes. 30-50 mg of tissue was added to a falcon tube with 600 µl of RLT lysis buffer containing 1% beta-mercaptoethanol. The tissue was homogenized using a tissuemiser for one minute on the maximum setting. The total homogenate was pipetted into a micro centrifuge tube and centrifuged for three minutes at 15,000 revolutions per minute (rpms). Approximately a 1:1 volume addition of 70% ethanol was added to the supernatant sample. The solution was transferred to a RNeasy spin column and the steps of the Qiagen's RNeasy Plus mini kit protocol were then employed. The samples were eluted with 30 µl of RNase-free water. Total RNA was quantified using the nanospectrophotometer and the total RNA concentration and the absorbance ratio of 260:280 nm (Table 2.2) were measured. The RNA sample was stored at -80 ℃ until cDNA was made.

2.2.3 cDNA synthesis

The complementary DNA (cDNA) synthesis was carried out by using the Quantabio qScript^{TM} cDNA Synthesis Kit (Qiagen, Beverly, MA. Lot 6645677) according to the manufacturer's instructions. Briefly, 1 mg total RNA was added to 4 µl qScript Reaction mix (5x) and 1 µl qScript reverse transcriptase and brought final volume of 20 µl with nuclease-free water. All samples were then added to a Thermo-Cycler that was programed at 22 ℃ for 5 minutes, 42 ℃ for 30 minutes, 85 ℃ for 5 mins, and hold the samples at 4 ℃ until they are taken out. All the cDNA samples were stored at -20 ℃ until use.

2.2.4 RT-PCR

Four different targets were selected for the RT-PCR: Beta -Actin, Bag 4, Heat-shock Protein 70 B2T2 and Krüppel 5. The primer sequence for the Beta -Actin, Bag 4, Heat-shock Protein 70 B2T2 and Krüppel 5 are shown in Table 2.3. A mastermix was made for each primer that contained the primer, SYBER green, and RNAse-free. In each well of the PCR plate, 1 µl of cDNA was added along with 19 µl of the mastermix. Each Primer and cDNA were run in triplicate. After each well was loaded, a clear optical film was placed over the plate and smoothed out to secure it. The plate was then placed in the BioRad CFX96 Real Time PCR machine where it ran for 40 amplification cycles with the following cycle parameters: 95 ℃ for 10 seconds and 58 ℃ for 30 seconds (Luo et al. 2014). The RT-PCR concluded the protocol with a melt curve. The expression levels of the genes were standardized to the house keeping gene selected, Beta-actin. Expression data was analyzed using Bio-Rad CFX manager 3.1.

2.2.5 Water, Tissue and Sediment Analysis for PFOS

Experimental water: Experimental water samples were thawed to room temperature before analysis. Duplicate water samples from each time interval were combined, spiked with an internal standard (MPFOS, 1 ng/mL) and subjected to cleanup using a solid phase extraction (SPE) approach. Prior to loading the sample, SPE cartridges were preconditioned with 0.1% ammonium hydroxide in methanol (4 mL) followed by methanol (4 mL) and the portions were discarded. The cartridges were then rinsed with liquid chromatography (LC) grade water (4 mL) and sample were loaded and eluted by adjusting the flow to one drop per second. After the sample loading step was complete, SPE cartridges were rinsed with water to eliminate salts that may have stayed on the column. Also, SPE columns were washed with 25mM ammonium acetate in water (4mL) to adjust the pH to 4.0 and the fraction was discarded. Finally, cartridges were dried under vacuum and then eluted with extraction solvents methanol (2 mL) followed by 0.1% ammonium hydroxide in methanol (3 mL). Both the solvent fractions were combined and filtered through Agilent nylon glass-fiber syringe filters (0.2 µm).

Unionid samples: The weighed and freeze-dried unionid samples were thawed to room temperature, spiked with an internal standard (MPFOS, 1 ng/mL) and extracted with 30 ml extraction solvent (methanol/water, 70:30, v/v). Samples were placed on an orbital shaker at 250 rpm for 3 hours. The samples were removed from the shaker and allowed to stand for 2 minutes. Unionid extracts were then sonicated at 60 Hz for 2 hours and were centrifuged at 13,000 rpm, 4 ℃ for 10 minutes. The supernatant extracts were transferred into 500 mL HDPE bottles and diluted with DI water (500 mL). Diluted extracts were purified using the SPE method mentioned above and eluted with extraction solvents methanol (2 mL) followed by 0.1% ammonium

hydroxide in methanol (2 mL). Combined extracts were filtered through Agilent nylon glass-fiber syringe filters (0.2 µm).

Sediment samples: Freeze-dried sediments were thawed to room temperature, weighed accurately, and spiked with an internal standard (MPFOS, 1 ng/mL). The samples were extracted on an orbital shaker at 250 rmp for 3 hours. The samples were allowed to stand for 2 minutes and then sonicated for 2 hours. The extracts were centrifuged at 13,000 rpm, 4 ℃ for 10 minutes. The supernatants extracts were diluted with DI water to make a final dilution to 500 mL. Diluted extracts were purified using the SPE method mentioned above and eluted with extraction solvents methanol (2 mL) followed by 0.1% ammonium hydroxide in methanol (2 mL). Combined extracts were filtered through Agilent nylon glass-fiber syringe filters (0.2 µm).

Ultra-high performance liquid chromatography-triple quadrupole mass spectrometry (UHPLC-MS/MS) Analysis: Prior to analysis, all the extracts were spiked with an internal standard (MPFOS) to adjust the final concentration to 2 ng/mL and analyzed using UHPLC-MS/MS according to a published method (Mulabagal et al. 2018). Samples were quantified with a PFOS calibration curve developed using standard concentrations ranging from 0.05 to 50 ng/mL in 80% methanol in water.

2.2.6 Statistical Analysis

All data were presented as means ± standard error. The expression levels were first normalized against the housekeeping gene Beta-actin and analyzed using a modified $\Delta\Delta C_T$ method (Vandesompele et al. 2002). The data were log-transformed and differences between means were tested by a t-test assuming equal variances followed by the Mann-Whitney Rank

Sum test for the data that failed the normality test (SigmaPlot version 13.0). The significance level was $\alpha = 0.05$ in all cases.

2.3 Results

2.3.1 PFOS concentrations in water, sediment, and unionid soft tissues.

The Instrument Detection Limits (IDLs) for PFOS for the Ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry and JetStream ion source (UHPLC-QqQ-MS) is 1.7 pg/injection. Initial unionids and sediment did not show any peak(s) corresponding to PFOS. The unionids were exposed to a mean of 213.41 ng/L of PFOS during the 168 hours (Fig. 2.2). The lowest concentration was 128.41 ng/L of PFOS and the highest concentration was 295.37 ng/L of PFOS (Fig. 2.2). At the end of the exposure period, the female mean body burden of PFOS was 9.117 ng/L with a standard error of 1.672 (Fig. 2.3). The male mean body burden of PFOS was 9.185 ng/L with a standard error of 2.922 (Fig. 2.3). There was no significant different in the means of the female and the male tissue accumulation of PFOS (t-test: $t_8 = -0.0201$, $p=0.984$).

2.3.2 RT-PCR

The RT-PCR results are quantified as a fold-change in gene expression. The male gills did not show a significant response to PFOS exposure by any of the three targeted genes. (Table 2.4). Gene expression was not significantly upregulated in the BAG 4 gene target (t-test: $t_6 = 0.140$, $p=0.893$). The relative normalized expression mean of the control sample was 1.06 with a standard error of 0.109. (Fig. 2.4). Gene expression was not significantly up regulated in the HSP 70 target (t-test: $t_6 = -1.804$, $p=0.121$) (Fig. 2.4). The relative normalized expression mean of the control sample was 1.14 with a standard error of 0.169. Gene expression was not significantly

upregulated in the Krüppel 5 target (t-test: t_6 = -1.511, p=0.182) (Fig. 2.4). The relative normalized expression mean of the control sample was 2.32 with a standard error 0.865.

The female gills did not show a significant change in the selected gene expression after PFOS exposure (Table 2.5). Gene expression was not significantly altered in the BAG 4 gene target (t-test: t_7 = 1.991, p=0.087) (Fig. 2.5). The relative normalized expression mean of the control sample was 1.22 with a standard error of 0.225. Gene expression was not significantly changed in the HSP 70 target (t-test: t_7 = 0.362, p=0.728) (Fig. 2.5). The relative normalized expression mean of the control sample was 1.13 with a standard error of 0.166.

The male mantle did show a significant response to PFOS in the Krüppel 5 target, but in the other targets there were no significant responses (Table 2.4). Gene expression was not significantly down regulated in the BAG 4 gene target (t-test: t_6 = 1.011, p=0.351) (Fig. 2.6). The relative normalized expression mean of the control sample was 1.28 with a standard error of 0.263. Gene expression was not significantly up regulated in the HSP 70 target (t-test: t_6 = -1.690, p=0.142) (Fig.2.6). The relative normalized expression mean of the control sample was 1.009 with a standard error of 0.0405. Gene expression was significantly upregulated in the Krüppel 5 target (t-test: t_6=-3.313, p=0.0161) (Fig. 2.6). The relative normalized expression mean of the control sample was 1.045 with a standard error of 0.0926.

The female mantle did not show a significant response to PFOS exposure by any of the targeted genes (Table 2.5). Gene expression was not significantly down regulated in the BAG 4 gene target (Mann-Whitney test: U = 4.00, n1 = n2 = 4, p = 0.343) (Fig. 2.7). The relative normalized expression mean of the control sample was 1.01 with a standard error of 0.0327. Gene expression was not significantly downregulated in the HSP 70 target (t-test: t_6 = 1.101, p=0.313) (Fig. 2.7). The relative normalized expression mean of the control sample was 1.06

with a standard error of 0.113. Gene expression was not significantly upregulated in the Krüppel 5 target (t-test: t_6=-0.717, p=0.870) (Fig. 2.7). The relative normalized expression mean of the control sample was 1.15 with a standard error of 0.175.

2.4 Discussion

Many studies examined on the effects of PFOS on mammals and fishes but there are few on unionids. PFOS has been reported in the Tennessee River in Alabama at concentrations of 220 ng/L of PFOS (Newton et al. 2017). Studies have shown that PFOS exposure can reduce glochidia viability, induce antioxidant enzymes in the gills, and cause DNA damage at concentrations well above that in the present study (Hazelton et al. 2012, Li et al. 2014, Amraoui et al. 2018). Gene expression studies typically look for a > 2-fold change from the control to signal a significant biological change (Dalman et al. 2012). A change in gene expression does not always mean that there is a change in the amount of proteins translated (Hu et al. 2005). In the present study we used *Ligumia subrostrata* to test for significant changes in gene expression in gill or mantle tissue in response to environmentally relevant PFOS exposure and to determine if gravid females appear to be more strongly affected than males in terms of gene expression in response to environmentally relevant PFOS exposure.

Contrary to our expectations, there was no evidence that gill tissue was more sensitive to PFOS exposure than mantle tissue in the present study. Rather, mantle tissue appeared to be slightly more sensitive. Gill tissue did not exhibit any statistically or biologically significant changes in gene expression in response to PFOS exposure. In contrast, the male mantle tissue exhibited a 2.37-fold change in expression in the KLF5 gene target. KLF5 has been found in many different types of tissues and has an essential role in the inflammatory stress response as

well as cell proliferation, and cell differentiation (Dong and Chen 2009, Diakiw et al. 2013). Cancer and other diseases can cause an upregulation of the expression of the KLF5 (Noto et al. 2013). In humans the upregulation of KLF5 is common in lung cancer and individuals with high levels of KLF5 in breast cancer have shorter survival rates (Zheng et al. 2009, Li et al. 2014). Studies suggest that, in human lung cancer, KLF5 acts as a tumor suppressor in lung cancer (Diakiw et al. 2013) and as an oncogene in breast cancer (Zheng et al. 2009). Whereas our data is preliminary, increased expression of KLF5 in mantle tissue suggest that environmentally relevant PFOS concentrations can influence pathways regulated by KLF5 (Dong and Chen 2009) in unionids.

In the present study, there was no evidence that gravid females were more sensitive than males to environmentally relevant concentrations of PFOS exposure. In the gravid female gills and mantle tissue, there was not a statistically significant or biologically significant change in the targets that were tested. Because the females incubate their glochidia inside their gills, there could be a protective molecular mechanism that helps protect her and offspring from stress (Ugge et al. 2020). Also, in response to pollution, bivalves can close their shell to minimize exposure (Hazelton et al. 2012), which may have reduced exposure to PFOS in this study. If females closed more frequently than males to reduce exposure of the gills and brooded glochidia, this might explain the lack of changes in gene expression when exposed to PFOS. However, because body burden of PFOS did not differ between male and female unionids, there is no evidence that exposure of brooding females was less than that of males.

Gene expression studies are a useful tool in evaluating potential toxic effects of PFOS to an organism. Our study indicated that when *Ligumia subrostrata* was exposed to

environmentally relevant concentrations of PFOS there was little change in gene expression except in the male mantle tissue where that was a significant upregulation of KLF5.

The present study had a few limitations such as sample size, time, and financial resources. A greater sample size could help determine the effects of environmentally relevant concentrations of PFOS on gene expression because in the male and female tissues the power of the tests performed were below 0.8. In a power analysis using the parameters of the current data, 380 (190 for control and 190 for experimental) individuals would be needed to raise the power to at least 0.8 in all tissue types. This would require considerable investment of time and money and may not be practical for many labs. Future studies could expand on the current data by conducting a western plot on the KLF5 target to determine if there is a change in the proteins being produced after PFOS exposure. Longer-term studies to determine if chronic exposure to environmentally relevant PFOS concentration induces a greater change in gene expression than the short-term exposures of this study would also be useful.

2.5 References

Amraoui, I., N. Khalloufi, and S. Touaylia. 2018. Effects to perfluorooctane sulfonate (PFOS) on the mollusk Unio ravoisieri under laboratory exposure. Chemistry and Ecology **34**:324-339.

Beere, H. M., and D. R. Green. 2001. Stress management–heat shock protein-70 and the regulation of apoptosis. Trends in cell biology **11**:6-10.

Carella, F., G. Villari, N. Maio, and G. De Vico. 2016. Disease and disorders of freshwater unionid mussels: a brief overview of recent studies. Frontiers in physiology **7**:489.

Cope, W. G., R. B. Bringolf, D. B. Buchwalter, T. J. Newton, C. G. Ingersoll, N. Wang, T. Augspurger, F. J. Dwyer, M. C. Barnhart, and R. J. Neves. 2008. Differential exposure, duration, and sensitivity of unionoidean bivalve life stages to environmental contaminants. Journal of the North American Benthological Society **27**:451-462.

Dalman, M. R., A. Deeter, G. Nimishakavi, and Z.-H. Duan. 2012. Fold change and p-value cutoffs significantly alter microarray interpretations. Pages 1-4 *in* BMC bioinformatics. BioMed Central.

Dheda, K., J. F. Huggett, S. A. Bustin, M. A. Johnson, G. Rook, and A. Zumla. 2004. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. Biotechniques **37**:112-119.

Di Nisio, A., M. Rocca, I. Sabovic, M. D. R. Ponce, C. Corsini, D. Guidolin, C. Zanon, L. Acquasaliente, A. Carosso, and L. De Toni. 2020. Perfluorooctanoic acid alters progesterone activity in human endometrial cells and induces reproductive alterations in young women. Chemosphere **242**:125208.

Diakiw, S. M., R. J. D'Andrea, and A. L. Brown. 2013. The double life of KLF5: Opposing roles in regulation of gene-expression, cellular function, and transformation. IUBMB life **65**:999-1011.

Dong, J.-T., and C. Chen. 2009. Essential role of KLF5 transcription factor in cell proliferation and differentiation and its implications for human diseases. Cellular and molecular life sciences **66**:2691-2706.

Gehrmann, M., J. Marienhagen, H. Eichholtz-Wirth, E. Fritz, J. Ellwart, M. Jäättelä, T. Zilch, and G. Multhoff. 2005. Dual function of membrane-bound heat shock protein 70 (Hsp70), Bag-4, and Hsp40: protection against radiation-induced effects and target structure for natural killer cells. Cell Death & Differentiation **12**:38-51.

Giesy, J. P., J. E. Naile, J. S. Khim, P. D. Jones, and J. L. Newsted. 2010. Aquatic Toxicology of Perfluorinated Chemicals. Pages 1-52 Reviews of Environmental Contamination and Toxicology. Springer New York, New York, NY.

Gómez-Mendikute, A., M. Elizondo, P. Venier, and M. P. Cajaraville. 2005. Characterization of mussel gill cells in vivo and in vitro. Cell and Tissue Research **321**:131-140.

Hagenaars, A., D. Knapen, I. Meyer, K. Van der Ven, P. Hoff, and W. De Coen. 2008. Toxicity evaluation of perfluorooctane sulfonate (PFOS) in the liver of common carp (Cyprinus carpio). Aquatic Toxicology **88**:155-163.

Haney, A., H. Abdelrahman, and J. A. Stoeckel. 2020. Effects of thermal and hypoxic stress on respiratory patterns of three unionid species: implications for management and conservation. Hydrobiologia **847**:787-802.

Hazelton, P. D., W. G. Cope, T. J. Pandolfo, S. Mosher, M. J. Strynar, M. C. Barnhart, and R. B. Bringolf. 2012. Partial life-cycle and acute toxicity of perfluoroalkyl acids to freshwater mussels. Environmental Toxicology and Chemistry **31**:1611-1620.

Hinds, P. W., and R. A. Weinberg. 1994. Tumor suppressor genes. Current opinion in genetics & development **4**:135-141.

Hu, W., P. D. Jones, T. Celius, and J. P. Giesy. 2005. Identification of genes responsive to PFOS using gene expression profiling. Environmental Toxicology and Pharmacology **19**:57-70.

Kabbage, M., and M. Dickman. 2008. The BAG proteins: a ubiquitous family of chaperone regulators. Cellular and molecular life sciences **65**:1390-1402.

Kaczynski, J., T. Cook, and R. Urrutia. 2003. Sp1-and Krüppel-like transcription factors. Genome biology **4**:1-8.

Kang, D.-H. 2002. Oxidative stress, DNA damage, and breast cancer. AACN Advanced Critical Care **13**:540-549.

Krøvel, A. V., L. Søfteland, B. Torstensen, and P. A. Olsvik. 2008. Transcriptional effects of PFOS in isolated hepatocytes from Atlantic salmon Salmo salar L. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **148**:14-22.

Kültz, D. 2005. Molecular and evolutionary basis of the cellular stress response. Annu. Rev. Physiol. **67**:225-257.

Li, Q., Z. Dong, F. Zhou, X. Cai, Y. Gao, and L.-W. Wang. 2014. Krüppel-like factor 5 promotes lung tumorigenesis through upregulation of Sox4. Cellular Physiology and Biochemistry **33**:1-10.

Luo, Y., C. Li, A. G. Landis, G. Wang, J. Stoeckel, and E. Peatman. 2014. Transcriptomic profiling of differential responses to drought in two freshwater mussel species, the giant

floater Pyganodon grandis and the pondhorn Uniomerus tetralasmus. PLoS One **9**:e89481.

Mane, V. P., M. A. Heuer, P. Hillyer, M. B. Navarro, and R. L. Rabin. 2008. Systematic method for determining an ideal housekeeping gene for real-time PCR analysis. Journal of biomolecular techniques: JBT **19**:342.

Mayer, M., and B. Bukau. 2005. Hsp70 chaperones: cellular functions and molecular mechanism. Cellular and molecular life sciences **62**:670-684.

McConnell, B. B., A. M. Ghaleb, M. O. Nandan, and V. W. Yang. 2007. The diverse functions of Krüppel-like factors 4 and 5 in epithelial biology and pathobiology. Bioessays **29**:549-557.

Modesto, V., M. Ilarri, A. T. Souza, M. Lopes-Lima, K. Douda, M. Clavero, and R. Sousa. 2018. Fish and mussels: importance of fish for freshwater mussel conservation. Fish and Fisheries **19**:244-259.

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

Newton, S., R. McMahen, J. A. Stoeckel, M. Chislock, A. Lindstrom, and M. Strynar. 2017. Novel Polyfluorinated Compounds Identified Using High Resolution Mass Spectrometry Downstream of Manufacturing Facilities near Decatur, Alabama. Environmental Science & Technology **51**:1544-1552.

Noto, J. M., T. Khizanishvili, R. Chaturvedi, M. B. Piazuelo, J. Romero-Gallo, A. G. Delgado, S. S. Khurana, J. C. Sierra, U. S. Krishna, and G. Suarez. 2013. Helicobacter pylori

promotes the expression of Krüppel-like factor 5, a mediator of carcinogenesis, in vitro and in vivo. PLoS One **8**:e54344.

Pockley, A. G. 2003. Heat shock proteins as regulators of the immune response. The lancet **362**:469-476.

San-Segundo, L., L. Guimarães, C. F. Torija, E. M. Beltrán, L. Guilhermino, and M. V. Pablos. 2016. Alterations in gene expression levels provide early indicators of chemical stress during Xenopus laevis embryo development: A case study with perfluorooctane sulfonate (PFOS). Ecotoxicology and environmental safety **127**:51-60.

Shi, X., Y. Du, P. K. Lam, R. S. Wu, and B. Zhou. 2008. Developmental toxicity and alteration of gene expression in zebrafish embryos exposed to PFOS. Toxicology and applied pharmacology **230**:23-32.

Smith, M. E., J. M. Lazorchak, L. E. Herrin, S. Brewer-Swartz, and W. T. Thoeny. 1997. A reformulated, reconstituted water for testing the freshwater amphipod, Hyalella azteca. Environmental Toxicology and Chemistry: An International Journal **16**:1229-1233.

Sohail, M., M. N. Khan, A. S. Chaudhry, and N. A. Qureshi. 2016. Bioaccumulation of heavy metals and analysis of mineral element alongside proximate composition in foot, gills and mantle of freshwater mussels (Anodonta anatina). Rendiconti Lincei **27**:687-696.

Sørensen, J. G., T. N. Kristensen, and V. Loeschcke. 2003. The evolutionary and ecological role of heat shock proteins. Ecology letters **6**:1025-1037.

Thellin, O., W. Zorzi, B. Lakaye, B. De Borman, B. Coumans, G. Hennen, T. Grisar, A. Igout, and E. Heinen. 1999. Housekeeping genes as internal standards: use and limits. Journal of biotechnology **75**:291-295.

Ugge, G. M. O. E., A. Jonsson, B. Olsson, R. Sjöback, and O. Berglund. 2020. Transcriptional and biochemical biomarker responses in a freshwater mussel (Anodonta anatina) under environmentally relevant Cu exposure. Environmental Science and Pollution Research:1-12.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome biology **3**:1-12.

Yang, Z., X. Cao, Y. Ma, J. Cheng, C. Song, R. Jiang, X. Wang, Y. Huang, C. Buren, and X. Lan. 2020. Novel copy number variation of the BAG4 gene is associated with growth traits in three Chinese sheep populations. Animal biotechnology:1-9.

Zhang, X., L. Chen, X.-C. Fei, Y.-S. Ma, and H.-W. Gao. 2009. Binding of PFOS to serum albumin and DNA: insight into the molecular toxicity of perfluorochemicals. BMC molecular biology **10**:1-12.

Zheng, H.-Q., Z. Zhou, J. Huang, L. Chaudhury, J.-T. Dong, and C. Chen. 2009. Krüppel-like factor 5 promotes breast cell proliferation partially through upregulating the transcription of fibroblast growth factor binding protein 1. Oncogene **28**:3702-3713.

Tables

Table 2.1: The length and weight measurements for the *Ligumia subrostrata*. The initial and final wet weight are the total body (with shell) weight. The dry weight represents freeze-dried soft tissues. Tag numbers that begin with a P are female and tag numbers that begin with Y are male.

Tag Number	Length (mm)	Assignment	Initial wet weight (g)	Final wet weight (g)	Dry Weight (g)
P271	56.75	Control	18.9	20.3	0.6196
P272	60.07	Experimental	17.6	17	0.4023
P274	59.94	Initial	18.4	18.4	0.5240
P276	61.69	Initial	22	22	0.4400
P278	66.24	Initial	27	27	0.5526
P279	65.89	Experimental	27.2	28.3	0.5347
P280	60.3	Control	18.4	19	0.5350
P282	50.45	Control	12.3	12.3	0.2932
P284	58.84	Control	19	18.7	0.5591
P286	71.76	Experimental	30.3	32.5	1.0562
P289	65.11	Initial	26.7	26.7	1.0184
P290	60.18	Experimental	22.9	21.5	0.4303
P291	66.87	Initial	23.6	23.6	0.7686
P292	73.09	Control	34.4	36.4	1.3343
P302	69.32	Experimental	31.4	32.1	0.8601
Y611	65.12	Control	22.1	22.2	0.55
Y614	70.53	Initial	26.5	26.5	0.6339
Y615	62.52	Initial	21.6	21.6	0.7038
Y616	69.77	Experimental	27.2	27.1	0.7353
Y617	74.2	Initial	27.3	27.3	0.6052
Y618	61.64	Experimental	26.5	25.8	0.7333
Y619	68.3	Initial	24.6	24.6	0.4929
Y620	75.47	Experimental	32.1	31.7	0.3845
Y622	71.7	Control	27.4	28.1	0.3653
Y623	63.13	Control	17.5	18.2	0.3242
Y624	64.81	Experimental	21.6	21.5	0.3582
Y625	73.88	Control	25.9	27.3	0.5314
Y627	62.14	Control	18.4	18.3	0.6056
Y628	67.6	Experimental	22.4	21.9	0.4238
Y629	72.32	Initial	29	29	0.7193

Table 2.2: RNA quantification results for the mantle and gill tissue for the male and female *Ligumia subrostrata* The C represents a control sample, and the E represents an exposed sample. The samples that start with a P are female and the samples that start with a Y are male.

	Mantle tissue		**Gill tissue**	
Sample	Concentration (ng/µl)	260/280 Ratio	Concentration (ng/µl)	260/280 Ratio
C.P280	149.029	2.052	547.552	2.022
C.P271	86.345	1.908	542.015	2.027
C.P282	200.377	2	405.773	2.051
C.P284	63.045	2.105	308.428	2.07
C.P292	20.405	2.066	372.109	2.047
C.Y611	61.53	2.087	193.575	2.046
C.Y622	162.18	2.092	81.084	1.941
C.Y623	73.757	2.065	259.919	2.059
C.Y625	123.661	2.168	165.621	2.065
C.Y627	95.919	1.95	492.659	2.067
E. P272	77.887	2.086	358.15	2.052
E. P279	117.543	2.147	343.007	2.044
E. P286	27.085	2	420.999	2.051
E. P290	148.617	2.083	401.89	2.06
E. P302	160.036	2.053	131.527	2.051
E.Y616	22.837	2.072	379.727	2.11
E.Y618	243.076	2.107	353.431	2.085
E.Y620	143.76	2.119	431.712	2.048
E.Y624	196.858	2.068	370.129	2.05
E.Y628	78.144	2.09	416.638	2.085

Table 2.3: Primers used for RT-PCR. Primers are listed in the 5’ to 3’ orientation. Sequences were previously published by Luo et al. (2014).

Gene Name	Forward (5’-3)	Reverse
Beta-actin	ACTCTGGTGATGGTGTGA	AGCAGTGGTTGTGAAGGA
HSP 70 B2T2	CCTGTCTCTGTGAATCGTTA	GAAGAAGTCTCCTCAATGGT
Bag 4	AACAGCAGTCAGCGTCTCA	GTTGTGGTGGTGTCATTGGT
Krüppel 5	CGAGAAAGCCAAACAAGG	TGTCCTCCCACAACGAAT

Table 2.4: The means of the relative normalized expression of each stress gene target for the experimental *Ligumia subrostrata* males.

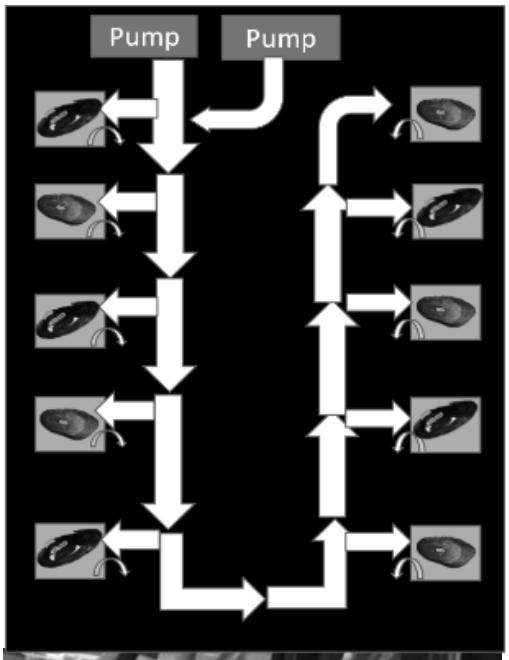
Male Gills				**Male Mantle**			
	Relative normalized expression				Relative normalized expression		
Individual	Bag 4	HSP 70	Krüppel 5	Individual	Bag 4	HSP 70	Krüppel 5
Y628	0.752	2.447	2.030	Y618	0.998	1.256	3.376
Y618	0.930	1.632	3.955	Y620	0.404	0.893	1.914
Y620	1.160	1.140	3.843	Y624	1.331	1.557	2.779
Y624	1.195	2.227	3.813	Y628	0.426	1.548	1.412
Mean	1.009	1.862	3.410	**Mean**	0.789	1.313	2.370
Standard Error	0.070	0.202	0.559	**Standard Error**	0.135	0.078	0.226

Table 2.5: The means of the normalized relative expression of each stress gene target for the experimental *Ligumia subrostrata* females.

Female Gills			**Female Mantle**			
	Relative normalized expression			Relative normalized expression		
Individual	Bag 4	HSP 70	Individual	Bag 4	HSP 70	Krüppel 5
P279	0.488	1.508	P290	0.487	0.533	0.502
P272	0.603	1.144	P272	0.369	0.976	1.325
P286	0.678	0.932	P279	0.392	1.116	1.009
P290	0.840	1.070	P302	1.754	0.645	2.365
P302	0.475	0.395				
Mean	0.617	1.010	**Mean**	0.750	0.818	1.300
Standard Error	0.049	0.113	**Standard Error**	0.151	0.079	0.213

Figures

A)



B)

Figure 2.1: A) A depiction of the manifold system that was built for the experiment. The white arrows indicate water flow. Every cup (the grey boxes) had 226.8 grams of wet sediment. One unionid was placed in each cup, alternating between males and females. The water flowed from a sump, into each cup, and back into the sump at a flow rate of 12 L/hr. B) An actual photograph of the experimental system with the sediment, unionids, and water in the cups.

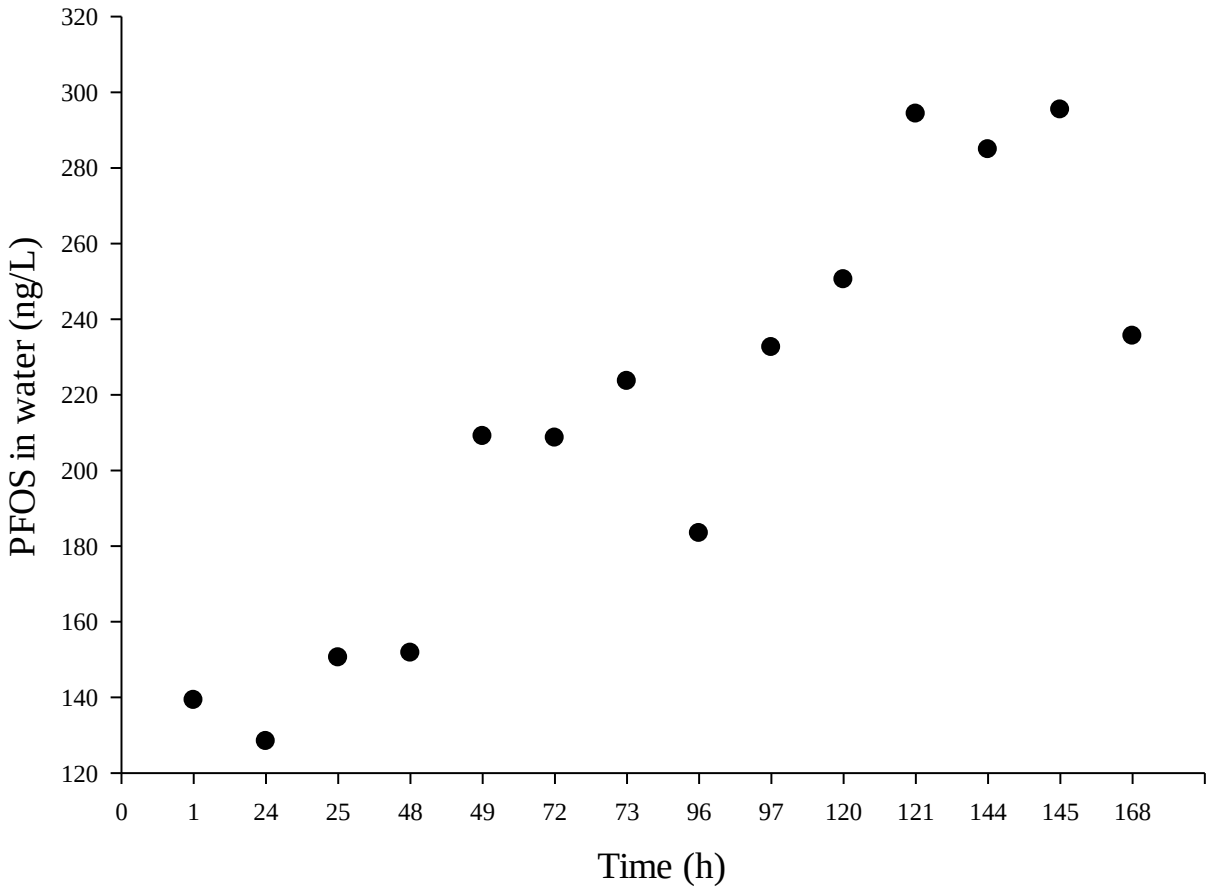


Figure 2.2: Change in PFOS concentration over time in the water of the experiemental system.

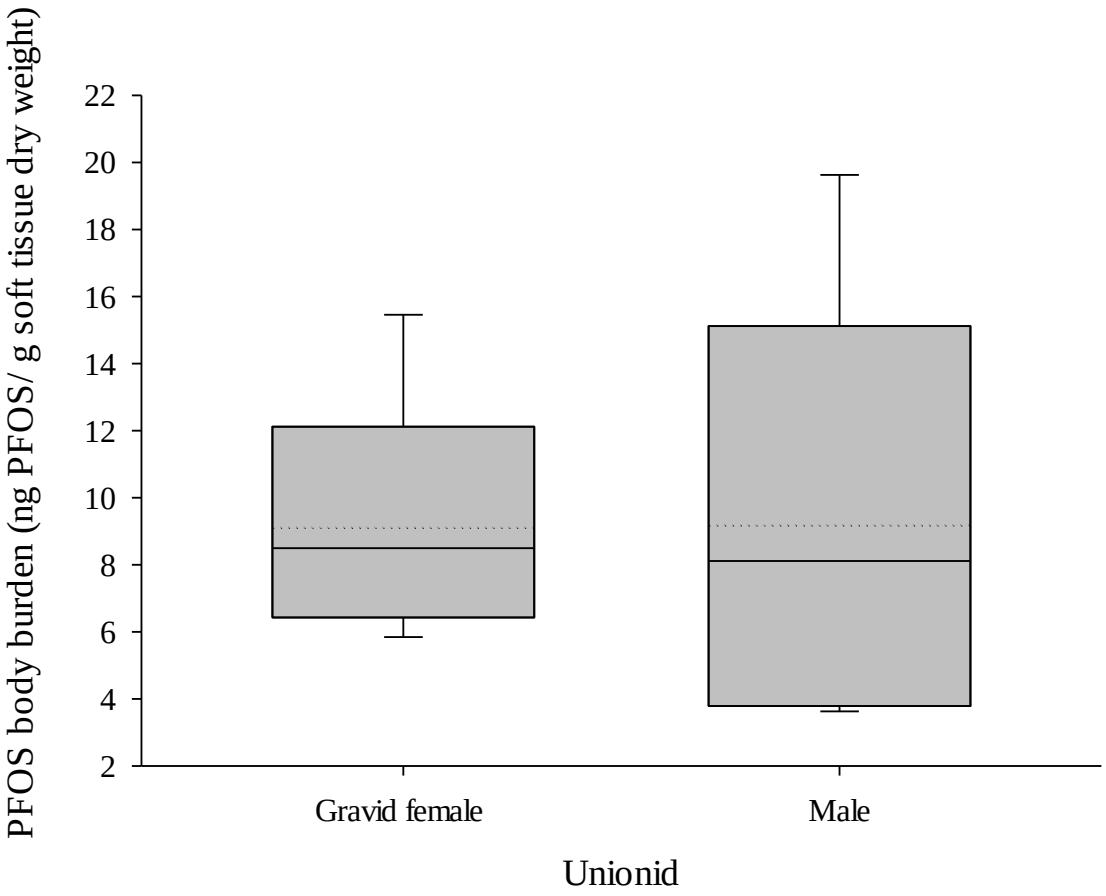


Figure 2.3: Box plot of mean PFOS concentration (ng/g dry weight) in visceral mass of male and gravid female unionids. No significant differences were found between gravid female and male visercal mass PFOS concentrations. Boxes represent the upper and lower quartiles of data.. The solid line is the median and the dotted line is the mean.

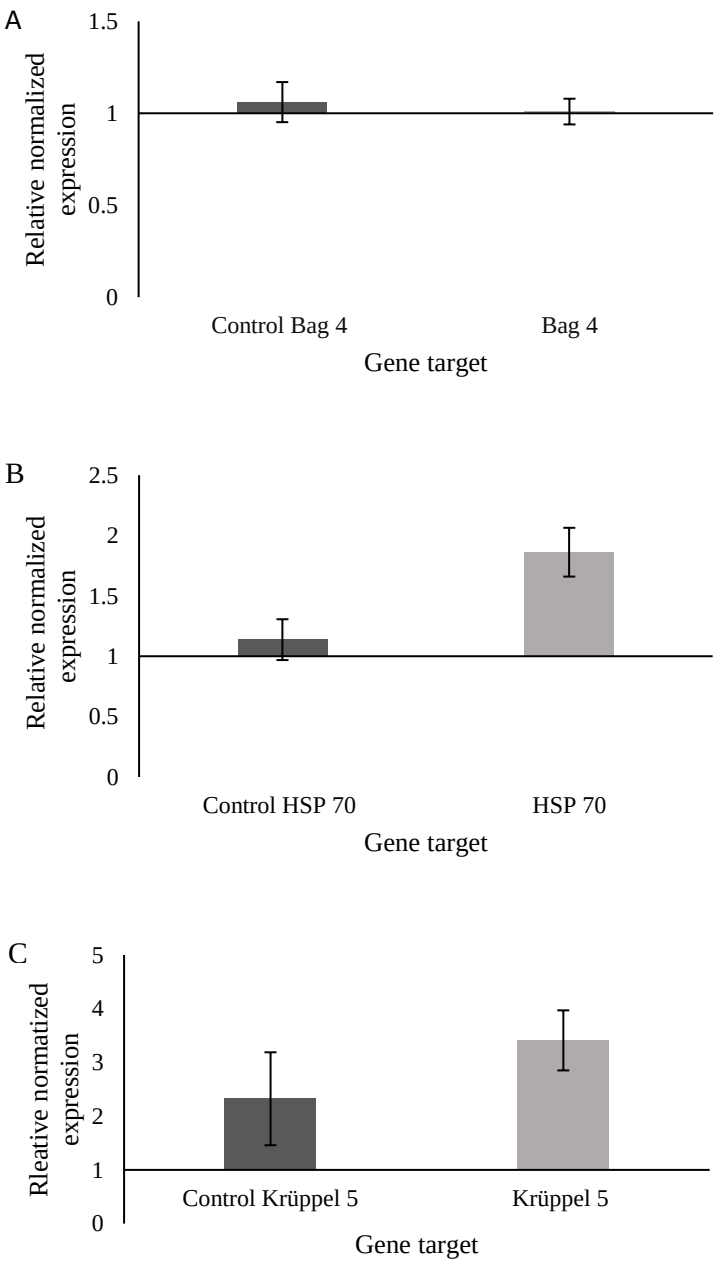


Figure 2.4: Relative normalzied expression of the targeted genes in the control (N=4) and PFOS exposed gills of male unionids (N=4). A) The relative normalized expression of the Bag 4 gene target. B) The relative normalized expression of the HSP 70 gene target. C) The relative normalized gene expression for the Krüppel 5 gene target. Beta-Actin was used as a reference gene. Bars represent the means and the error bars represent the standard error.

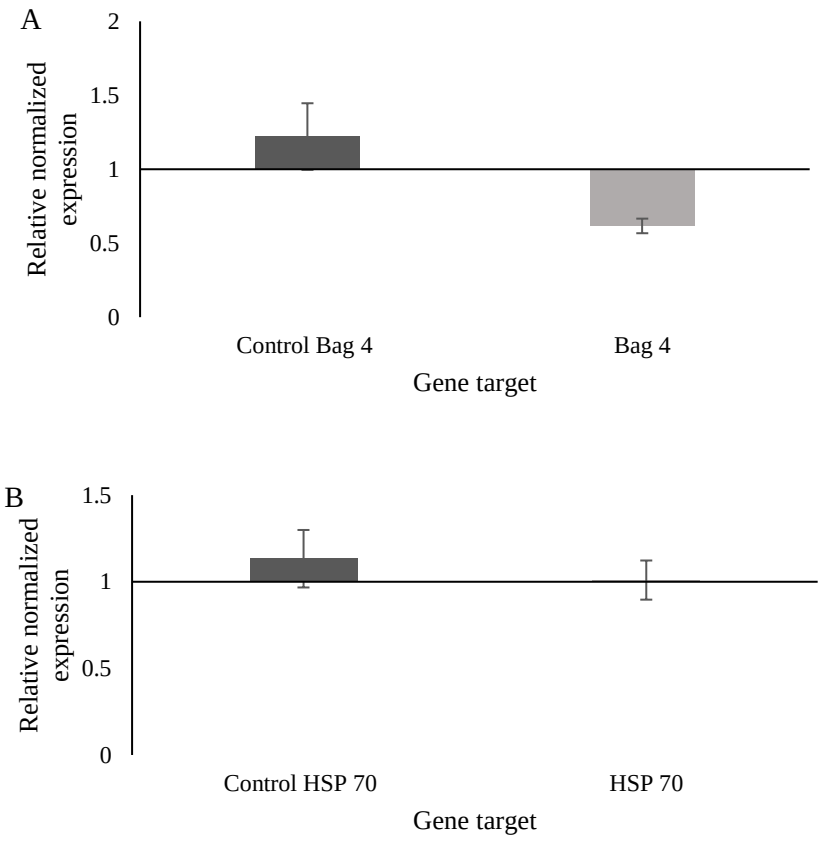


Figure 2.5: Relative normalized expression of the targeted genes in the control (N=4) and PFOS exposed gills of gravid female unionids (N=5). A) The relative normalized expression of the Bag 4 gene target. B) The relative normalized expression of the HSP 70 gene target. Beta-Actin was used as a reference gene. Bars represent the means and the error bars represent the standard error.

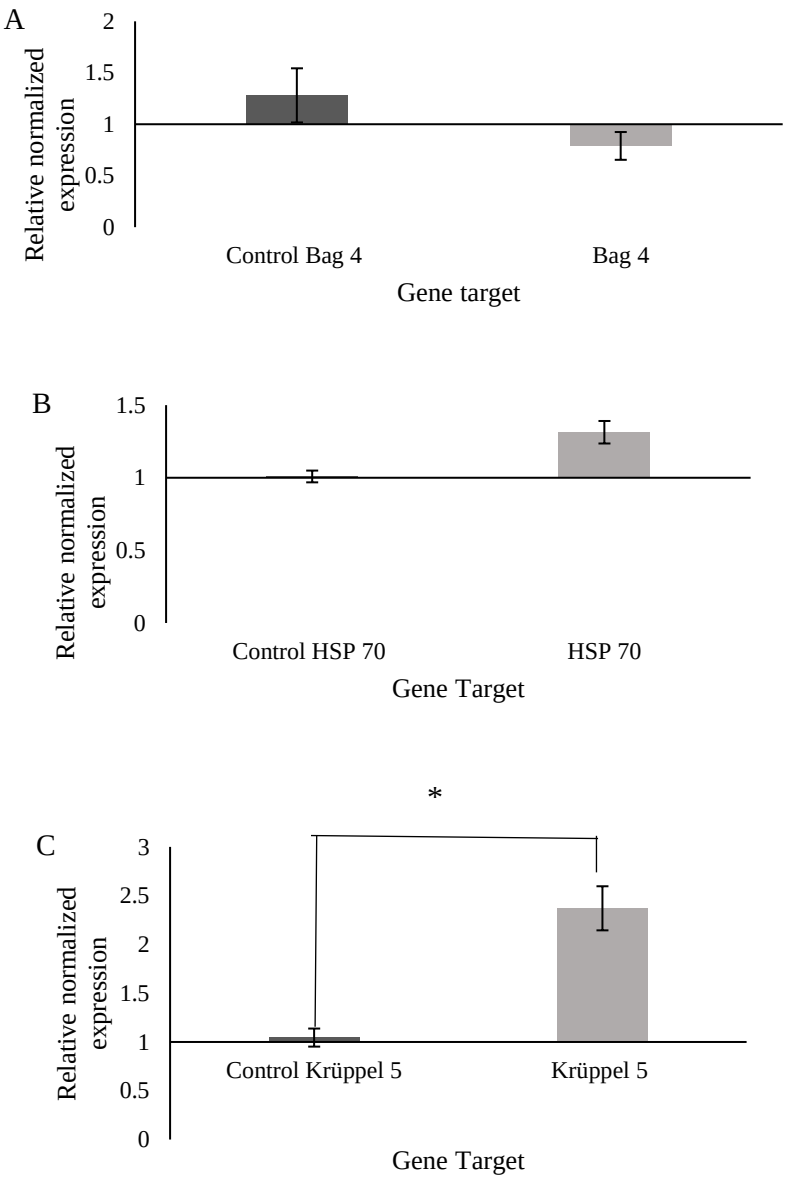


Figure 2.6: Relative normalized expression of the targeted genes in the control (N=4) and PFOS exposed mantle tissue of male unionids (N=4). A) The relative normalized expression of the Bag 4 gene target. B) The relative normalized expression of the HSP 70 gene target. C) The relative normalized gene expression for the Krüppel 5 gene target. Beta-Actin was used as a reference gene. Bars represent the means and the error bars represent the standard error. Asterix indicates a significant difference from control ($p<0.05$).

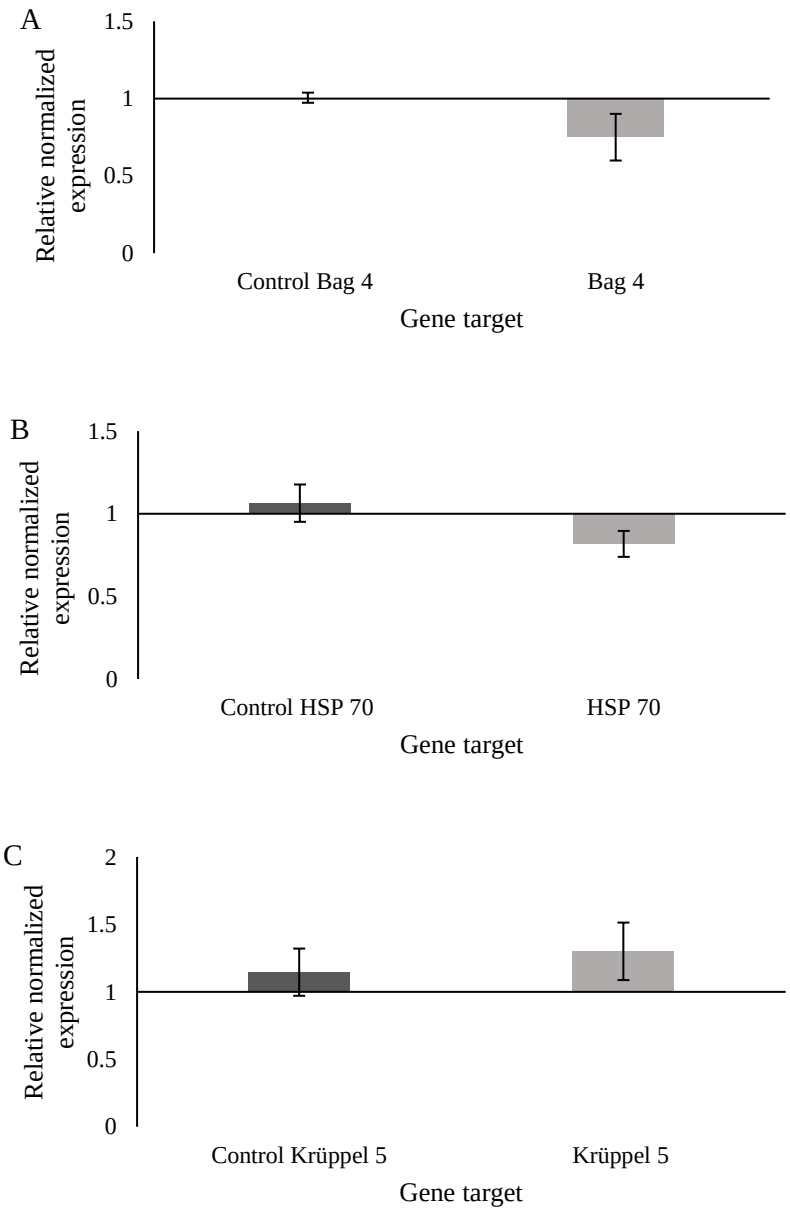


Figure 2.7: Relative normalized expression of the targeted genes in the control (N=4) and PFOS exposed mantle tissue of gravid female unionids (N=4). A) The relative normalized expression of the Bag 4 gene target. B) The relative normalized expression of the HSP 70 gene target. C) The relative normalized gene expression for the Krüppel 5 gene target. Beta-Actin was used as a reference gene. Bars represent the means and the error bars represent the standard error.