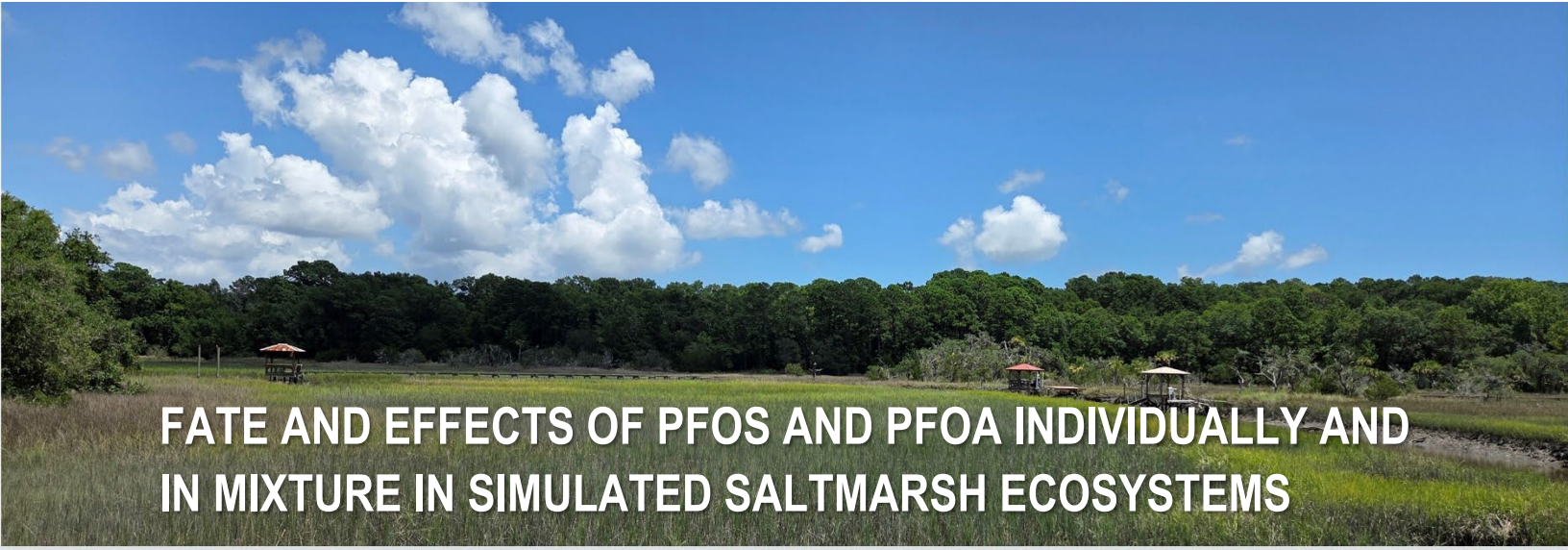
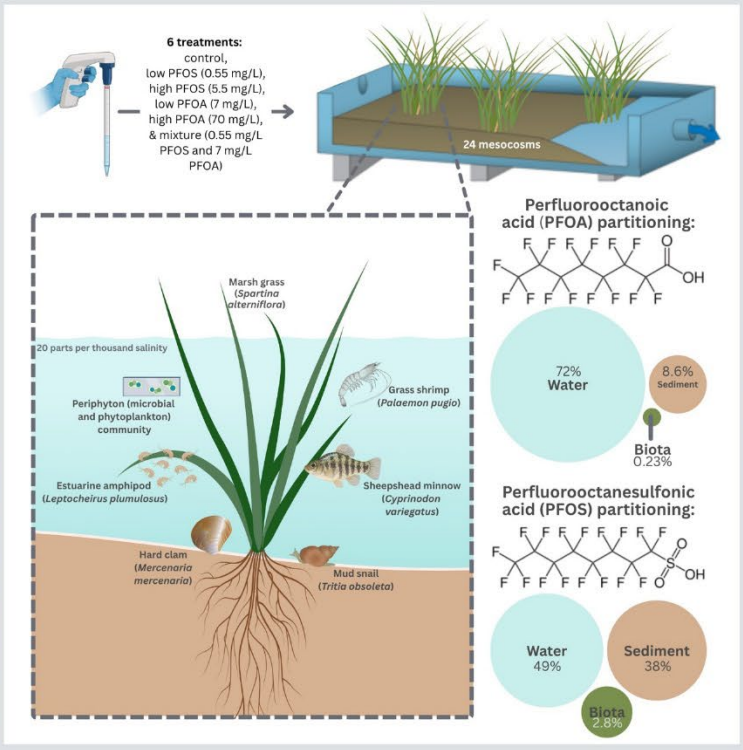


FATE AND EFFECTS OF PFOS AND PFOA INDIVIDUALLY AND IN MIXTURE IN SIMULATED SALTMARSH ECOSYSTEMS



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NOAA TECHNICAL MEMORANDUM NOS NCCOS 368

National Centers for Coastal Ocean Science
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U.S. Department of Commerce

COVER GRAPHIC DESCRIPTION

Illustrates the experimental design and subsequent partitioning of PFAS compounds in a mesocosm experiment simulating the tidal creeks of the southeastern U.S. Top: the 5 PFAS treatments (low perfluorooctanesulfonic acid (PFOS) at 0.55 mg/L, high PFOS at 5.5 mg/L, low perfluorooctanoic acid (PFOA) at 7 mg/L, high PFOA at 70 mg/L, and a mixture treatment with 0.55 mg/L PFOS and 7 mg/L PFOA) and control treatments were added to 24 mesocosm units. Inset: the ecosystem components of the mesocosm: natural seawater, sediments, marsh grass (*Spartina alterniflora,* or *Sporobolus alterniflorus*), microbial community, phytoplankton community, grass shrimp (*Palaemon pugio*), mud snails (*Tritia obsoleta,* or *Ilyanassa obsoleta*), clams (*Mercenaria mercenaria*), amphipods (*Leptocheirus plumulosus*), and sheepshead minnows (*Cyprinodon variegatus*). Bottom right: the full names of the PFOA and PFOS, their chemical structures, and labeled circle charts showing how each compound partitioned across water (blue), sediment (brown), and biota (green). The diameters of the circles are proportional to the percentage of the initial mass of PFAS that were present in each compartment at the end of the experiment.

CITATION

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DISCLAIMER

Fate and Effects of PFOS and PFOA Individually and in Mixture in Simulated Saltmarsh Ecosystems

Prepared by

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EXECUTIVE SUMMARY

Per- and polyfluoroalkyl substances (PFAS) are widespread environmental contaminants known for their persistence and mobility. While much research has focused on freshwater systems, their fate and ecotoxicological effects in marine and estuarine environments remain less understood. This mesocosm study aimed to investigate the partitioning behavior and impacts of two common PFAS, perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), individually and in mixture, within a simulated salt marsh ecosystem. The study assessed partitioning into water, sediment, and various biota, as well as effects on animal survival, growth, and physiological endpoints.

The results revealed differential sensitivities among species. Fish (*Cyprinodon variegatus*) and mud snails (*Tritia obsoleta*) showed resilience to PFOS and PFOA exposure, with no significant effects on survival. In contrast, grass shrimp (*Palaemon pugio*) exhibited 66% mortality in the High PFOS treatment, while amphipods (*Leptocheirus plumulosus*) demonstrated the greatest sensitivity, with 96% and 100% mortality in the Low PFOA and High PFOA treatments, respectively. Clams (*Mercenaria mercenaria*) also experienced significant mortality (92%) in the High PFOA treatment. The mixture treatment showed less than additive effects on mortality.

Regarding physiological endpoints, *P. pugio* exposed to Low PFOA, High PFOA, and Mixture treatments exhibited significantly reduced glutathione (GSH) levels, indicating oxidative stress. While *T. obsoleta* GSH levels were not significantly affected by treatment, and neither species showed significant changes in lipid peroxidation (LPX) as measured by malondialdehyde (MDA) levels among treatments, these biomarkers highlight potential sub-lethal effects. *S. alterniflora* growth and nutrient concentrations (ammonia, phosphate, nitrite + nitrate) remained

largely unaffected by PFAS treatments. However, periphyton chlorophyll-*a* concentrations were significantly reduced across all PFAS treatments compared to controls, suggesting a broader impact on primary producers and ecosystem health. Further analysis indicated a negative correlation between periphyton chlorophyll-*a* concentration and total PFAS levels in their tissues.

PFAS fate and partitioning analysis showed that both PFOS and PFOA distributed primarily into water and sediment. PFOS demonstrated a greater propensity for sediment accumulation and biotic uptake compared to PFOA, with PFOS bioconcentration factors (BCFs) generally higher than PFOA BCFs across most organisms. Notable exceptions included *L. plumulosus*, which had higher PFOA BCFs, potentially due to their benthic, detritivorous feeding habits. *C. variegatus* exhibited the highest PFOS tissue concentrations and BCFs among fauna, while periphyton showed significant uptake for both PFAS. *S. alterniflora* also exhibited uptake of both compounds, with PFOS BCFs generally higher than PFOA BCFs. Mass balance calculations indicated that a proportion of the dosed PFAS remained unrecovered, possibly due to adsorption to tank materials or partitioning into deeper sediment layers.

The EPA Saltwater Acute Aquatic Life Criteria (USEPA, 2024a; USEPA, 2024b) is 0.55 mg/L and 7 mg/L for PFOS and PFOA, respectively. The findings highlight that existing acute EPA criteria for PFOA may not be fully protective for sensitive benthic invertebrates like amphipods. The study underscores the importance of chronic testing and a holistic approach to assessing ecological risks. Future research should focus on developing chronic criteria for PFOA in estuarine environments, investigating mechanisms of periphyton impact, exploring ingestion as a key exposure route for bioaccumulation in certain species, and examining the observed isomer-specific uptake in *M. mercenaria*. A more comprehensive understanding of PFAS mass

balance, including analysis of deeper sediment layers and plant root systems, is also crucial for better predicting their long-term environmental fate.

Key words: PFOS, PFOA, mesocosm, estuarine, bioaccumulation, toxicity, mass balance

INTRODUCTION

Per- and polyfluoroalkyl substances (PFAS) are a broad group of fluorinated chemicals marketed for widespread use in common products such as food packaging, oil, water and stain resistant products, personal care products, paints and industrial products, and firefighting foams. The branched chain structure with fluorine-carbon bonds is very stable under environmental and contemporary treatment conditions. PFAS are both mobile and persistent contaminants of groundwater, drinking water, surface water, and human and animal tissues. The presence of PFAS in tissues of aquatic and terrestrial species has been documented in studies conducted across every continent, including remote regions far from direct sources, such as the high arctic, Antarctica, and oceanic islands (Giesy and Kannan, 2001; Houde et al., 2006). PFAS has been measured in South Carolina, USA, coastal fish species (Fair et al., 2019) and in bottlenose dolphins (Lynch et al., 2019). Exposure to PFAS has been linked to multiple adverse health effects including liver, kidney, and thyroid diseases and cancers, autoimmune diseases, and developmental and reproductive impairments.

Much of the research on PFAS has focused on freshwater environments (Ehsan et al., 2025), with fewer studies on marine systems (Tansel, 2024) and PFAS exposure and toxicity thresholds in marine species. Differences in physicochemical properties, and subsequent bioavailability, may cause the fate and toxicity of PFAS to differ in marine vs. freshwater systems. Global surface water concentrations of PFAS compounds are found to be highest in rivers followed by coastal areas (Muir and Miaz, 2021) with the potential for PFAS to accumulate in large open ocean gyres (Tansel, 2024). Several studies report comparable PFAS levels in marine and freshwater fish (Barbo et al., 2023; Buzby et al., 2021; Fair et al., 2019) while another reports that levels in marine fish were much lower than freshwater fish (Yamada et

al., 2014). It is also important to note that there is considerable variability among sites in close proximity within marine environments (Fair et al., 2019).

Perfluroroalkyl sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are two PFAS commonly measured in coastal ecosystems (Fair et al., 2019) and throughout many other ecosystems as well as in humans (Ingelido et al., 2025). These compounds are found to be highly persistent in many ecosystems including the marine environment. Partitioning coefficients must be modeled because lab-based determinations are essentially impossible due to the surface active properties of the compounds (Brooke et al., 2004). The modeled Log K_{ow} (octanal/water partitioning coefficient) values range from 4.49 to 6.43 and 4.81 to 5.30 for PFOS and PFOA (EPA, 2025), respectively.

For comparison, these PFAS Log Kow values are in the same range or slightly lower than other organic contaminants such as DDT [6.79 (Swann et al., 1981)], fluoranthene [5.16 (Hansch et al., 1995)], and dioxin [6.8 (Shiu et al., 1988)]. Despite these elevated Log K_{ow} levels, these compounds are found to predominantly bioaccumulate in proteinaceous tissues (e.g. blood serum and liver tissue) rather than in lipid stores in aquatic organisms (Wang et al., 2025). These compounds have been shown to elicit reproductive toxicity, neurotoxicity, developmental toxicity, immunotoxicity, and metabolic toxicity in invertebrate organisms (Zhang et al., 2025). Studies in fish have demonstrated developmental toxicity, changes in gene expression, and behavior modification (Ivantsova et al., 2025).

Ecotoxicologists face significant challenges when extrapolating precise laboratory data to dynamic aquatic environments due to the complexity of natural ecosystems. While laboratory toxicity tests provide controlled conditions to assess the effects of specific compounds on individual species, they often fail to account for the intricate physical, chemical, and biological

interactions present in real-world settings. Field studies, though more environmentally relevant, are limited by high costs, time constraints, and lower statistical reliability. To bridge this gap, aquatic mesocosms have been developed as intermediate-scale systems that replicate and manipulate key ecological parameters (Touart, 1994), offering a more holistic approach to ecotoxicology. Mesocosms integrate various environmental factors such as temperature, nutrient interactions, and food web dynamics, providing more realistic insights into fate and effects (Pennington et al., 2007).

The purpose of this study was to examine the fate of two common PFAS in a simulated salt marsh ecosystem and determine impacts to a representative marine community. We tested PFOS and PFOA individually and in mixture to 1) measure partitioning from water into sediment, plant, and animal tissues, and 2) assess effects on plant and animal survival, growth, and physiological endpoints.

METHODS

CHEMICALS

Perfluoroalkyl sulfonate (also known as Heptadecafluorooctanesulfonic acid or PFOS) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The chemical formula was $C_8HF_{17}O_3S$ (CAS 1763-23-1) and the molecular weight was 500.13 g/mol with a purity ≥ 95%. It was comprised of a near 50:50 mix of linear and branching molecules. Perfluorooctanoic acid (also known as Pentadecafluorooctanoic acid, C8, or PFOA) was obtained from Millipore Sigma (St. Louis, MO, USA). The chemical formula was $C_8HF_{15}O_2$ (CAS 335-67-1) and the molecular weight was 414.07 g/mol with a stated purity of 95%. It was comprised of linear molecules. Individual working stock solutions of these chemicals were made by dissolving them in deionized (DI) water. The stocks were stored at room temperature in a dark environment.

MESOCOSM TEST SETUP

Testing occurred in simulated southeastern tidal creek mesocosms located at NOAA's National Centers for Coastal Ocean Science (NCCOS) Hollings Marine Laboratory. The facility consists of 24 mesocosm units enclosed in a greenhouse. Each mesocosm consisted of two tanks, one upper and one lower in accordance with procedures outlined in NOAA Technical Memorandum NOS NCCOS 62 (Pennington et al., 2007). The systems received near-ambient lighting and temperature, and incorporated tidal flux via an upper and lower tank fitted with pump and timer facilitating two high tide and two low tide events within each 24 h period. The ecosystem components included natural seawater, sediments, marsh grass (*Spartina alterniflora,* also known as *Sporobolus alterniflorus*), microbial community, phytoplankton community, grass shrimp (*Palaemon pugio*), mud snails (*Tritia obsoleta,* also known as *Ilyanassa obsoleta*), clams

(*Mercenaria mercenaria*), amphipods (*Leptocheirus plumulosus*), and sheepshead minnows (*Cyprinodon variegatus*).

Seawater was collected from the Charleston Harbor estuary (32.75324, -79.89969), filtered (5 μm), UV-sterilized, activated carbon filtered, and diluted to 20 ppt. The salinity was held constant at 20 ppt by adding deionized water as needed. Intertidal sediment was collected for each mesocosm from a relatively undeveloped reference site at Leadenwah Creek, a tributary of the North Edisto River, Wadmalaw Island, SC (32.64754, -80.22161). Each tank contained approximately 443 L of seawater and 52 kg of sediment. *P. pugio* and *T. obsoleta* were collected from the same Leadenwah Creek site, while the *C. variegatus* were reared in the laboratory (original brood stock was from Aquatic BioSystems Inc., Fort Collins, CO, USA). *L. plumulosus* (Aquatic Research Organisms Inc., Hamptons, NH, USA) and *M. mercenaria* (Bay Shellfish Co., Terra Ceia, FL, USA) were acquired commercially. The sediments were sieved through a 3 mm sieve to remove larger benthic fauna and other debris and were then placed into mesocosm sediment trays (high density polyethylene material, 20 cm x 20 cm x 20 cm depth, approximately 12.75 kg of mud/tray, 4 trays/tank). Sediment trays were completely submerged at high tide and 70% exposed at low tide allowing water to drain as the tide receded. *S. alterniflora* plugs (5 cm x 5 cm; initial shoot height approximately 35 cm; four plugs/tray, acquired from Environmental Concern, Inc. St. Michaels, MD) were added ten weeks prior to the start of the test. *C. variegatus* (27-39 mm, 17/tank), *P. pugio* (20-30 mm, 100/tank) and *T. obsoleta* (15-18 mm, 30/tank) were added 10 days prior to the start of the test. Juvenile *M. mercenaria* (1-2 mm) and *L. plumulosus* (2-4 mm) were placed in plastic exposure containers made from 500 mL polypropylene cups with meshed sides (30 animals each with 100 mL of sediment, 4 cups/tank) and added one week prior to the start of the test. An additional 100 *M. mercenaria* were added to a round, plastic

planter tray (20.3 cm diameter) filled with approximately 1 L of sediment (1 tray/tank). Sediment used for *M. mercenaria* and *L. plumulosus* was further sieved through a 1 mm and 500 µm stainless steel sieve, respectively. Standard glass microscope slides (10 slides/tank) to collect periphyton were added four days prior to the start of the test. With the seawater, sediment, and biota considered to be distinct compartments of each system, the relative sizes of those compartments based on mass were estimated. Figure 1 shows a representation of those compartments and their relative sizes.

Six tanks (one in each treatment) were monitored continuously (sampling every 15 minutes) throughout the experiment with a YSI 5200A Continuous Aquaculture Monitor for water quality parameters (temperature, pH, dissolved oxygen, salinity), and daily, discrete water quality measurements were taken from all 24 tanks using a YSI handheld instrument (YSI 556) once or twice each day.

Figure 1: Mesocosm compartment sizes based on total mass (g) and relative percentages. The values within each box represent the mass (g) of that compartment and the boxes are sized proportionally relative to one another. The lower box is expanding out the biota compartment of the upper box (BGB = Below Ground Biomass [S. alterniflora rhizomes and roots], AGB = Above Ground Biomass [S. alterniflora stems]).

TREATMENTS AND SAMPLING

The experiment consisted of six treatments with four replicate mesocosms each: Control, Low PFOS (0.55 mg/L), High PFOS (5.5 mg/L), Low PFOA (7 mg/L), High PFOA (70 mg/L), and Mixture (PFOS [0.55 mg/L] and PFOA [7 mg/L]). All concentrations listed here and throughout the paper are nominal. Actual measured concentrations were used in the calculations for biouptake and are found in Table 1. The lower levels of PFOS and PFOA were selected to match the EPA Saltwater Aquatic Life Criteria (EPA, 2024a; EPA, 2024b), whereas the 10 fold higher levels represented the acute effects range determined for the sheepshead minnow, *C. variegatus* (Beach et al., 2006; Tanabe et al., 2024). Each chemical stock was prepared in deionized water and dosed into the upper mesocosm tank at low tide. The water was then circulated throughout the system by pumping the seawater up and down between the upper and lower tanks thrice before collecting an initial water sample for chemistry (T=0 h).

Water column samples (approximately 250 mL) were collected for PFAS chemistry, nutrients, chlorophyll *a*, and phytoplankton pigment composition at the following timepoints: pre-dose, T=0 (after dosing and the three mixing exchanges), T=24 h, T=48 h, T=72 h, T=7 d, T=14 d, T=21 d, and T=28 d. Nutrients (nitrates+nitrites, ammonia, phosphate) were analyzed by the Nutrient Analytical Services Laboratory at the Chesapeake Biological Laboratory (Solomons, MD, USA). Water column chlorophyll-*a* was quantified using a fluorometric method (Welschmeyer, 1994). Periphyton community was collected from the glass slides suspended in the water column at T=28d. Phytoplankton and periphyton pigment analyses were performed by the University of South Carolina Estuarine Ecology Lab (Columbia, SC) using an acetone-sonication extraction process followed by High Performance Liquid Chromatograph (HPLC) separation (Pinckney et al., 2002). CHEMTAX software was subsequently used to analyze

pigment data and estimate microalgal class or group abundances (Mackey et al., 1996; Pinckney et al., 2001).

Table 1: Time=0 Water PFOS and PFOA Concentrations. Mean and standard error of PFAS concentrations measured 1 hour after dosing. Percent of target dose was calculated based on the doses in the treatment column. Values in bold are measurements of the dosed compound. Unbolded values represent background levels in systems not dosed with that compound.

Treatment	Concentration (mg/L)		Percent of Nominal Dose (%)
	Mean PFOA (SE)	Mean PFOS (SE)	Based on Average
Control	0.000072 (0.000034)	0.00028 (0.000028)	NA
Low PFOA (7 mg/L)	**4.38 (0.56)**	0.067 (0.026)	PFOA: 62.57
High PFOA (70 mg/L)	**52.12 (1.73)**	0.00 (0)	PFOA: 74.46
Low PFOS (0.55 mg/L)	0.15 (0.029)	**0.19 (0.050)**	PFOS: 34.55
High PFOS (5.5 mg/L)	0.46 (0.40)	**3.90 (1.96)**	PFOS: 70.91
PFOS + PFOA (0.55 mg/L + 7 mg/L)	**4.70 (0.26)**	**0.31 (0.023)**	PFOA: 67.14 PFOS: 56.36

PFOA and PFOS were quantified in sediments (approximately 10 g surface scrape) at pre-dose, T= 0 h, 24 h, 48 h, 72 h, 7 d, 14 d, 21 d, and 28 d. Tissues were collected for PFAS analysis at T=7 d (*M. mercenaria* and *L. plumulosus*) and T=28 d (*M. mercenaria*, *P. pugio*, *C. variegatus*, *S. alterniflora*, and *L. plumulosus*). Periphyton was only assessed for PFAS concentrations at T=28 d.

Animal survival was assessed at T=28 d. *C. variegatus* length and weight was determined and *M. mercenaria* weight was determined. Surviving animals were preserved for chemical analysis and species with sufficient tissue mass remaining (*T. obsoleta* and *P. pugio*) were preserved for biomarker analysis (lipid peroxidation and glutathione). *S. alterniflora* was measured for stem height and stem density.

CHEMICAL ANALYSIS

Water sample aliquots (10 mL for dosed water and 250 mL for controls) were analyzed for PFOS and PFOA at all time points. Dosed water samples were diluted gravimetrically with methanol to the working calibration range of the instrument (1-10,000 ng). Diluted samples were spiked with an internal standard (13C PFAS mix [MPFAC-24ES], Wellington Laboratories) and stored at -20°C prior to analysis. Control water samples were extracted according to EPA method 533 using weak anion exchange solid phase extraction (SPE) (EPA, 2019). Both dosed and control water samples were analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS) according to methods detailed in Tanabe et al. (2024).

Sediment samples collected from each time point were analyzed according to standard protocols developed by the National Institute of Standards and Technology (NIST, 2021; Reiner, 2023). Briefly, 0.5 g of sediment material was weighed into a 50 mL polypropylene tube. Sediment aliquots were spiked with internal standards, vortexed and allowed to equilibrate for one hour. Next, 5 mL of 0.1 M potassium hydroxide in methanol was added and samples were sonicated for 30 min. Samples were then centrifuged (2500 rpm, 5 min), and the supernatant was transferred to a new 50 mL polypropylene tube. The extraction was repeated one more time and supernatants were combined and then run through pre-conditioned ENVI-carb SPE (Supelco

Supelclean; 3 mL, 250 mg). Samples were eluted with methanol, evaporated to 1 mL and analyzed using LC-MS/MS.

Biological tissues (*C. variegatus*, *P. pugio*, *M. mercenaria*, *T. obsoleta*, *L. plumulosus* and periphyton) were all analyzed for PFOS and PFOA. With the exception of *M. mercenaria* and *L. plumulosus*, treatment replicates were analyzed individually. Organisms (*C. variegatus*, *P. pugio*, *M. mercenaria*, *T. obsoleta*, *L. plumulosus*) were first homogenized (Table 2) then extracted using QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) according to the US Food and Drug Administration's method C-010.01 (FDA, 2024; UCT, 2020). For periphyton samples, glass slides (n=1 per replicate) were weighed (wet weight) and recorded, placed into 50 mL polypropylene tubes with 50 mL of methanol, spiked with internal standard and sonicated for 20 min. Samples were then decanted through GF/F paper, concentrated to 1 mL, and cleaned-up using ENVI-carb SPE as detailed earlier. To determine how much periphyton mass was extracted, glass slides were wiped clean of all residual algae post-extraction and re-weighed. The final mass of the slide was subtracted from the initial mass to determine how much periphyton mass (m_{ps}) was on each slide. All biological samples were analyzed for PFOS and PFOA using LC-MS/MS.

Marsh grass blade (*S. alterniflora*) samples (20 cm in length) were collected at the end of the test and frozen at -80°C until analysis. For analysis, each sample was thawed, carefully cut into 1 mm pieces using dissecting scissors (~ 1 g material), and placed in a glass mortar bowl. One gram of silica powder was added and the sample was ground with a pestle until homogenized. Sample material was then transferred into a 50 mL polypropylene tube, spiked with internal standard and equilibrated for 20 min. Ten milliliters of methanol was added to the sample, vortexed and then sonicated for 30 min. Samples were then centrifuged (3000 rpm, 5

min), decanted and extracted one additional time. Extracts were combined and concentrated to 3 mL then cleaned-up using ENVI-carb SPE as detailed earlier. *S. alterniflora* samples were analyzed using LC-MS/MS.

Table 2: Sample preparation for tissue analysis

Organism	**Tissue Analysis**	**Homogenization**	**Homogenization Notes**
sheepshead minnow *(C. variegatus)*	whole body	cutting and maceration via scalpel	n=1 from each replicate
grass shrimp *(P. pugio)*	whole body	maceration via stainless steel spatula	n=10 pooled, from each replicate
mud snail *(T. obsoleta)*	whole body, shell removed	maceration via stainless steel spatula	n=10 pooled, from each replicate
amphipod *(L. plumulosus)*	whole body	maceration via stainless steel spatula	combined treatment replicates
clams *(M. mercenaria)*	whole body, shell included	maceration via stainless steel spatula	combined treatment replicates
cordgrass *(S. alterniflora)*	20 cm grass blade of above-ground biomass	cut into pieces via scalpel and ground with mortar and pestle	n=1 from each replicate

MASS BALANCE

Mass balance calculations were conducted to estimate the distribution of PFOS and PFOA within the environmental compartments (water, sediment, and biota) of each treatment tank after 28 days of exposure (Equations 1 and 2), allowing for the assessment of partitioning behaviors.

$$PFAS_{dosed} - (PFAS_{water} + PFAS_{sediment} + PFAS_{biota}) = PFAS_{recovered}$$

Equation 1: Calculation for recovered mass of PFAS

$$PFAS_{dosed} - PFAS_{recovered} = PFAS_{unrecovered}$$

Equation 2: Calculation for unrecovered mass of PFAS

The total mass (mg) of PFAS in the water compartment of each mesocosm was estimated by multiplying the measured concentration of PFAS (mg/L) in seawater by the number of liters of seawater in each system (440 L). Likewise, the total mass (mg) of PFAS in the sediment was estimated by multiplying the measured concentration (mg/kg) in the sediment by the number of kg of sediment in each system (78.7 kg). The total mass (mg) of PFAS in the biotic compartment was estimated by summing the total mass of PFAS in each species of fauna, *S. alterniflora*, and the periphyton. For each species of fauna, the total mass of PFAS (mg) found in each species ($m_{PFAS:\ faunal\ species}$) was estimated by multiplying the measured tissue concentration of $PFAS_{cf}$ (ng/g) of an individual from each mesocosm by the tissue mass (m_{fs}) of that individual and then multiplying by the number of individuals (n) therein (Equation 3). All individuals were of the same relative size. That number, in ng, was then converted to mg by dividing by 1×10^6 ($1\ mg \equiv 1 \times 10^6\ ng$).

$$m_{PFAS:\,faunal\ species} = \frac{\left[PFAS_{cf}\right] \times m_{fs} \times n}{1 \text{ x } 10^6}$$

Equation 3: Estimation of the total mass of PFAS in each species of fauna per mesocosm

The total mass (mg) of PFAS in *S. alterniflora* AGB in each mesocosm ($m_{PFAS:\,S.alterniflora}$) was estimated according to Equation 4:

$$m_{PFAS:\,S.alterniflora} = \frac{[PFAS_{cs}] \times h\ x\ n\ x\ (S_{conv})}{1\ x\ 10^6}$$

Equation 4: Estimation of total mass of PFAS in S. alterniflora per mesocosm

Where $PFAS_{cs}$ (ng/g) equals the measured concentration of PFAS in a 20 cm AGB cutting; h equals the average measured height (cm) of the *S. alterniflora* stems from that mesocosm; n equals the total number of stems from that mesocosm; and S_{conv} (g/cm) is the conversion factor 0.0075 (where a 1 cm blade length of *S. alterniflora* ≡ average mass of 0.0075 g determined from random cuttings). The mass, in ng, was then converted to mg by dividing by 1 x 10^6. The total mass of PFAS (mg) found in below ground biomass (BGB) of *S. alterniflora* was estimated based on the total mass of PFAS (mg) of the AGB as direct measurements from BGB were not obtained. This estimate was made by multiplying the total mass (mg) of PFAS in AGB mass by 14 to get the approximate total mass (mg) of PFAS for the BGB. This is based on the study by Pennington et al. (2024) where the dry weight BGB of *S. alterniflora* of was found to be ~14x greater than AGB per estuarine mesocosm. The total mass of PFAS (mg) found in the periphyton compartment ($m_{PFAS:\,periphyton}$) was estimated using Equation 5:

$$m_{PFAS:\,periphyton} = \frac{\left(\frac{m_{ps}}{A_s} * A_m\right) * \left[PFAS_{cp}\right]}{1\ x\ 10^6}$$

Equation 5: Estimation of total mass of PFAS in periphyton per mesocosm

Where $PFAS_{cp}$ (ng/g) equals the measured concentration of PFAS sampled from the periphyton on the glass slide, m_{ps} equals the mass of periphyton recovered from the slide, A_s equals the combined surface of both sides of the slide, A_m equals the combined surface areas of all of the submerged and intertidal interior wetted surfaces of each mesocosm including the upper tanks, lower tanks, and sediment boxes. As in the previous biotic compartments, the mass is divided by 1 x 10^6 to convert from ng to mg.

BIOMARKER ANALYSIS

P. pugio and *T. obsoleta* were preserved at -80°C until analysis. *P. pugio* were preserved whole in groups of 10 from each mesocosm. The soft tissues of the *T. obsoleta* were preserved in groups of 5 from each mesocosm. The effects of PFOS/PFOA exposure in both species were measured using glutathione (GSH) and lipid peroxidation (LPX) assays. For each mesocosm, 10 *P. pugio* and 3 *T. obsoleta* tissue samples were used in the GSH and LPX assays. *P. pugio* were measured individually, and *T. obsoleta* tissues were randomly selected for analysis.

Glutathione is a tripeptide antioxidant involved in detoxification and maintaining homeostasis. Depletion of GSH after toxicant exposure could adversely impact organismal health. The GSH assay was completed following methods described by Ringwood et al. (2003). Samples were homogenized in cold 5% sulfosalicylic acid (SSA) at 10 times their weight and centrifuged at 13,000 x g at 4°C for 5 minutes. *T. obsoleta* were centrifuged for 10 minutes. Supernatants with a volume of 100 µL were combined with 100 µL of 5% SSA. Standards of

reduced L-glutathione were created ranging from 200 µM to 3.125 µM and serially diluted with 5% SSA. Aliquots of 25 µL for each sample, standard, or blank (5% SSA) were added to 0.238 mg/mL nicotinamide adenine dinucleotide phosphate (NADPH) buffer, 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and deionized water for a total volume of 1.0 mL. Subsamples of 900 µL were added to cuvettes with 15 µL of 50 U/mL glutathione reductase and read at 405 nm for 90 seconds using an Agilent BioTek Epoch 2 spectrophotometer. Changes in absorbance were taken every 15 seconds using Agilent BioTek Gen 6 software to calculate GSH concentrations in nmol/g wet weight (w.w.).

The LPX assay measures the levels of malondialdehyde (MDA), an end-product of lipid peroxidation. Increased levels of MDA can indicate cellular membrane damage after exposure to toxicants. The LPX assay was completed following methods by Ringwood et al. (2003). Samples were homogenized in cold 50 mM potassium phosphate (K_2PO_4) buffer at 4 times their weight and centrifuged at 13,000 x g at 4°C for 5 minutes. *T. obsoleta* were centrifuged for 10 minutes. Standards of MDA were created ranging from 3200 to 25 µM for *P. pugio* and from 800 to 6.25 µM for *T. obsoleta*. Standards were serially diluted with 50 mM K_2PO_4 buffer. For analysis, 75 µL aliquots of either sample supernatant, MDA standard, or blank (K_2PO_4 buffer) were combined with 1050 µL of 0.375% thiobarbituric acid and 10.5 µL of 2% butylated hydroxytoluene. Tubes were heated at 92°C for 15 minutes and centrifuged at 13,000 x g at room temperature for 5 minutes. Aliquots of 300 µL were plated in triplicate into a clear Corning 96-well plate and read at 532 nm using an Agilent BioTek Epoch 2 spectrophotometer. Agilent BioTek Gen 6 software was used to calculate MDA concentrations in nmol/g w.w.

Data Analysis

The effects of PFAS treatment on animal survival and growth were analyzed in SAS (SAS Institute, 2016) using ANOVAs. Dunnett's test was used, if the ANOVA p–value was significant ($p<0.05$), to determine which individual treatments were significantly different from the control. Biomarker data were analyzed using non-parametric Kruskal-Wallis tests followed by post-hoc tests utilizing a Dunnett's-like approach, as transformations did not improve the normality of the data (Zar, 1999).

S. alterniflora growth, nutrients, chlorophyll-*a*, periphyton, phytoplankton, water and sediment chemistry, and tissue chemistry were statistically analyzed in RStudio (Posit Team, 2024; R Core Team, 2024). For each set of data, an ANOVA was performed followed by a Dunnett's test, if warranted, to examine the effect of PFAS treatment on the various endpoints (Signorell et al., 2019). For all statistical analyses, there were four replicate mesocosms per treatment, unless otherwise noted. To ensure the data met ANOVA and Dunnett's assumptions, a Shapiro-Wilk test was used to ensure residual normality followed by a Bartlett's test to assess equality of variance. If the data did not meet assumptions, it was natural log or square root transformed, or non-parametric tests were used if transformations were unsuccessful. Non-parametric analyses included a Kruskal-Wallis test which, if significant, was followed by a post-hoc Dunn's test comparing treatments to the control (Pohlert, 2024). All figures were built in R or Excel with the color-blind friendly Okabe-Ito, Dark2, or viridis palettes used for multicolor graphs (Garnier et al., 2024; Neuwirth, 2022; Okabe and Ito, 2008).

For periphyton analyses and figures, data from one control mesocosm and one Low PFOA (7 mg/L treatment) mesocosm were excluded due to being anomalously large. These points were determined to be influential outliers according to studentized residuals (values > |3|) and Cook's distance (more than 4/sample size).

Time-weighted average (TWA) concentrations for the water and sediment PFOS and PFOA samples were calculated in a similar methodology to that of (Belgers et al., 2011; Watanabe et al., 2018; Wirth et al., 2024; Zimmer et al., 2018) according the following equation (6) found in Belgers et al. (2011).

$$\boldsymbol{TWA} = \frac{\sum_{n=1}^{N}(\Delta \boldsymbol{t_n} \frac{\boldsymbol{c_{n-1}} + \boldsymbol{c_n}}{\boldsymbol{2}})}{\sum_{n=1}^{N}(\Delta \boldsymbol{t_n})}$$

Equation 6: Time-weighted average (TWA) concentration.

Where n=time interval number; N=total number of intervals; c=concertation of PFOS or PFOA in the water (mg/L) or sediment (mg/kg); Δt = time interval between measurements: and TWA= the time-weighted average concertation in units of mg/L or mg/kg for water and sediments, respectively.

Mass balance estimates were compared across treatments using single factor ANOVAs (SAS Proc GLM) for each compartment (water, sediment, biota, and accounted PFAS) as well as a Multivariate ANOVA (or MANOVA) with contrasts (SAS Proc GLM, MANOVA statement) for comparing compartmental partitioning patterns between treatments. The MANOVA used the Type I Sum of Squares and Cross Products (SSCP) matrix and characteristic roots (eigenvalues) and characteristic vectors (eigenvectors) were reported. The p-value for the Wilks' Lambda statistic was reported where significant differences in mass balance partitioning patterns were detected.

RESULTS

PFAS EFFECTS

ANIMAL GROWTH AND SURVIVAL

After 28 d of PFAS exposure, there was no significant effect on *C. variegatus* or *T. obsoleta* survival in any of the treatments [survival 91% (SE: 3.42) to 124% (SE: 4.93) of control, Figure 2]. *P. pugio* exhibited 40% (SE: 3.20) survival compared to the controls in the High PFOS treatment. *M. mercenaria* had 8% (SE: 6.97) survival compared to controls in the High PFOA treatment, but no effect on survival was seen in the Low PFOA, PFOS treatments, or Mixture treatments. *L. plumulosus* were the most sensitive species tested with 9.3% (SE: 6.85) survival in the Low PFOA treatment, no survival in the high PFOA, and 16.6% (SE: 7.78) in the Mixture (Figure 2).

Like with survival, PFAS exposure had no significant effect on the growth of *C. variegatus* with average *C. variegatus* lengths ($p=0.39$) and weights ($p=0.31$) being similar amongst the different treatments (Figure 3). Dry weights of surviving *M. mercenaria* also did not differ significantly among treatments ($p=0.61$) (Figure 4).

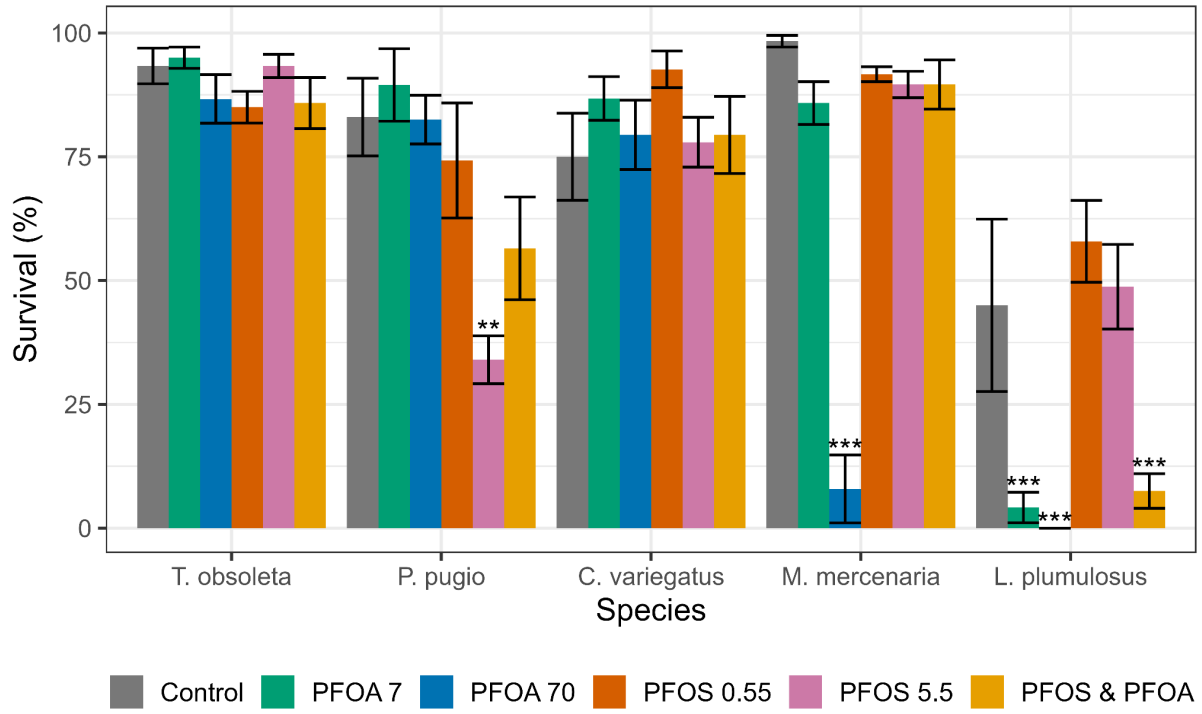

Figure 2: Fauna Survival. Error bars indicate standard error. Asterisks indicate a significant decrease in survival compared to a species' controls: p<0.05 '*', p<0.01 '**', p<0.001 ''.***

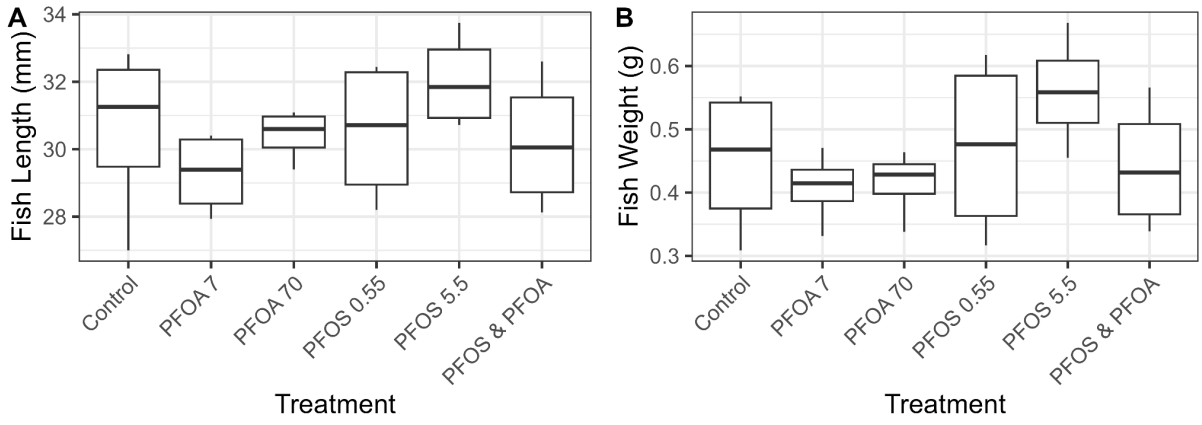

Figure 3: Size of C. variegatus. C. variegatus lengths and weights following 28 days of exposure. The horizontal line within each box designates the median, the lower and upper box edges indicate the first and third quartiles respectively, and each whisker extends up to 1.5 x the interquartile range. Datapoints beyond that value are represented as black dots.

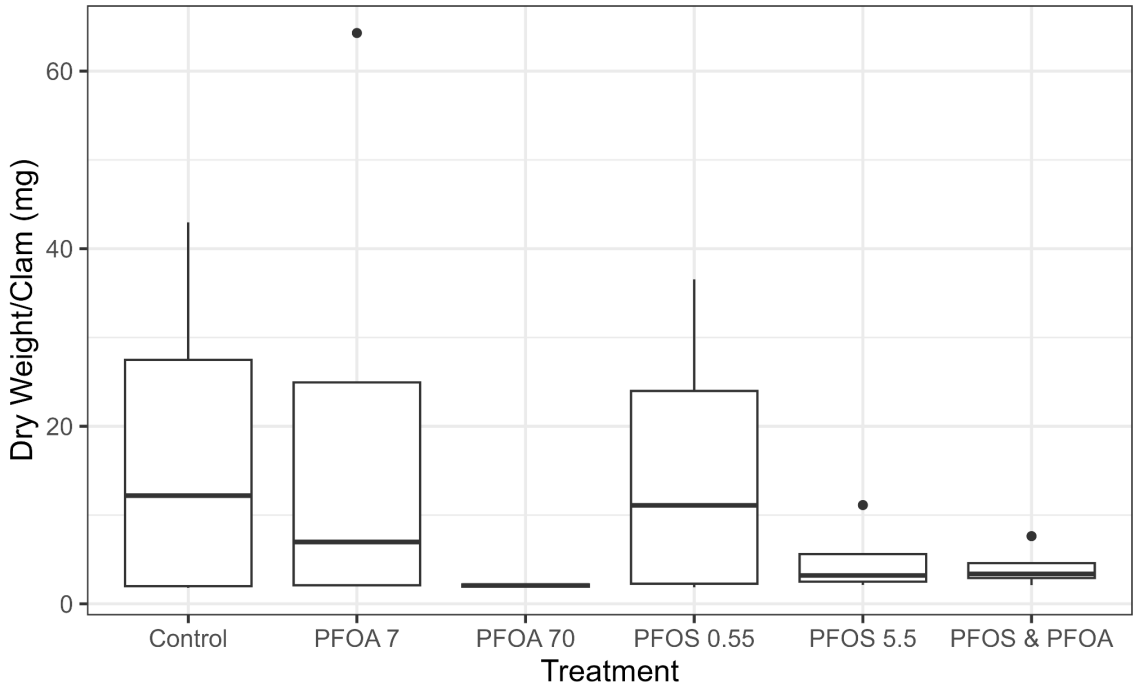

Figure 4: M. mercenaria Weights. Weight of clams following 28 days of exposure. Due to complete mortality, weights were not available for two of the mesocosms dosed with 70 mg/L PFOA. The horizontal line within each box designates the median, the lower and upper box edges indicate the first and third quartiles respectively, and each whisker extends up to 1.5 x the interquartile range. Datapoints beyond that value are represented as black dots.

S. ALTERNIFLORA GROWTH

S. alterniflora growth and survival was consistent across all control and PFAS treatments with no significant effect of treatment on live stem density (p=0.97), average stem height (p=0.30), or total stem height (p=0.86) 28 days after dosing (Table 3). PFAS exposure also had no significant effect on the change in those growth metrics from the beginning to end of the four-week exposure period (p=0.83 for stem density, 0.84 for average height, and 0.51 for total height). Across all mesocosms, average stem density and total stem height increased during the study by 120% (SE: 8.70) and 47.9% (SE: 4.49) respectively, while average stem height decreased by 30.9% (SE: 2.79) reflecting the production of new, shorter stems in the systems. No visible signs of plant chlorosis were observed.

Table 3: Growth of S. alterniflora. Mean and standard error for multiple S. alterniflora growth metrics measured 28 days after dosing. Every live stem was measured and counted across the six sediment trays in each mesocosm (four replicate mesocosms per treatment). Percent change of each measurement from before dosing to the conclusion of the study is also included. No significant effects of PFAS treatment were found (all p-values>0.05).

Treatment	28 Days After Dosing			Change During Study (%)		
	Stem Density per Mesocosm (SE)	Average Stem Height (cm) (SE)	Total Height per Mesocosm (cm) (SE)	Stem Density per Mesocosm (SE)	Average Stem Height (SE)	Total Height per Mesocosm (SE)
Control	219.00 (35.06)	25.71 (0.79)	5520.25 (746.32)	120.47 (18.52)	-34.33 (3.59)	45.46 (13.20)
Low PFOA (7 mg/L)	251.25 (30.16)	24.43 (1.23)	6030.50 (656.70)	117.64 (15.59)	-26.59 (4.53)	59.38 (13.45)
High PFOA (70 mg/L)	242.00 (31.16)	22.84 (0.55)	5524.75 (771.04)	148.53 (30.54)	-37.36 (9.32)	49.22 (4.78)
Low PFOS (0.55 mg/L)	242.00 (16.92)	24.40 (1.00)	5857.75 (152.12)	109.09 (20.99)	-26.39 (9.03)	49.43 (8.75)
High PFOS (5.5 mg/L)	242.00 (29.30)	25.61 (0.93)	6063.00 (537.82)	116.39 (12.17)	-27.52 (7.15)	54.87 (14.20)
PFOS + PFOA (0.55 mg/L + 7 mg/L)	215.50 (57.36)	25.37 (3.02)	4856.75 (1162.88)	108.81 (31.89)	-33.04 (8.38)	28.98 (9.48)

ANIMAL BIOMARKERS

Average *P. pugio* GSH levels ranged from 54.71 (SE: 6.78) nmol/g w.w. in the Low PFOA treatment to 199.95 (SE: 18.19) nmol/g w.w. in the controls (Table 4). GSH levels were significantly different among mesocosms ($X^2 = 125.15$, df = 23, $p < 0.001$) and among

treatments ($X^2 = 65.98$, df = 5, $p < 0.001$). GSH levels were significantly lower in *P. pugio* from the Low PFOA, High PFOA, and Mixture treatments than control *P. pugio* (Dunnett's test; adjusted p-value < 0.05). Within these treatments, measured GSH levels were between 27.36% (Low PFOA) to 38.97% (High PFOA) of control *P. pugio*.

Table 4: Animal Biomarkers. Mean, standard error, and sample size (n) for glutathione (GSH) and malondialdehyde (MDA) in T. obsoleta and P. pugio. Asterisks indicate significant differences from the controls: p<0.05 '*', p<0.01 '**', p<0.001 ''.***

Species	Treatment	GSH			MDA		
		Mean	SE	n	Mean	SE	n
T. obsoleta	Control	93.33	22.28	12	83.33	24.27	12
	Low PFOA	50.61	12.19	12	126.31	9.96	12
	High PFOA	39.92	9.08	7	135.94	27.92	12
	Low PFOS	46.62	14.94	12	59.35	19.24	11
	High PFOS	33.21	8.65	12	97.81	28.24	12
	Mixture	54.51	13.38	10	99.75	7.70	12
P. pugio	Control	199.95	18.19	40	1957.94	140.94	40
	Low PFOA	54.71***	6.78	40	1753.66	87.87	40
	High PFOA	77.93***	6.62	40	1666.57	110.88	40
	Low PFOS	137.28	14.35	40	1899.15	112.10	40
	High PFOS	146.07	13.88	37	1674.63	95.37	35
	Mixture	73.97***	6.70	40	2024.52	143.98	40

Average *P. pugio* MDA levels (from the LPX assay) ranged from 1,666.57 (SE: 110.88) nmol/g w.w. in the High PFOA treatment to 2024.52 (SE: 143.98) nmol/g w.w. in the Mixture treatment (Table 4). MDA levels were significantly different among mesocosms ($X^2 = 82.72$, df = 23, $p < 0.001$) but not among treatments ($X^2 = 7.30$, df = 5, $p = 0.199$).

Average *T. obsoleta* GSH levels ranged from 33.21 (SE: 8.65) nmol/g w.w. in the High PFOS treatment to 93.33 (SE: 22.28) nmol/g w.w. in the control treatment (Table 4). GSH levels were significantly different among mesocosms ($X^2 = 36.90$, df = 22, $p = 0.024$) but not among treatments ($X^2 = 6.80$, df = 5, $p = 0.236$). No treatment mesocosms were significantly different from control mesocosms (Dunnett's test; adjusted p-value > 0.05 for all comparisons).

Average *T. obsoleta* MDA levels ranged from 59.35 (SE: 19.24) nmol/g w.w. in the Low PFOS treatment to 135.94 (SE: 27.92) nmol/g w.w. in the High PFOA treatment (Table 4). MDA levels were significantly different among mesocosms ($X^2 = 47.20$, df = 23, $p = 0.002$) and among treatments ($X^2 = 14.85$, df = 5, $p = 0.011$). No treatment mesocosms were significantly different from control mesocosms (Dunnett's test; adjusted p-value > 0.05 for all comparisons).

NUTRIENTS

Compared to pre-dose measurements, average ammonia concentrations had declined for each treatment by two weeks after dosing while phosphate levels remained consistent (Figure 5). Mean nitrite + nitrate concentrations were greater in the control than the treatments at every time point following dosing; however, there was a large amount of variability around the means (Figure 5). No effect of treatment was found on ammonia ($p=0.077$), phosphate ($p=0.56$), or nitrite + nitrate ($p=0.094$) levels when first measured after dosing. When nutrients were last measured 14 days after dosing, treatments still had no effect on phosphate ($p=0.98$) and nitrite +

nitrate (p=0.45), while a slight effect was found for ammonia (p=0.040) where a Dunn's test then found no individual treatments to be different from the control.

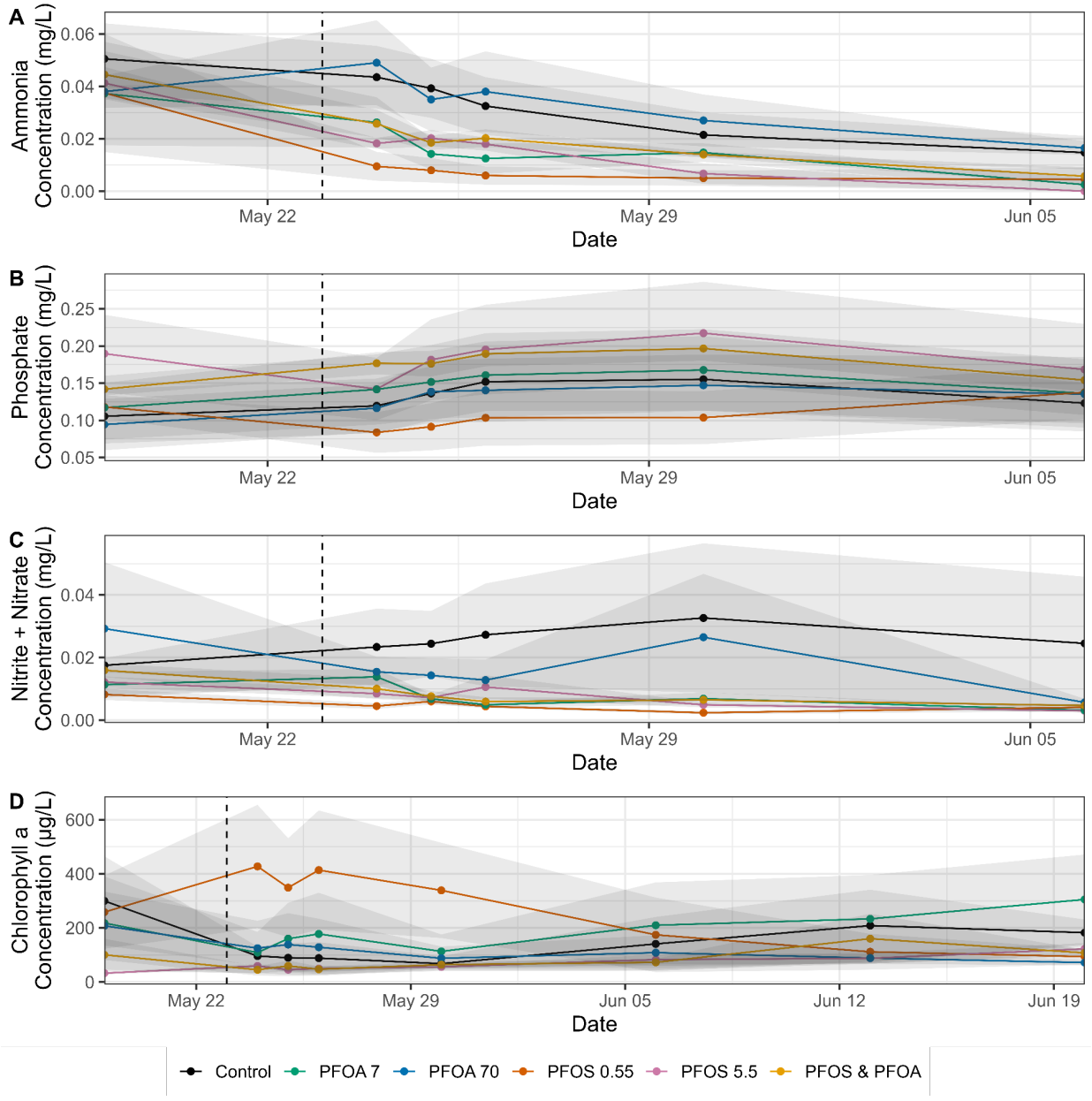

Figure 5: Nutrients and Chlorophyll a in Seawater Over Time. Vertical, dashed lines indicate the day of dosing. Grey shading represents standard error. Chlorophyll a was measured until 28 days after dosing, while nutrients were last analyzed two weeks after dosing.

PHYTOPLANKTON AND PERIPHYTON

Water column chlorophyll-*a* concentrations measured fluorometrically ranged from 67.1 µg/L (SE: 6.14) for the High PFOS treatment to 271 µg/L (SE: 31.1) for the Low PFOS treatment when averaged across all timepoints. An increase in chlorophyll-*a* for the Low PFOS treatment from 24 h to 7 d after dosing led to its higher average concentration (Figure 6); however, there was no significant effect of PFAS treatment on chlorophyll-*a* 24 hours after dosing (p=0.56) or at the conclusion of the study (p=0.073).

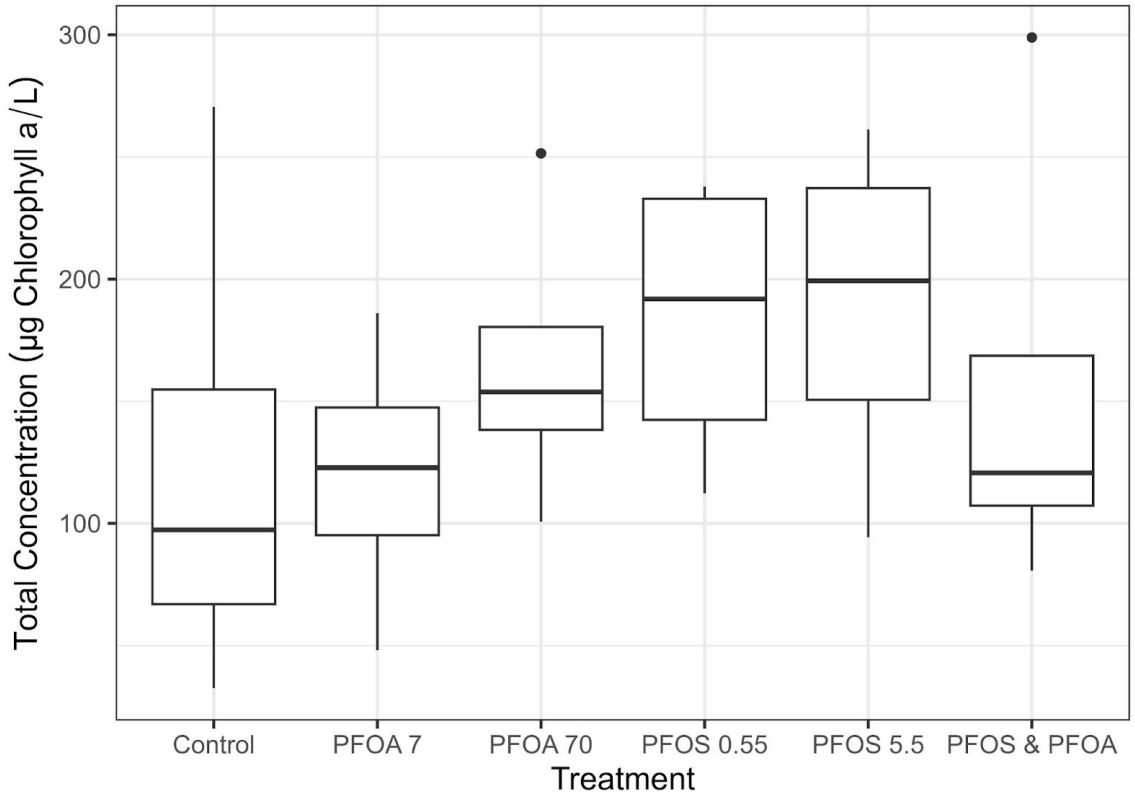

Figure 6: Total Phytoplankton Chlorophyll Concentrations. Total chlorophyll a measured from phytoplankton samples and used when determining concentrations of different phytoplankton groups. The horizontal line within each box designates the median, the lower and upper box edges indicate the first and third quartiles respectively, and each whisker extends up to 1.5 x the interquartile range. Datapoints beyond that value are represented as black dots.

Total chlorophyll-*a* concentrations from phytoplankton samples (determined via the HPLC method) collected 28 days after dosing ranged from 119.91 µg/L (SE: 28.60) for the Low PFOA treatment to 188.54 µg/L (SE: 36.73) for the High PFOS treatment (Figure 6). Despite differences in concentrations when measuring with HPLC instead of fluorometry (Figure 7), there was still no significant effect of PFAS treatment (p=0.74).

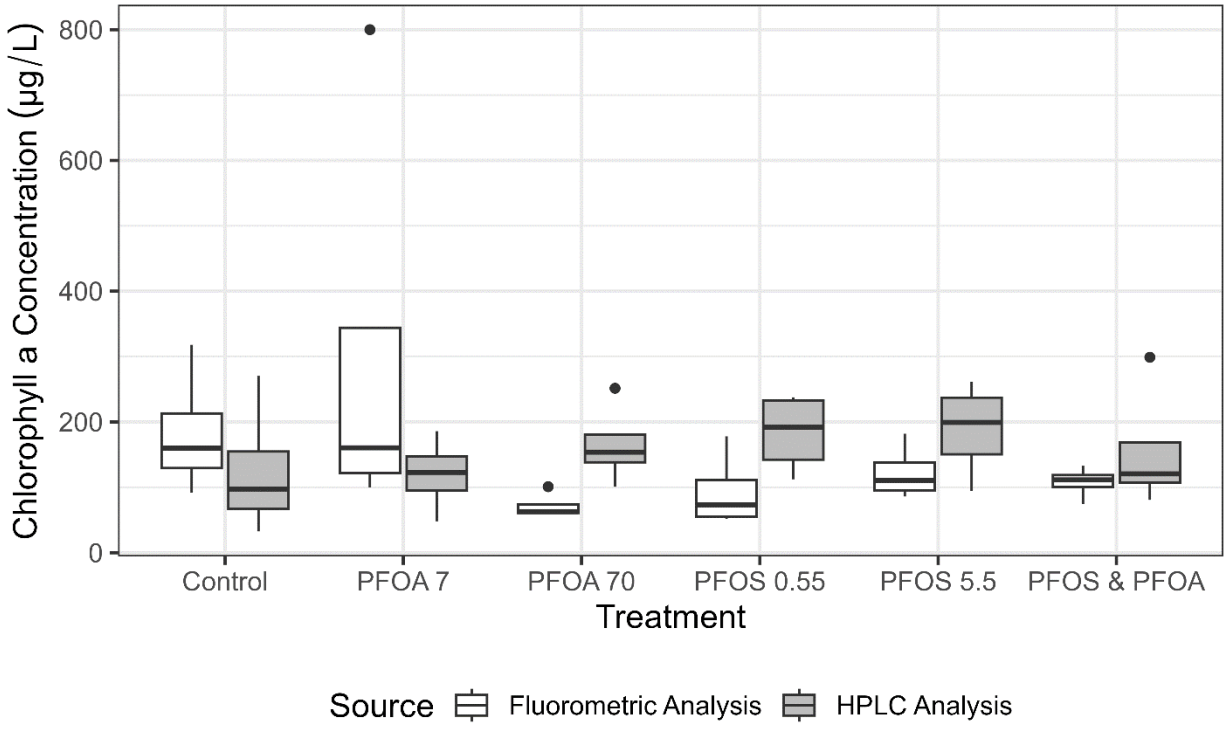

Figure 7: Phytoplankton Chlorophyll-a concentrations via fluorometry and HPLC.

The total chlorophyll-*a* concentrations along with additional pigments were used to estimate concentrations of cryptophytes, cyanobacteria, diatoms, dinoflagellates and green algae (Figure 8). The most abundant groups across every treatment were diatoms and green algae which composed 86.07%-99.31% of the periphyton and 94.62%-99.33% of the phytoplankton in each sample. For phytoplankton, there was only a significant effect of treatment on cryptophytes (p=0.021) with concentrations being higher than the control in the High PFOA (p=0.026), High

PFOS (p=0.022), and mixture (p=0.0093) treatments; however, cryptophytes accounted for just 0-1.15% of the phytoplankton in each mesocosm.

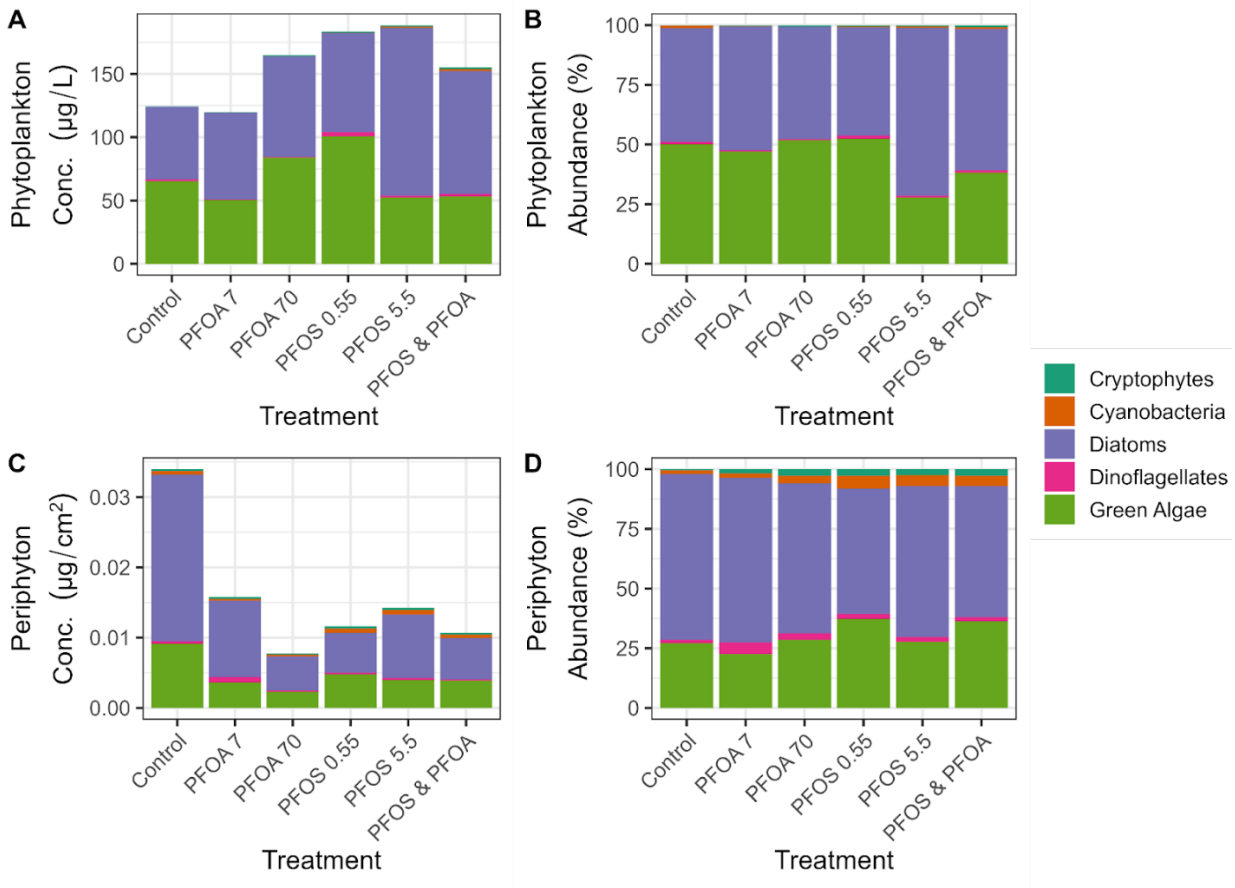

Figure 8: Phytoplankton and Periphyton Community Composition. Bars indicate average values across the replicate mesocosms. A and C show the total concentrations of chlorophyll a found in the samples collected 28 d after dosing. The concentrations are further split into five groups based on additional pigments in the samples. B and D indicate the relative abundance of each group within each treatment.

With outliers removed, periphyton chlorophyll-*a* concentrations were 0.0078 µg/cm^2 (SE: 0.0011, High PFOA treatment) to 0.034 µg/cm^2 (SE: 0.0095, control) (Figure 9). Every treatment had significantly lower chlorophyll-*a* concentrations than the control (p=0.0022 overall or 0.042 for Low PFOA, <0.001 for High PFOA, 0.0030 for Low PFOS, 0.012 for High PFOS, and 0.0020 for mixture). When looking at periphyton groups, PFAS treatment significantly affected diatom pigment concentration (p<0.001). On average, all treatments—except for Low PFOA—

had significantly lower concentrations (62–80% less than the control). The differences were statistically significant for High PFOA ($p<0.001$), Low PFOS ($p<0.001$), and the mixture ($p<0.001$), and for High PFOS ($p=0.018$) (Figure 8).

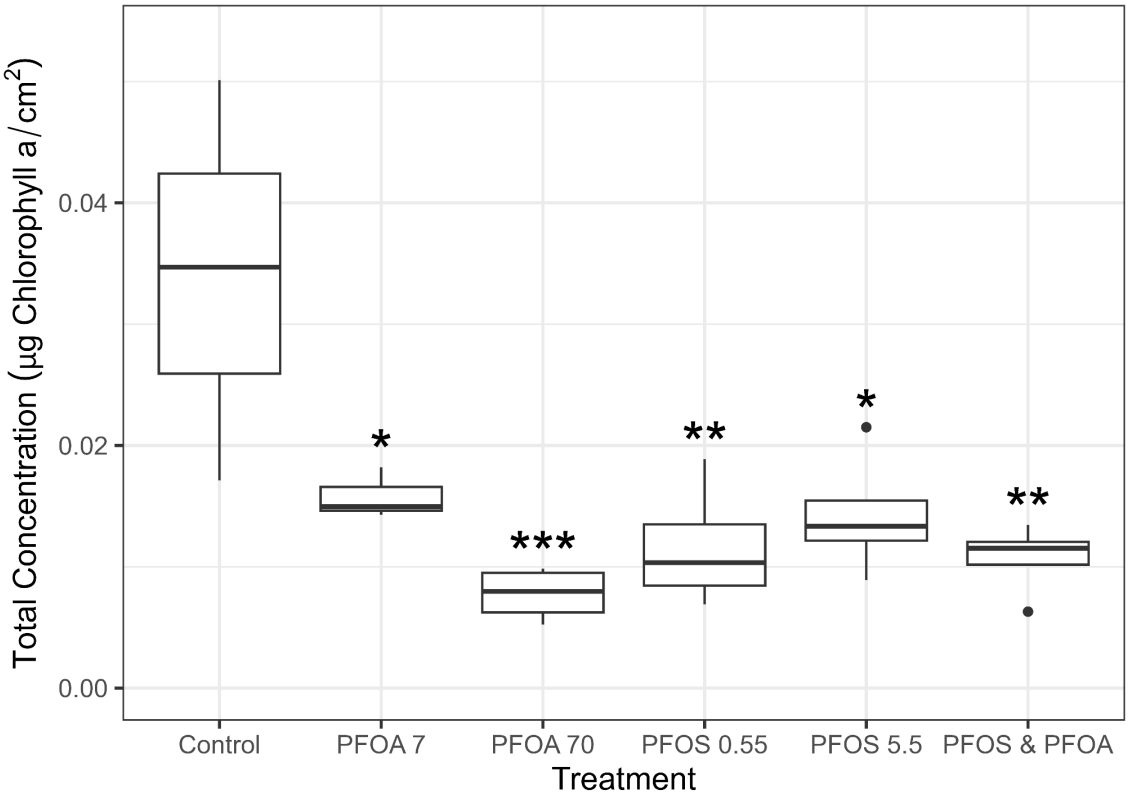

Figure 9: Total Periphyton Concentrations. Total chlorophyll a measured from periphyton seawater samples and used when determining concentrations of different periphyton groups. The horizontal line within each box designates the median, the lower and upper box edges indicate the first and third quartiles respectively, and each whisker extends up to 1.5 x the interquartile range. Datapoints beyond that value are represented as black dots.

There was also a significant effect on dinoflagellate pigment concentrations ($p=0.0071$) where no treatments were then found to be significantly different from the control. For relative abundance, only cyanobacteria pigment concentrations were affected ($p=0.049$) by PFAS exposure. Cyanobacteria pigment composed up to 8.01% of the total periphyton and had relative

abundances 240.22% (SE: 72.62) higher in mesocosms dosed at the Low PFOS level (p=0.0417) compared to the mean control value.

PFAS FATE

WATER AND SEDIMENT CHEMISTRY

Pre-dose water samples had <1 µg/L PFOS and <4 µg/L PFOA. T=0 h water concentrations ranged from 35-74% of target doses (Table 1). Following dosing, PFOA water and sediment concentrations were highest in the High PFOA treatment and slightly elevated in the Low PFOA and Mixture treatments compared to the controls (Figure 10). Throughout the 28 d study, PFOA concentrations in mesocosms dosed with PFOA remained stable and elevated compared to the controls. Concentrations of PFOS in water and sediment showed similar trends to PFOA (Figure 10) with waterborne concentrations being a bit more stable than sediment concentrations. Concentrations were highest for the High PFOS treatment and also elevated in the Low PFOS and Mixture treatments throughout the study compared to controls.

Time-weighted average (TWA) concentrations over the 28 d exposure duration were calculated for PFOS and PFOA in water and sediment and are shown in Table 5. The 28 d TWA concentrations for PFOA were 5 mg/L (SE: 0.044) and 56 mg/L (SE:2.2) for the Low (7 mg/L) and High (70 mg/L) PFOA dosed systems, respectively at 71.4% and 80.0% of nominals. Low (0.5 mg/L) and High (5.5 mg/L) PFOS TWAs concentrations were much lower than their respective nominals at 0.18 mg/L (32.7%) and 2.5 mg/L (45.5 %).

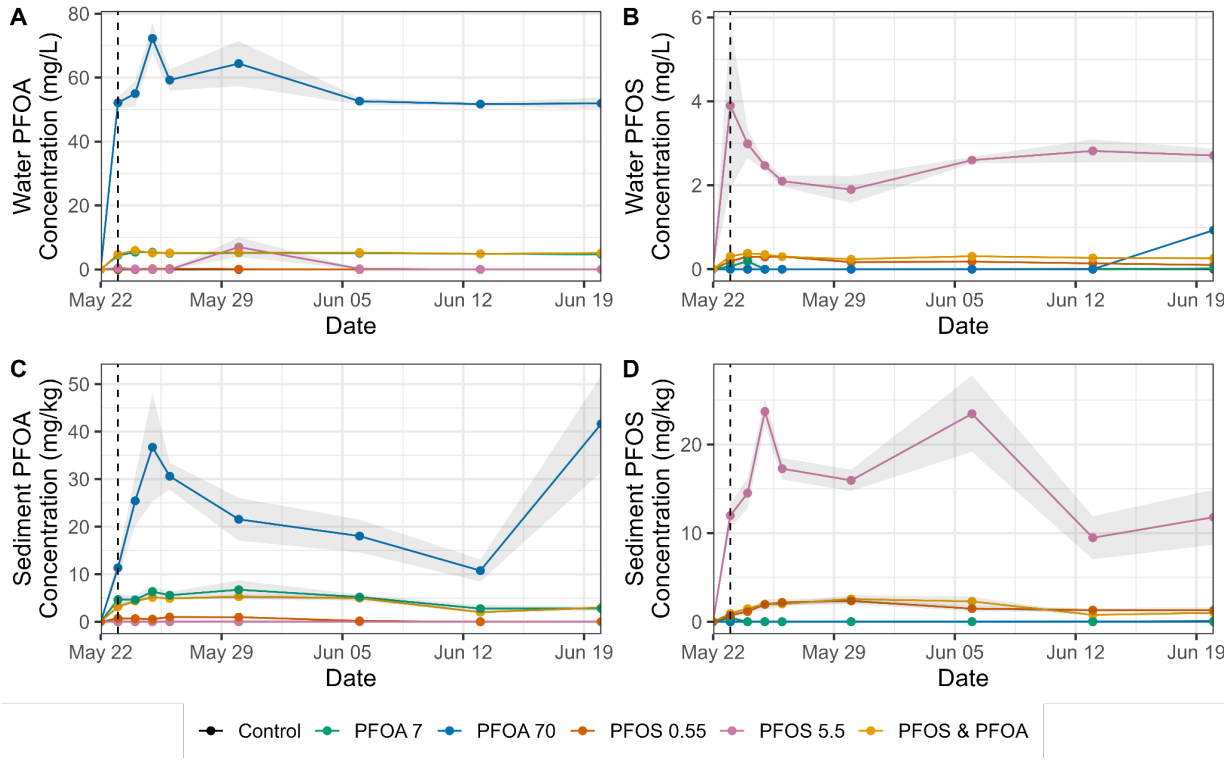

Figure 10: PFAS in Water and Sediment Over Time. Points indicate average values across the replicate mesocosms, and standard error is indicated by grey shading. Dashed lines indicate the first measurements following dosing. Four subfigures showing average concentrations with standard error shading of PFOS and PFOA in the water and sediment. Change over time for the control and five PFAS treatments is shown from May 22, 2023, (one day before dosing) to June 20, 2023.

TISSUE CHEMISTRY

Pre-dose, baseline measurements of *P. pugio* and *T. obsoleta* showed PFOA tissue concentrations of <1.67 ng/g and PFOS concentrations of <2.72 ng/g. After dosing, measured PFOS and PFOA in animal tissues showed uptake for both chemicals in all species (Figure 11). PFAS treatment was found to have a significant effect on PFOS and PFOA tissue concentrations for *T. obsoleta*, *P. pugio*, *C. variegatus*, and periphyton following 28 days of exposure (Table 6; Figures 12-15). For PFOA concentrations, *T. obsoleta* and periphyton in the High PFOA treatment had significantly higher concentrations than the control. For *P. pugio* and *C.*

variegatus, every treatment dosed with PFOA (Low PFOA, High PFOA, and mixture) produced animals with significantly higher PFOA tissue concentrations than the control.

Table 5: Time-Weighted Average (TWA) concentrations of PFAS in sediment and water. Asterisks indicate significant differences from the controls: p<0.05 '*', p<0.01 '**', p<0.001 ''. The percent of the nominal dose is shown for water concentrations. A sediment/water partitioning coefficient (Kd) based on TWAs from this study is shown in brackets under the sediment columns. Values in bold are measurements of the dosed compound. Unbolded values represent background levels in systems not dosed with that compound.***

	Water		Sediment	
Treatment (nominal)	PFOA mg/L (SE) *% of nominal dose*	PFOS mg/L (SE) *% of nominal dose*	PFOA µg/g (SE) [K_d]	PFOS µg/g (SE) [K_d]
Control (0 mg/L)	0.0017 (0.00034)	0.0024 (0.00057)	0.0071 (0.00086)	0.016 (0.0041)
Low PFOA (7 mg/L)	**5 (0.044)** ***71.4 %***	0.01 (0.0068)	**3 (0.29)** **[0.60]**	0.013 (0.0032)
High PFOA (70 mg/L)	**56 (2.2)** *** ***80.0 %***	0.12 (0.0042) ***	**14 (2.5)** ** **[0.25]**	0.0025 (0.0011)
Low PFOS (0.55 mg/L)	0.067 (0.025)	**0.18 (0.012)** *** ***32.7 %***	0.29 (0.054)	**2.4 (0.49)** **[13.33]**
High PFOS (5.5 mg/L)	1.4 (0.62)	**2.5 (0.098)** *** ***45.5 %***	0.0005 (0.00007)	**10 (0.7)** * **[4.00]**
PFOA (7 mg/L) + PFOS (0.55 mg/L)	**5.2 (0.074)** * ***74.3 %***	**0.28 (0.013)** *** ***50.9 %***	**2.7 (0.098)** **[0.52]**	**1.2 (0.13)** **[4.29]**

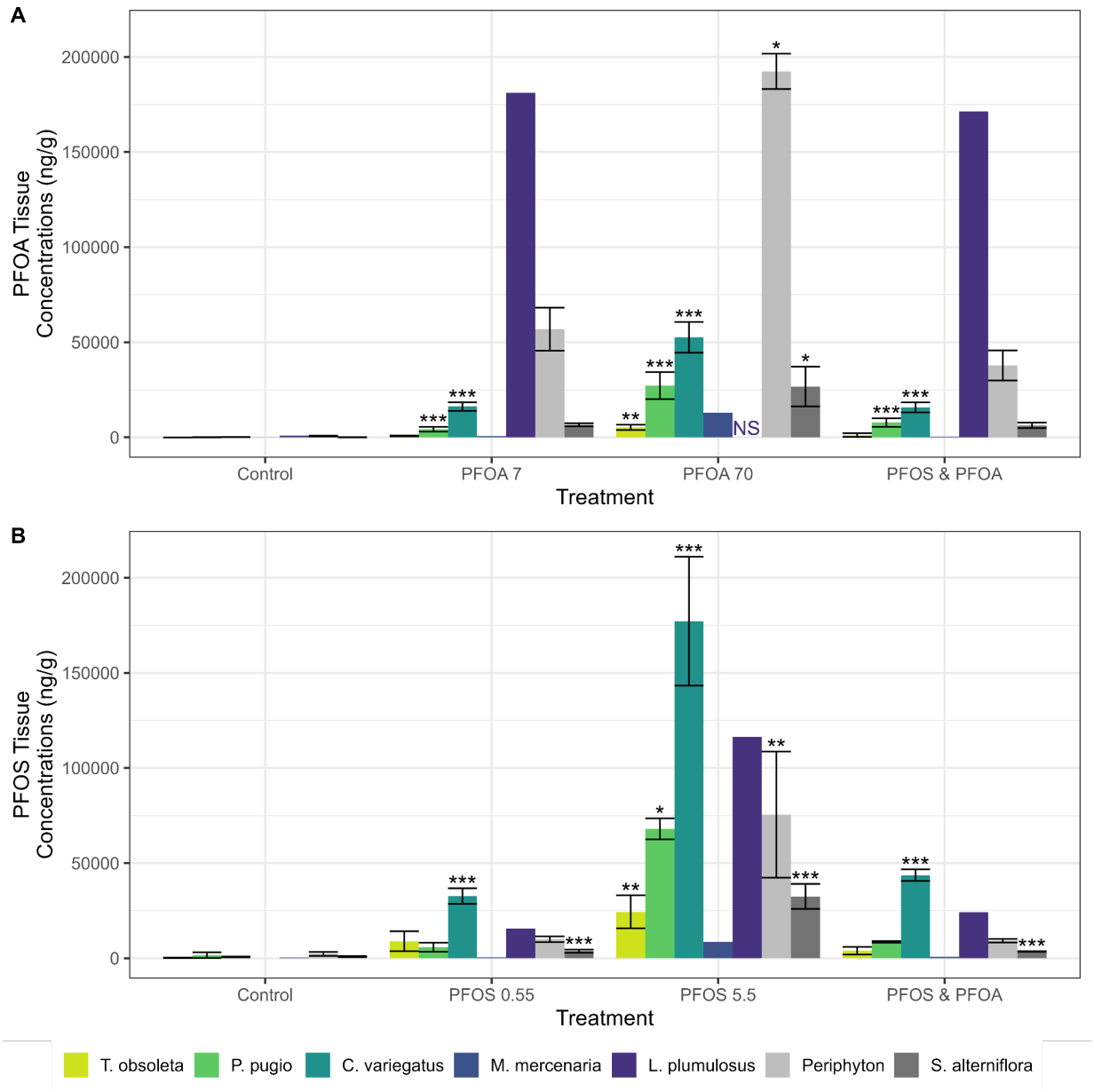

Figure 11: PFOS and PFOA Tissue Concentrations. Mean concentrations following 28 days of exposure are shown for each species with standard error indicated by error bars. Due to space constraints and low-levels of PFOA in the other treatments, A only shows PFOA concentrations from the treatments dosed with PFOA along with the control for comparison. For the same reasons, only the control and PFOS treatments are shown in B. Asterisks indicate a significant decrease in survival compared to a species' controls: p<0.05 '*', p<0.01 '**', p<0.001 ''. Tissue concentrations from composite samples (M. mercenaria and L. plumulosus) do not have error bars and were not analyzed statistically. Additionally, the PFOA and PFOS values for M. mercenaria are very low for the controls and appear non-existent on the graph given the scale. Due to complete mortality (NS=no survival), there is no data from L. plumulosus at the high PFOA concentration.***

Table 6: Organism Tissue Concentrations (µg/g or ppm) at 28 d. Mean and standard error of PFOA and PFOS tissue concentrations for each species and treatment. Asterisks indicate a significant difference in tissue concentrations for a treatment compared to the controls: p<0.05 '*', p<0.01 '**', p<0.001 ''. P-values were determined using either Dunnett's (parametric) or a modified Dunn's (non-parametric) test. Values in bold are measurements of the dosed compound. Unbolded values represent background levels in systems not dosed with that compound.***

Chemical	Treatment	Species				
		T. obsoleta µg/g (SE)	*P. pugio* µg/g (SE)	*C. variegatus* µg/g (SE)	Periphyton µg/g (SE)	*S. alterniflora* µg/g (SE)
PFOA	Control	0.0034 (0.002)	0.051 (0.024)	0.11 (0.013)	0.64 (0.25)	0.049 (0.017)
	Low PFOA	**0.73 (0.21)**	**4.3 *** (1.3)**	**16 *** (2.3)**	**57 (11)**	**6.6 (0.81)**
	High PFOA	**5.3 ** (1.4)**	**27 *** (7.1)**	**53 *** (8.1)**	**190 * (9.3)**	**27* (10)**
	Low PFOS	0.084 (0.075)	0.063 (0.028)	0.16 (0.052)	0.32 (0.044)	0.021 (0.0085)
	High PFOS	0.0084 (0.0039)	0.036 (0.016)	0.06 (0.013)	0.1 (0.047)	0.18 (0.073)
	PFOS + PFOA	**1.3 (0.98)**	**7.8 *** (2.3)**	**16 *** (2.6)**	**38 (7.9)**	**6.3 (1.5)**
PFOS	Control	0.17 (0.15)	1.6 (1.5)	0.38 (0.18)	2.3 (1)	0.88 (0.27)
	Low PFOA	0.18 (0.034)	0.089 (0.037)	0.73 (0.24)	21 (6.5)	0.88 (0.24)
	High PFOA	0.13 (0.017)	2.8 (2.8)	0.36 (0.11)	0.74 (0.24)	2.6 ** (0.46)
	Low PFOS	**8.9 (5.3)**	**5.8 (2.4)**	**33 *** (4.1)**	**10 (1.5)**	**3.7 *** (0.87)**
	High PFOS	**24 ** (8.6)**	**68 * (5.5)**	**180 *** (34)**	**76 ** (33)**	**33 *** (6.6)**
	PFOS + PFOA	**4 (2)**	**8.5 (0.41**	**44 *** (3.1)**	**9.2 (0.93)**	**3.5 *** (0.16)**

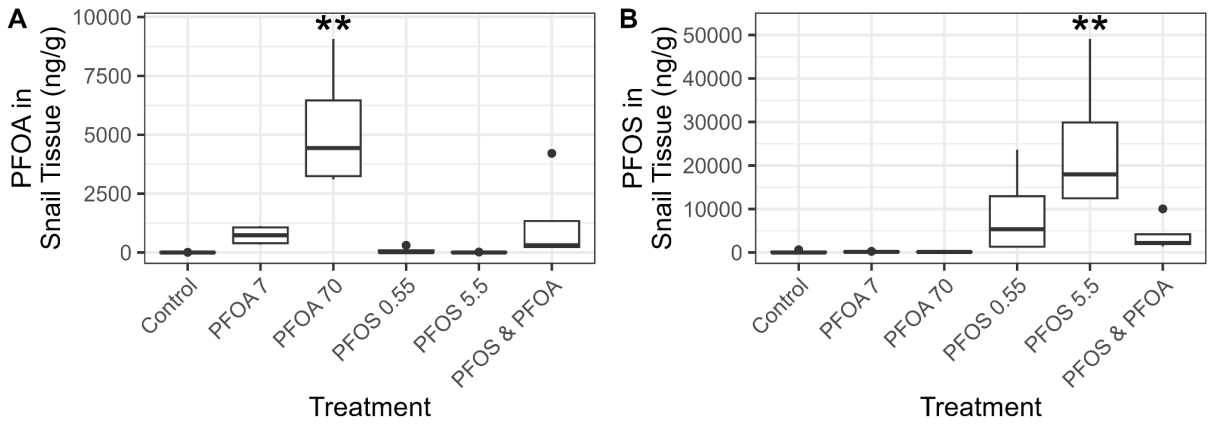

Figure 12: T. obsoleta PFOA and PFOS Tissue Concentrations. Asterisks indicate a significant difference compared the controls: p<0.05 '*', p<0.01 '**', p<0.001 ''.***

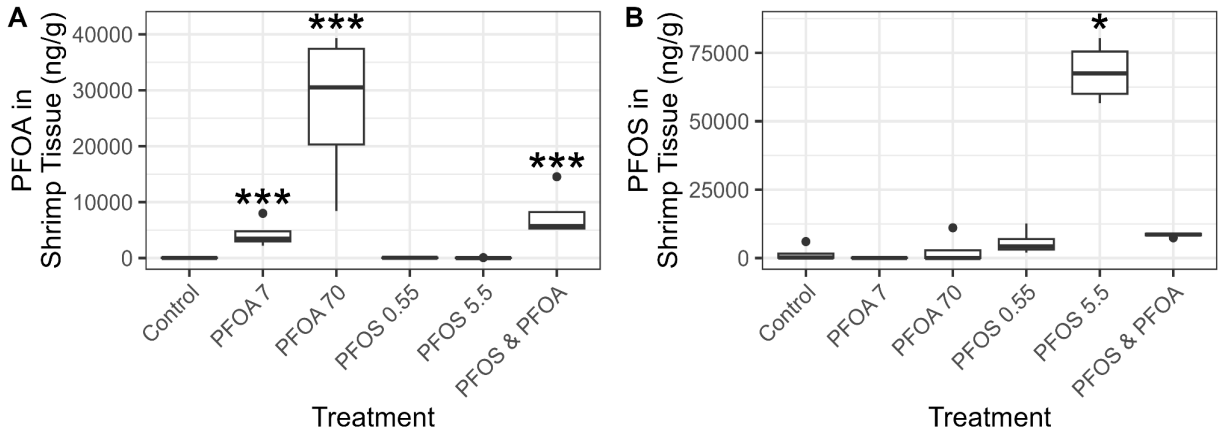

Figure 13: P. pugio PFOA and PFOS Tissue Concentrations. Asterisks indicate a significant difference compared the controls: p<0.05 '*', p<0.01 '**', p<0.001 ''.***

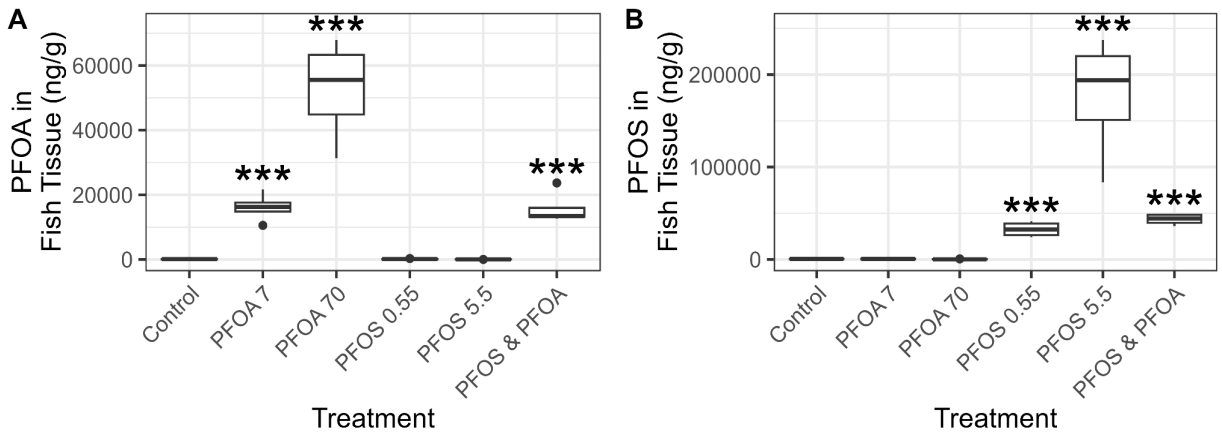

Figure 14: C. variegatus PFOA and PFOS Tissue Concentrations. Asterisks indicate a significant difference compared the controls: p<0.05 '*', p<0.01 '**', p<0.001 ''.***

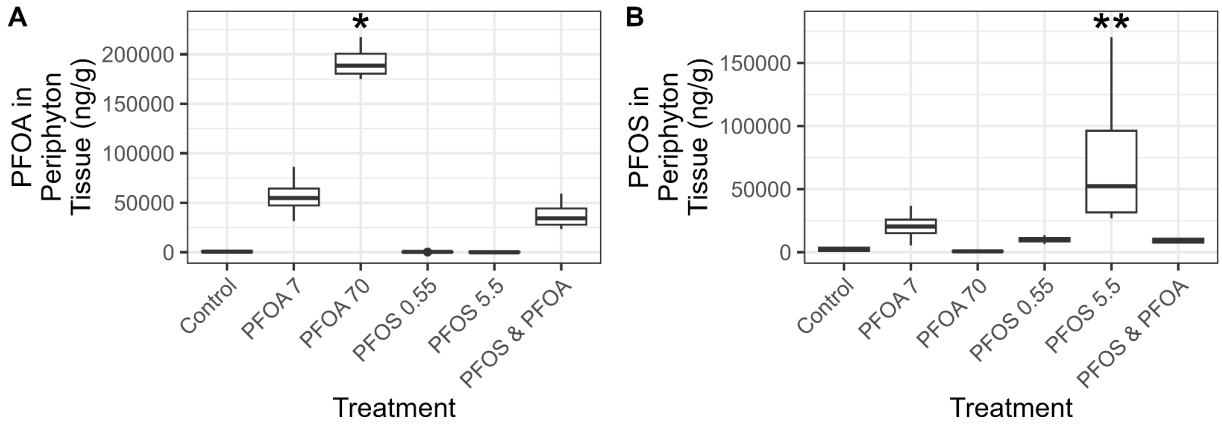

Figure 15: Periphyton PFOA and PFOS Tissue Concentrations. Asterisks indicate a significant difference compared the controls: p<0.05 '*', p<0.01 '**', p<0.001 ''.***

For PFOS, only *T. obsoleta*, *P. pugio*, and periphyton in the High PFOS treatment had significantly higher tissue concentrations than the control whereas *C. variegatus* once again had significantly higher concentrations in every treatment dosed with that PFAS. Due to the lack of replicates from using composite samples, *M. mercenaria* and *L. plumulosus* concentrations were not analyzed statistically; however, elevated PFAS levels were still seen in the pooled samples from dosed mesocosms compared to controls (Figures 11, 16, and 17). *M. mercenaria* PFOA

tissue concentrations showed 82.8-, 1,494-, and 50.6-fold increases for Low PFOA, High PFOA, and the mixture, respectively compared to controls.

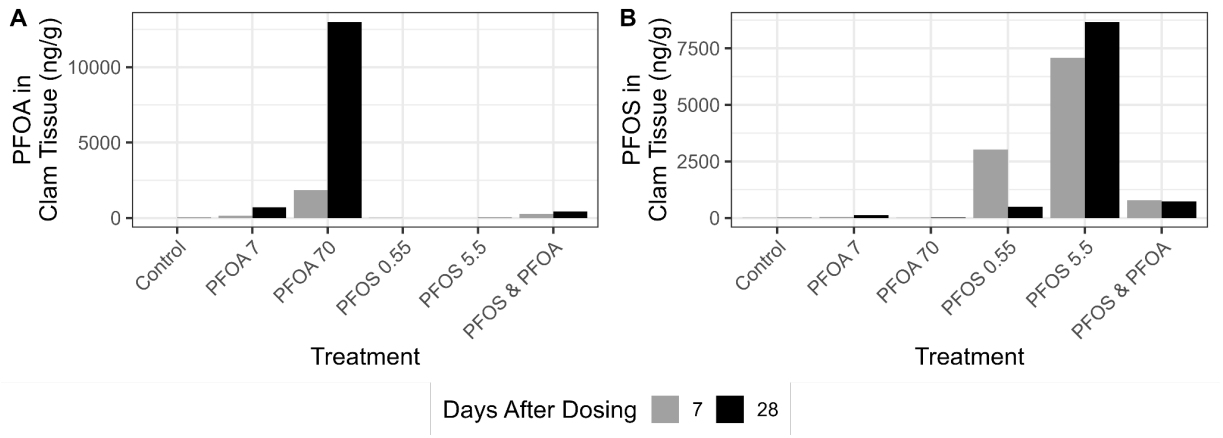

Figure 16: M. mercenaria PFOA and PFOS Tissue Concentrations. Following 7 and 28 days of exposure, samples from replicate mesocosms were combined for analysis due to small tissue amounts.

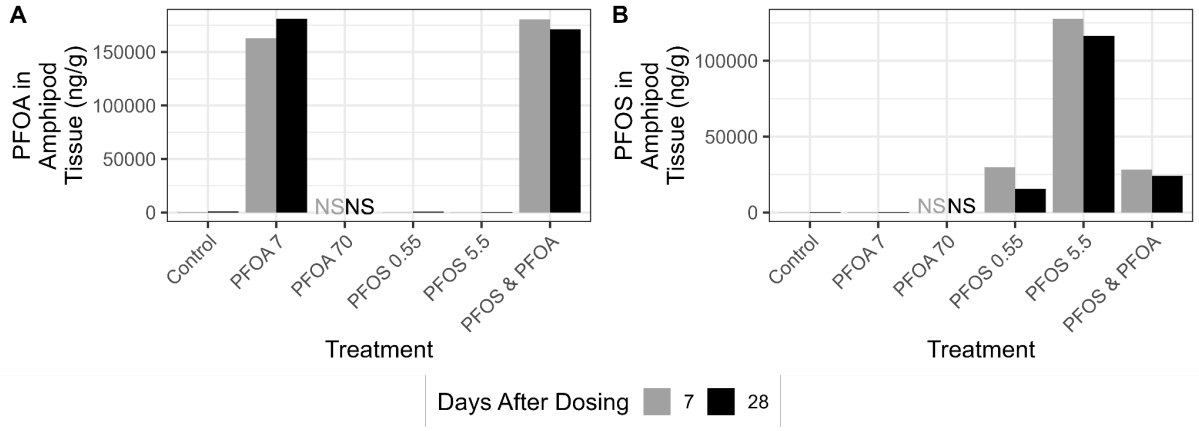

Figure 17: L. plumulosus PFOA and PFOS Tissue Concentrations. Following 7 and 28 days of exposure, samples from replicate mesocosms were combined for analysis due to small tissue amounts. NS indicates no survival.

That same species showed 40.8-, 725-, and 61.7-fold increases for Low PFOS, High PFOS, and the mixture, respectively compared to controls. *L. plumulosus* PFOA tissue concentrations were 189.5- and 178.9-fold higher for Low PFOA and the mixture, respectively compared to controls.

For PFOS, *L. plumulosus* tissue concentrations were 45.7-, 342.9-, and 68.6-fold higher than Low PFOS, High PFOS, and the mixture, respectively compared to controls.

Mean bioconcentration factors (BCF) for each species were calculated as the 28 d tissue concentration/time-weighted average water or sediment concentration (Table 7). Mean BCF values for PFOS ranged from 0.2 (composite *M. mercenaria* sample) to 190 (SE: 27) across species, with *C. variegatus* having the highest BCF for PFOS (190, SE: 27, for the 0.55 mg/L treatment and 70, SE: 13, for the 5.5 mg/L treatment). BCF values for PFOA were lower than PFOS, ranging from 0.24 (composite *M. mercenaria* sample) to 61.0 (composite *L. plumulosus* sample) in the non-mixture treatments, with *L. plumulosus* having the highest BCF (61.0 for the 7 mg/L PFOA treatment. There was complete mortality for *L. plumulosus* in the 70 mg/L PFOA treatment, thus a BCF could not be calculated). The highest mean BCF for PFOS in the Mixture treatment was 160 (SE: 18, *C. variegatus*), whereas the highest BCF for PFOA in the Mixture treatment was 64.0 (*L. plumulosus*).

S. alterniflora above-ground biomass also showed significant uptake of PFOA ($p=0.002$) and PFOS ($p<0.001$) (Figures 11 and 18). For PFOA, *S. alterniflora* from mesocosms dosed with 70 mg/L of that chemical had significantly higher concentrations at the conclusion of the study than the control ($p=0.019$). For PFOS, concentrations were significantly increased for every treatment except Low PFOA ($p=0.0051$ for PFOA 70 and $p<0.001$ for PFOS 0.55, PFOS 5.5, and mixture). *S. alterniflora* BCFs showed similar trends compared to most of the animals with mean PFOS BCFs being higher than PFOA BCFs (Table 7). For *S. alterniflora*, the largest PFOS BCF was 3.2 (SE: 0.73) in the mixture treatment and the largest PFOA BCF was 2.3 (SE: 0.4) from the 7 mg/L PFOA treatment.

Table 7: Mean Bioconcentration factors (BCF) with standard error (SE) by treatment. Composite samples (M. mercenaria and L. plumulosus) do not have standard error. Bioconcentration factors were calculated by dividing the 28 d tissue concentrations by the 28 d time-weighted average (TWA) concentrations of PFOS or PFOA in the water (P. pugio, C. variegatus, and periphyton) or sediment (T. obsoleta, M. mercenaria, L. plumulosus, and S. alterniflora). BCF values > 1 (in bold) indicate possible biomagnification. (NS = No survival).

Species	PFOA BCF (SE)		PFOS BCF (SE)		Mixture PFOA and PFOS BCF (SE)	
	Low PFOA	High PFOA	Low PFOS	High PFOS		
	(7 mg/L Treatment)	(70 mg/L Treatment)	(0.55 mg/L Treatment)	(5.5 mg/L Treatment)	7 mg/L PFOA	0.55 mg/L PFOS
BCFs based on TWA water concentrations:						
P. pugio	0.85 (0.25)	0.49 (0.13)	**32 (13)**	**27 (1.9)**	**1.5 (0.43)**	**30 (2.2)**
C. variegatus	**3.2 (0.44)**	0.93 (0.14)	**190 (27)**	**70 (13)**	**3.1 (0.52)**	**160 (18)**
Periphyton	**11 (2.2)**	**3.4 (0.17)**	**56 (5.1)**	**29 (12)**	**7.3 (1.5)**	**33 (3.9)**
BCFs based on TWA sediment concentrations:						
T. obsoleta	0.27 (0.09)	0.46 (0.2)	**5.6 (4.4)**	**2.5 (0.94)**	0.51 (0.41)	**4.2 (2.7)**
M. mercenaria	0.24	0.91	0.2	0.83	0.17	0.64
L. plumulosus	**61.0**	NS	**6.4**	**11.0**	**64.0**	**21.0**
S. alterniflora	**2.3 (0.4)**	**2.1 (0.81)**	**2 (0.87)**	**3.2 (0.73)**	**2.4 (0.53)**	**3.2 (0.54)**

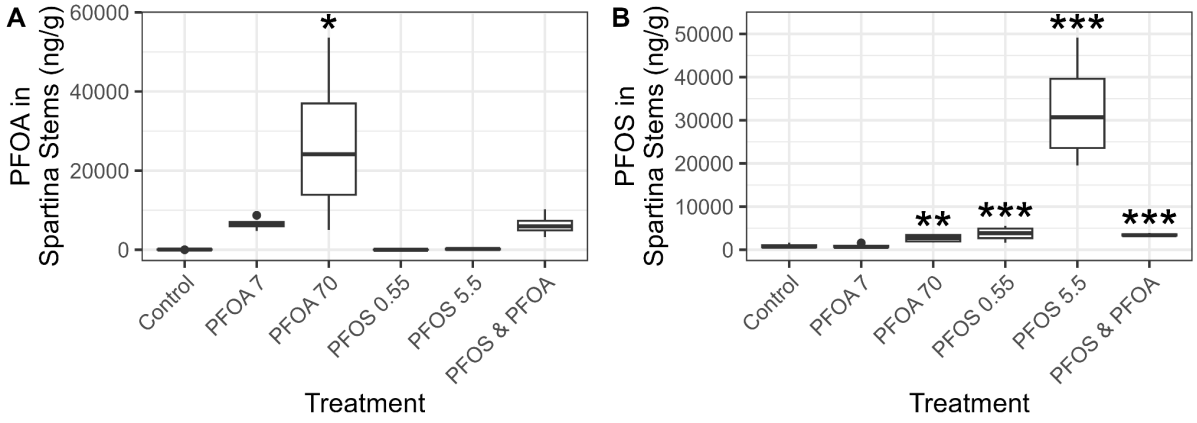

Figure 18: S. alterniflora PFOA and PFOS Tissue Concentrations. Asterisks indicate a significant difference compared the controls: $p<0.05$ '*', $p<0.01$ '**', $p<0.001$ '***'.

MASS BALANCE

Mass balance estimation showed similarities in the partitioning behavior between PFOS and PFOA (Figure 19). In looking at the four major compartments of the system (water, sediment, biota, and unrecovered PFAS), the majority of PFAS compounds partitioned into water and sediment compartments. For those two compartments combined, total PFAS partitioning was quite consistent ranging from 74.4% for the Low PFOA treatment to 87.5% for the High PFOS treatment. The only exception was the Low PFOS treatment which was at 57.3%. For the sediment compartment, the PFOS treatments had significantly great partitioning of total PFAS into sediments when contrasted against PFOA treatments ($p<0.001$). While partitioning into biota was quite low among all treatments (Figures 20-25) with a mean of 1.5% (SE: 0.65) total PFAS, the PFOS treatments had significantly greater ($p < 0.001$) partitioning of total PFAS (at 2.8%, SE:0.69) into biota when contrasted against the PFOA treatments (0.23% SE:0.031).

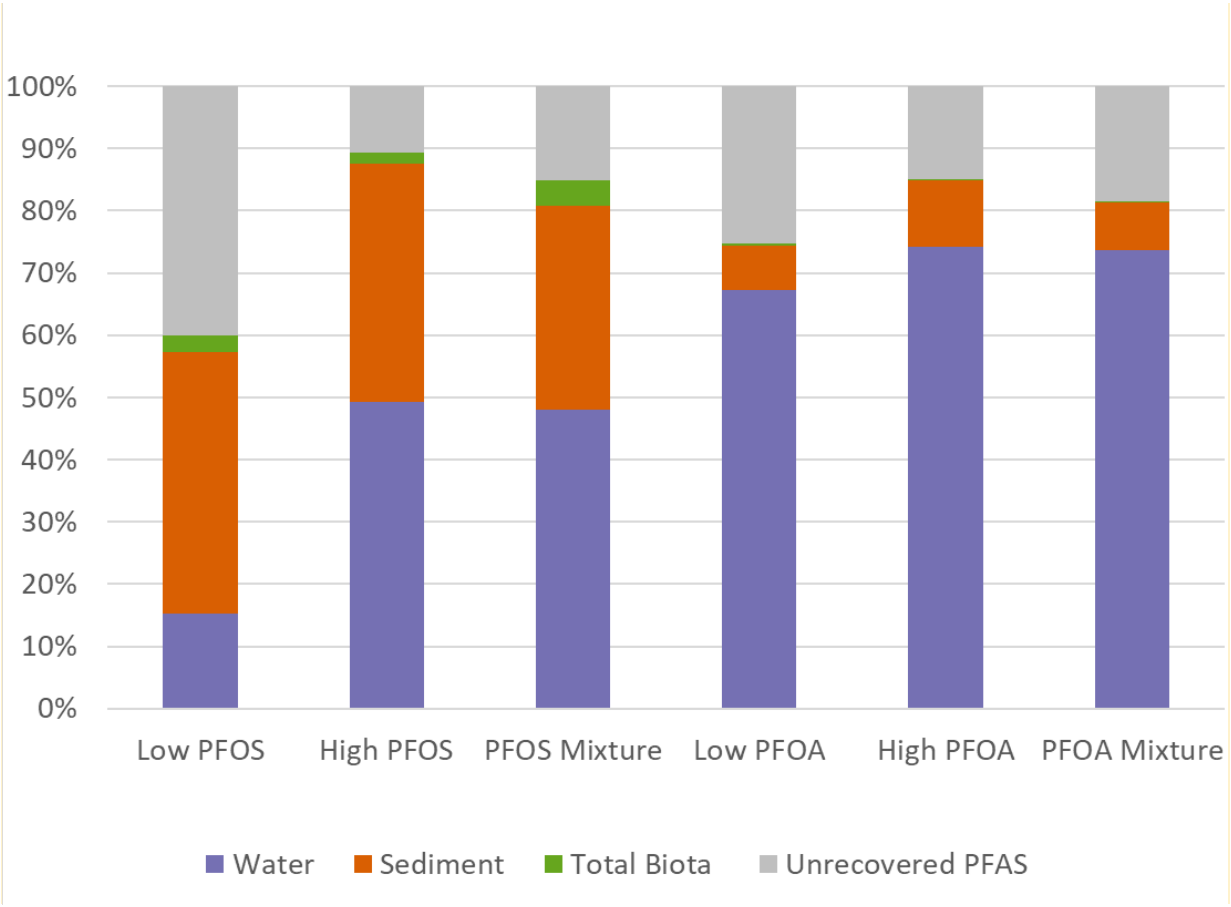

Figure 19: Mass Balance Summary of PFOS and PFOA. Average proportions are reported within the major environmental compartments (water, sediment, biota, and unrecovered) of each mesocosm treatment after 28 days of exposure.

The overall mass balance of PFAS partitioning between the four major compartments (water, sediment, biota, and unrecovered PFAS) was compared across all treatments using a MANOVA with a Type I Sum of Squares and Cross Products (SSCP) matrix. The top two characteristic roots (eigenvalues) accounted for >98% of the explained variability with the first root explaining 84.6%. The water compartment was the greatest driver in the model as it had the highest characteristic vector (eigenvector) in the first root at 74.9 followed by biota (72.2), unaccounted PFAS (71.1), and sediment (70.1).

Figure 20: PFOS Mass Balance for the Low PFOS (0.55 mg/L) treatment. The values within each box represent the mass (mg) of PFOS in each compartment and the boxes are sized proportionally relative to one another. The lower box is expanding out the biota compartment of the upper box (BGB = Below Ground Biomass [S. alterniflora rhizomes and roots], AGB = Above Ground Biomass [S. alterniflora stems]).

Figure 21: PFOS Mass Balance for the High PFOS (5.5 mg/L) treatment. The values within each box represent the mass (mg) of PFOS in each compartment and the boxes are sized proportionally relative to one another. The lower box is expanding out the biota compartment of the upper box (BGB = Below Ground Biomass [S. alterniflora rhizomes and roots], AGB = Above Ground Biomass [S. alterniflora stems]).

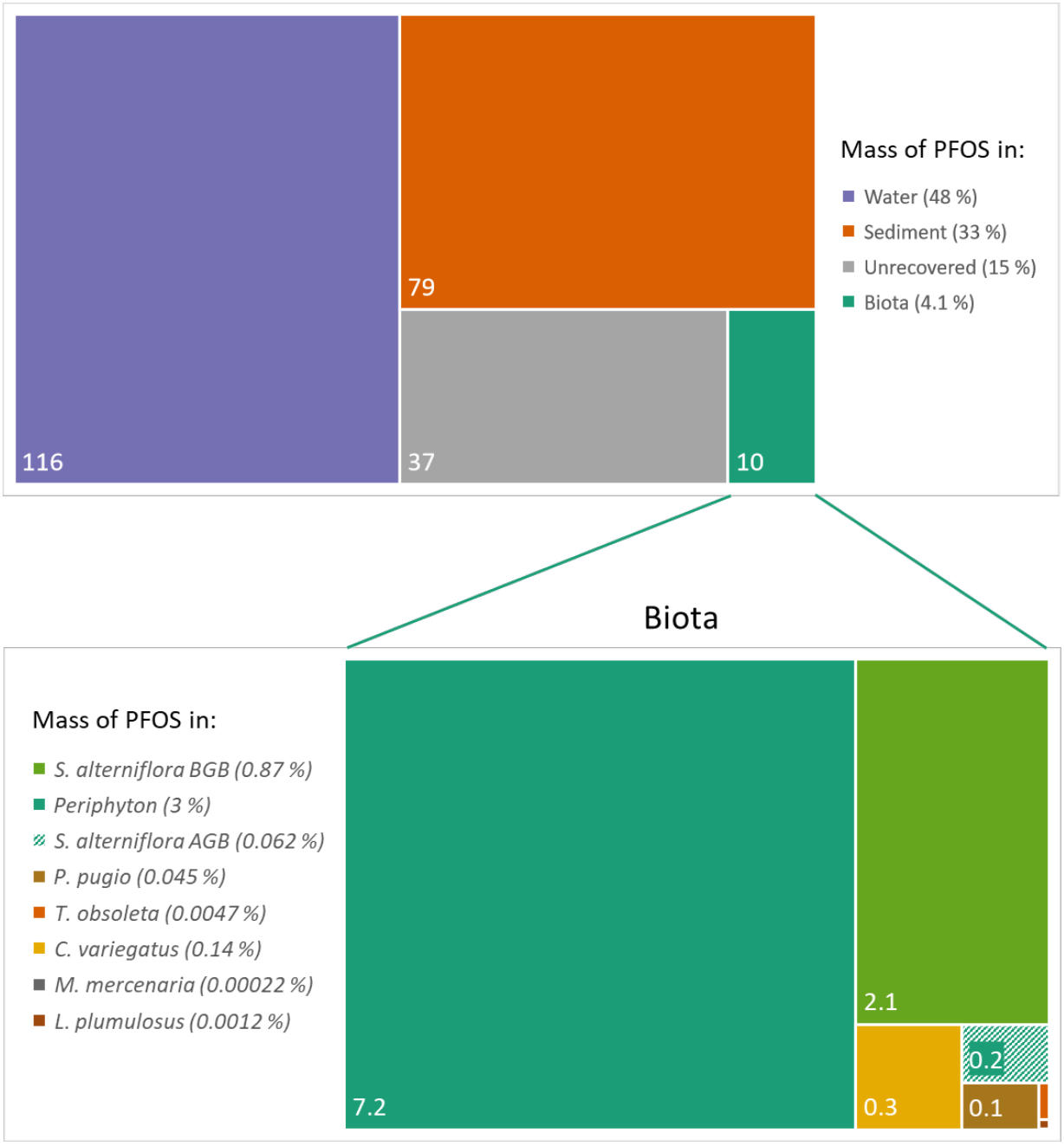

Figure 22: PFOS Mass Balance for the Mixture (0.55 mg/L PFOS / 7.0 mg/L PFOA). The values within each box represent the mass (mg) of PFOS in each compartment and the boxes are sized proportionally relative to one another. The lower box is expanding out the biota compartment of the upper box (BGB = Below Ground Biomass [S. alterniflora rhizomes and roots], AGB = Above Ground Biomass [S. alterniflora stems]).

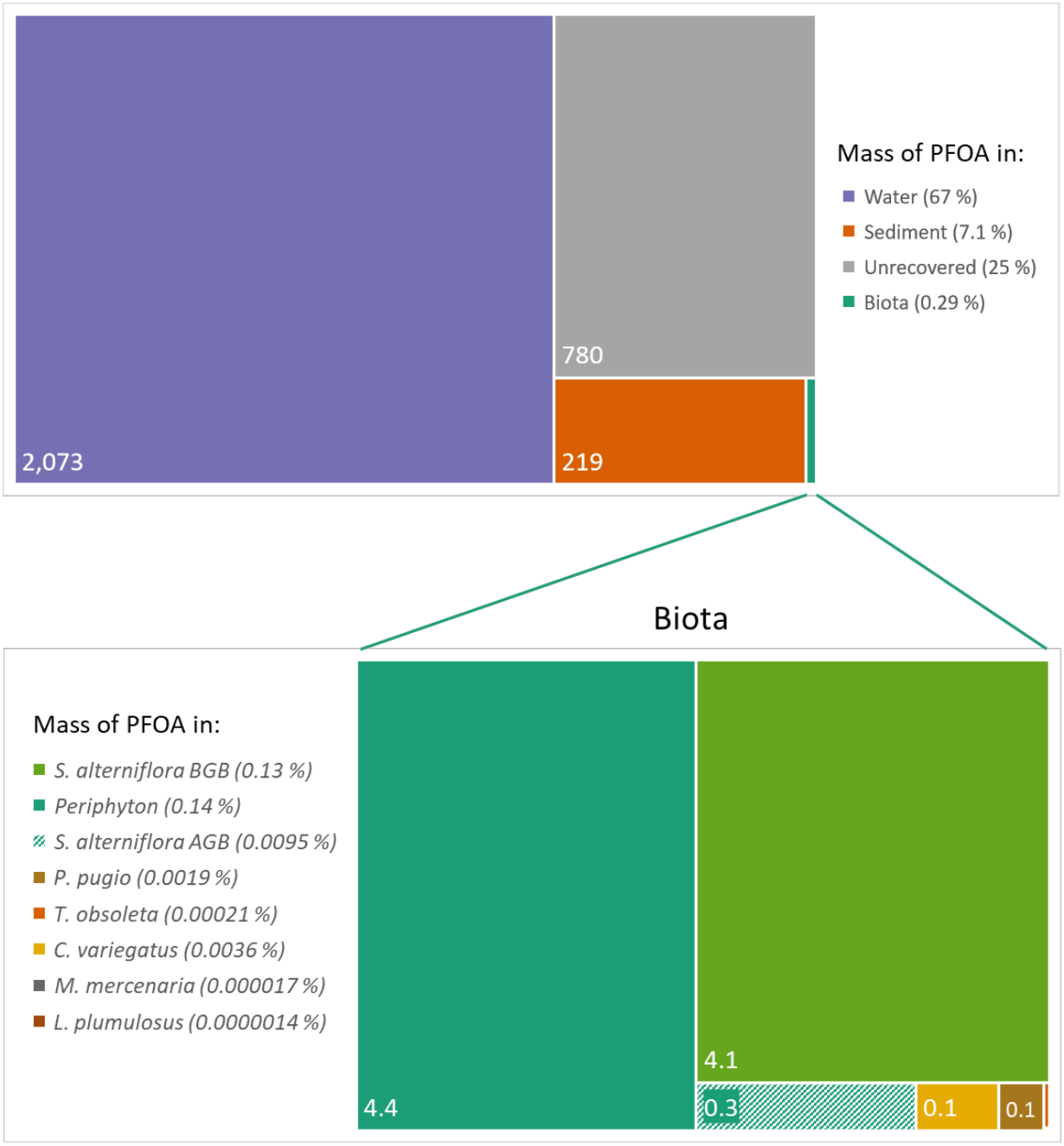

Figure 23: PFOA Mass Balance for the Low PFOA (7 mg/L) treatment. The values within each box represent the mass (mg) of PFOS in each compartment and the boxes are sized proportionally relative to one another. The lower box is expanding out the biota compartment of the upper box (BGB = Below Ground Biomass [S. alterniflora rhizomes and roots], AGB = Above Ground Biomass [S. alterniflora stems]).

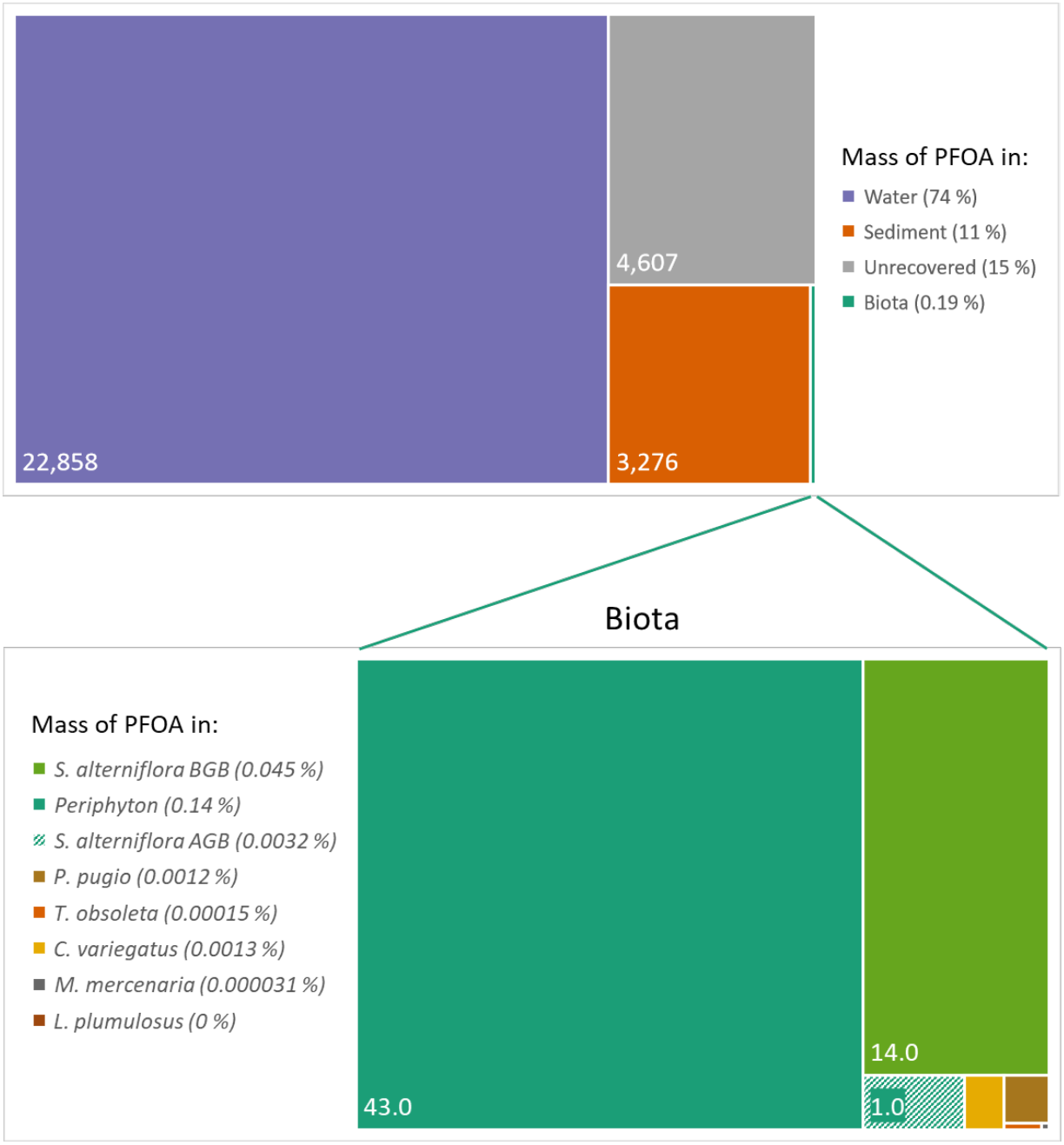

Figure 24: PFOA Mass Balance for the High PFOA (70 mg/L) treatment. The values within each box represent the mass (mg) of PFOS in each compartment and the boxes are sized proportionally relative to one another. The lower box is expanding out the biota compartment of the upper box (BGB = Below Ground Biomass [S. alterniflora rhizomes and roots], AGB = Above Ground Biomass [S. alterniflora stems]).

Figure 25: PFOA Mass Balance - Mixture (0.55 mg/L PFOS/7.0 mg/L PFOA) treatment. The values within each box represent the mass (mg) of PFOS in each compartment and the boxes are sized proportionally relative to one another. The lower box is expanding out the biota compartment of the upper box (BGB = Below Ground Biomass [S. alterniflora rhizomes and roots], AGB = Above Ground Biomass [S. alterniflora stems]).

Biota (82.9) was the primary driver in the second root followed by water (44.1), sediment (42.9), and (42.7). This analysis identified an overall treatment effect in total PFAS partitioning patterns ($p < 0.001$, Wilks' Lambda). Subsequent MANOVA contrasts revealed significant differences in partitioning patterns between Low PFOS and High PFOS treatments ($p < 0.001$, Wilks' Lambda), as well as the low PFOS and PFOS mixture treatments ($p < 0.001$, Wilks' Lambda). No significant differences in overall PFAS partitioning patterns were detected between any of the PFOA treatments. A proportion of the PFAS introduced into the systems was not recovered, and there were no significant differences detected between unrecovered PFAS proportions across treatments.

MESOCOSM MONITORING

Temperature, salinity, dissolved oxygen, specific conductivity, and pH values in each mesocosm remained within similar ranges throughout the study as measured by YSI 556s (Figure 26). However, there were some small, consistent differences among the YSI 5200A measurements, such as lower dissolved oxygen in the Low PFOA treatment and lower salinity values in the mixture treatment (Figure 27). Since those differences were not seen in the YSI 556 data, they likely reflect slight variations between the 5200A instruments or the six tanks chosen for those measurements and not an effect of treatment on water quality. Daily, cyclical changes in salinity values are due to evaporation and the daily addition of deionized water to combat it. Other changes over time, especially regarding temperature increases, reflect seasonal changes from spring to summer.

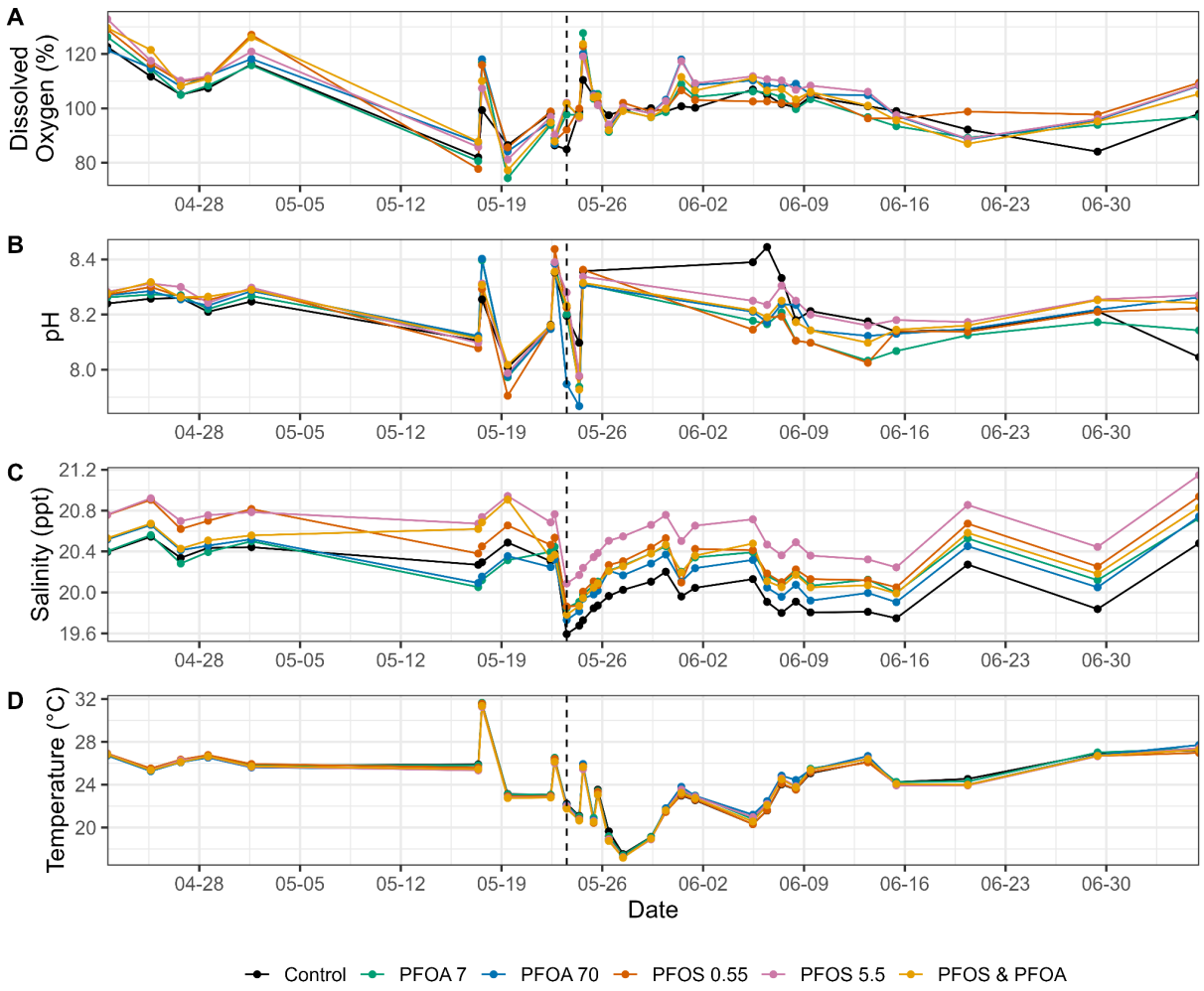

Figure 26: Discrete Water Quality Sampling Over Time. From top to bottom, average dissolved oxygen, pH, salinity, and temperature data recorded for each treatment by a YSI 556 over the course of the entire experiment. Vertical dashed lines indicate the first measurements following dosing.

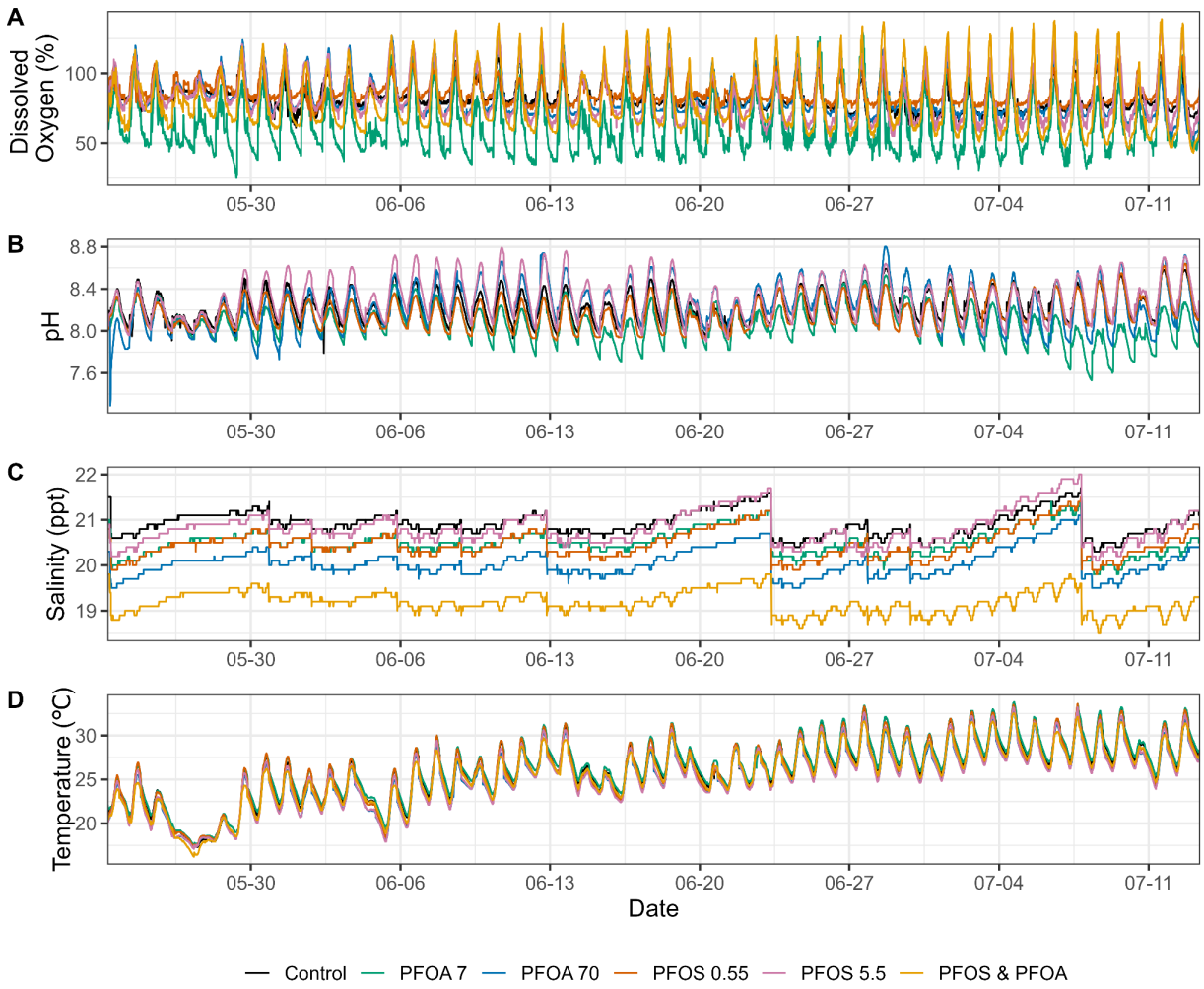

Figure 27: Continuous Water Quality Over Time. From top to bottom, dissolved oxygen, pH, salinity, and temperature data recorded by YSI 5200A in one mesocosm for each treatment. Measurements start ~2 hours prior to dosing.

DISCUSSION

TOXICITY

There was no significant effect of PFOS or PFOA on *C. variegatus* or *T. obsoleta* survival, whereas 66% (SE: 4.83) mortality was seen in *P. pugio* exposed to 5.5 mg/L PFOS, 96% (SE: 3.08) mortality was seen in *L. plumulosus* exposed to 7 mg/L PFOA, 100% (SE: 0) mortality was seen in *L. plumulosus* exposed to 70 mg/L PFOA, and 92% (SE: 6.85) mortality was seen in *M. mercenaria* exposed to 70 mg/L PFOA. Overall, amphipods were shown to be the most sensitive species to PFOA, whereas grass shrimp were the most sensitive species to PFOS. The mixture of PFOS (0.55 mg/L) and PFOA (7 mg/L) did not result in increased mortality compared to the compounds individually, with results indicating a less than additive effect. No significant effects were seen on growth of *C. variegatus*, *M. mercenaria*, or *S. alterniflora*.

The EPA Saltwater Aquatic Life Criteria (EPA, 2024a; EPA, 2024b) for PFOS of 0.55 mg/L resulted in no significant mortality for *C. variegatus*, *T. obsoleta*, and *M. mercenaria*, and *L. plumulosus* when compared to controls over the 28 d exposure. Thus, the EPA criteria for PFOS would be protective of the estuarine species tested. The EPA Saltwater Aquatic Life Criteria for PFOA of 7 mg/L resulted in no mortality for *C. variegatus*, *T. obsoleta*, and *M. mercenaria*, but yielded nearly complete mortality in *L. plumulosus* over the 28 d exposure. In this study the time-weighted average water exposure concentration was 4.97 mg/L over 28 d, compared to the acute EPA criteria of 7 mg/L. The sediment TWA of 4.24 mg/L was similar to the water TWA concentration. Thus, the *L. plumulosus* mortality occurred at concentrations below the EPA acute PFOA criteria, but the exposure criteria are for a duration of one hour average and a frequency not to be exceeded more than once in three years on average. Marine/estuarine chronic criteria for PFOS and PFOA have not yet been developed due to a lack

of toxicity data. Additional studies for estuarine benthic invertebrates may be warranted based on the results of this study.

In laboratory exposures, adult *C. variegatus* had a 96 h LC50 value for PFOS of >81 mg/L at 20°C, but a lower LC50 of 18.36 mg/L was determined at 30°C (Thornton, 2024). Likewise, Palmer et al. (2002) reported a 96 h LC50 exposure to PFOS for adult *C. variegatus* of >15 mg/L. In studies with the freshwater fathead minnow (*Pimephales promelas*), adults were more sensitive than *C. variegatus* to PFOS with a 96h LC50 of 9.1 mg/L (Drottar and Krueger, 2000a). Larval *C. variegatus* (24-48 h old) were more sensitive than adult *C. variegatus* in laboratory exposures with a 96 h LC50 for PFOS of 1.97 mg/L. In the same laboratory study, *C. variegatus* larvae were much less sensitive to PFOA with a 96 h LC50 >100 mg/L (Tanabe et al., 2024). The results of the present mesocosm study are in general agreement with the previous laboratory studies for adult *C. variegatus* with 28 d LC50 values being greater than the highest concentrations tested for both PFOS (> 5.5 mg/L) and PFOA (> 50 mg/L).

As in the mesocosm study, adult *P. pugio* in a 96 h laboratory study were more sensitive than *C. variegatus* with an LC50 for PFOS of 9.94 mg/L (Key, 2023). The chronic mesocosm exposure yielded greater mortality (66% at 5.5 mg/L) than that estimated in the acute laboratory exposure (50% at 9.94 mg/L). Larval *P. pugio*, like *C. variegatus* larvae, were more sensitive to PFOS with a 96 h LC50 of 2.01 mg/L at 25°C and 1.65 mg/L at 32°C (Chung et al., 2024). Another marine invertebrate, the mysid (*Americamysis bahia*) was also sensitive to PFOS exposure with a 96 h LC50 of 3.6 mg/L (Drottar and Krueger, 2000b). Similar to larval *C. variegatus* studies, larval *P. pugio* showed much less sensitivity to PFOA with a 96 h LC50 of 173.49 mg/L (Key et al., 2024), and no significant effects on adult *P. pugio* survival were seen in the 28 mesocosm exposure (7 or 70 mg/L PFOA).

Laboratory exposures were also conducted with *L. plumulosus* and *M. mercenaria* in 96 h tests in the presence of sediment. For *L. plumulosus* and *M. mercenaria*, no mortality was observed with PFOS at concentrations up to 200 mg/L. PFOA was more toxic, with a 96 h LC50 of 42.49 mg/L for *L. plumulosus* and 158.02 mg/L for *M. mercenaria* (Key et al., 2024). The mesocosm study confirmed that these two benthic species are more sensitive to PFOA than PFOS, and demonstrated that chronic exposure to PFOA significantly increases toxicity in clams and amphipods. These differences in sensitivity show the importance of chronic testing when researching persistent chemicals.

S. alterniflora growth was not affected by any PFAS treatment (Table 3). There is a general lack of published literature on the toxicological effects of PFAS chemicals on *Spartina* sp. While this study examined general growth metrics, it did not focus on physiological, cellular, or molecular effects. Similarly, no treatment effect was observed on nutrient concentrations or water column chlorophyll-*a* levels, as measured by fluorometric methods, during the experiment.

However, analysis of 28 d HPLC phyto-pigment samples revealed trends in overall chlorophyll-*a* concentrations and shifts in the abundance of major taxonomic groups for phytoplankton and periphyton (Figure 8). Most notably, periphyton chlorophyll-*a* concentration was significantly reduced in all treatments compared to the control ($p < 0.0027$). Further linear regression analysis showed a negative correlation between chlorophyll-*a* concentration and total PFAS levels (PFOS + PHOA) in periphyton tissues ($p < 0.0047$). While the coefficient of determination (r^2) was relatively weak at 0.34 (Figure 28), this finding warrants further investigation in a more controlled laboratory setting with increased replication. Decreases to periphyton biomass could lead to negative effects on grazers such as amphipods and snails.

FATE AND PARTITIONING

One-hour post-dosing, PFOA concentrations in the water column were 62.6% and 74.5% of the nominal doses for the Low and High PFOA treatments, respectively (Table 1). Similarly, measured PFOS concentrations were 34.6% and 70.9% of the nominal doses for the Low and High PFOS treatments, respectively. Interestingly, PFOA values remained consistent when based on 28 d time-weighted average (TWA) concentrations, at 71.4% and 78.6% of nominal values, respectively. This was not the case for PFOS; 28 d TWA concentrations for both PFOS treatments were somewhat lower, at 29.1% and 47.3% of nominal concentrations. It is also important to note that all un-dosed systems had detectable residual levels of PFOA and PFOS (Tables 1 and 5), indicating the ubiquitous environmental contamination of these compounds.

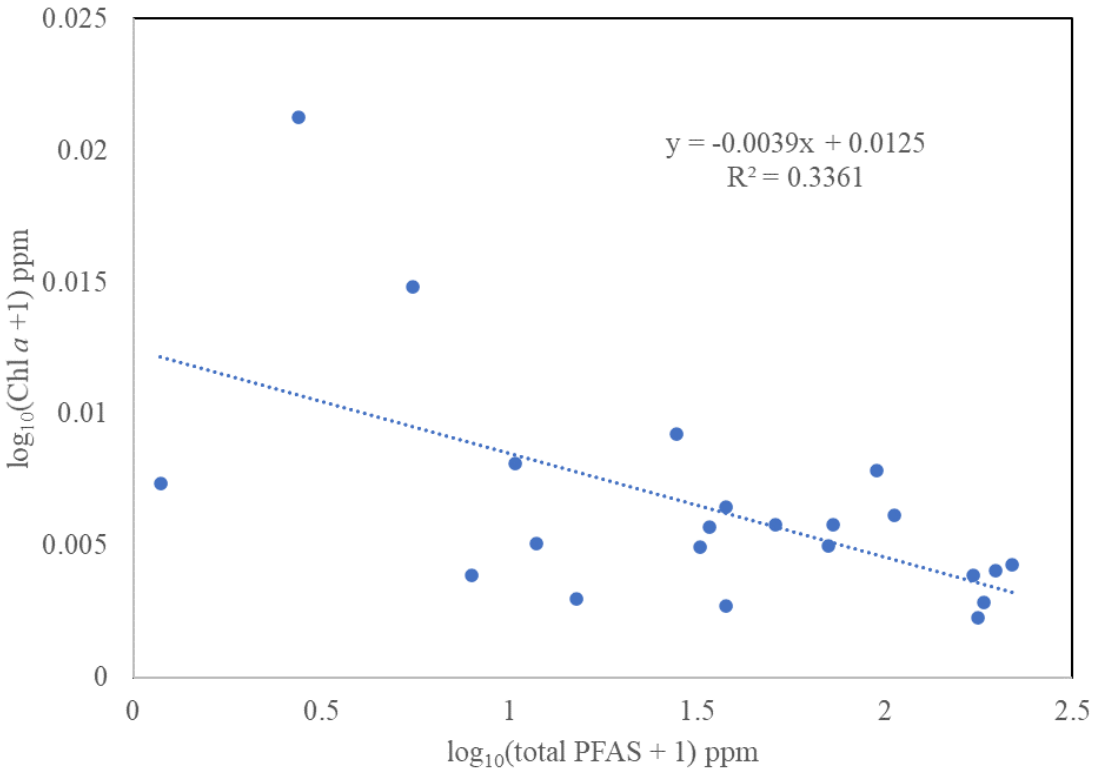

Figure 28: Linear regression of chlorophyll-a and total PFAS tissue concentration (PFOS + PFOA) in periphyton.

Sediment/water partitioning coefficients (K_d) were calculated using 28 d TWA concentrations for sediment and water (Table 5). Generally, $K_d < 1$ indicates highly mobile, hydrophilic chemical partitioning, whereas $K_d > 1$ signifies greater binding to sediments. Higher K_d values denote a stronger degree of sediment binding. While numerous factors can influence partitioning in complex, dynamic systems like mesocosms such as mineral content, electrostatic interactions, and organic carbon content (Ahrens et al., 2011; Higgins and Luthy, 2006), the 28 d TWA derived K_d values offer insight into the behavior of these compounds. For all PFOA treatments, including PFOA in mixture with PFOS, PFOA K_d values were < 1, indicating high chemical mobility. For PFOS, while the values were not as high as those from tightly controlled

laboratory K_d studies (Ahrens et al., 2011; Fagbayigbo et al., 2022; Higgins and Luthy, 2006; Jeon et al., 2011), they were all greater than 1, suggesting a higher degree of sediment binding.

The bioconcentration factor (BCF) and the bioaccumulation factor (BAF) are key metrics used to assess how chemicals concentrate in organisms. The BCF measures the uptake of a chemical directly from the surrounding medium, such as water, into an aquatic organism. In contrast, the more comprehensive BAF accounts for the total uptake from all sources, including water, food, and sediment. Therefore, the BAF is generally a more accurate predictor of a chemical's potential to accumulate in the food web. For the purposes of this paper, a mesocosm BCF calculation was based on the "laboratory BAF" by Burkhard (2021) for field measurements, with the mesocosm BCFs (Table 7) being calculated by dividing the 28 day tissue concentrations by the TWA concentration (over the 28 days) of PFOS or PFOA in the water (for *P. pugio, C. variegatus, and periphyton*) or sediment (for *T. obsoleta, M. mercenaria, L. plumulosus, and S. alterniflora*).

Bioconcentration factors (BCF) of 1 indicate that an organism's toxicant load is approximately in equilibrium with the surrounding matrix (water or sediment). BCF values > 1 suggest biomagnification, while values < 1 indicate that the organism's load is lower than its surrounding environment. In this mesocosm study, organisms generally had higher PFOS BCFs than PFOA BCFs (Table 7). The sole exception was *L. plumulosus* (Table 7), where PFOA BCFs exceeded PFOS BCFs for the same species. Additionally, *L. plumulosus* PFOA BCFs were higher than those of all other species. This is possibly attributed to the ecological niche of *L. plumulosus* in the benthos. These amphipods are highly mobile in sediment, actively burrowing and exhibiting flexible feeding behaviors, acting as both suspension feeders and deposit feeders (Yu and Fleeger, 2006). As suspension feeders, they can ingest planktonic and suspended benthic

microalgae, and as surface deposit feeders, they can ingest sediment, detritus, phytodetritus, and benthic microalgae. This complex behavior likely explains why PFOA tissue concentrations after 28 days (in the Low PFOA treatment) were highest for *L. plumulosus* (180 µg/g)—exceeding all other species in the treatment (Tables 6 and 8).

Alternatively, the approach described by Newman (1995) can be employed, where BCF is based on the concentration in the higher trophic level divided by the concentration in the next lower level. When the 28 d tissue concentration of *L. plumulosus* is divided by the 28 d tissue concentration of periphyton (used as a surrogate for all microalgae and phytodetritus), PFOA and PFOS BCFs are quite low (< 4.5; Table 9). Comparing *L. plumulosus* BCFs (Table 9) generated from 28 d TWA concentrations from water and sediment, as well as 28 d periphyton concentrations, yields somewhat mixed results compared to Tables 5 and 7.

Table 8: Composited Tissue Concentrations (L. plumulosus & M. mercenaria) at 28 d. For these species, samples were composited across replicates to gain enough tissue mass for analysis, thus there are no measures of variability. (* = no survival, Values in bold are measurements of the dosed compound. Unbolded values represent background levels in systems not dosed with that compound.)

Chemical	Treatment	Species	
		L. plumulosus µg/g or ppm	*M. mercenaria* µg/g or ppm
PFOA	Control	0.95	0.0087
	Low PFOA	**180**	**0.72**
	High PFOA	NS	**13**
	Low PFOS	0.67	0.0027
	High PFOS	0.34	0.0076
	PFOS + PFOA	**170**	**0.44**
PFOS	Control	0.35	0.012
	Low PFOA	0.35	0.13
	High PFOA	NS	0.016
	Low PFOS	**16**	**0.49**
	High PFOS	**120**	**8.7**
	PFOS + PFOA	**24**	**0.74**

On one hand, the TWA concentrations suggest that PFOA is more mobile and hydrophilic than PFOS, supporting findings that PFOA is more toxic to *L. plumulosus* when exposure is primarily from water rather than sediment. However, when examining BCF values, the highest for the Low PFOA treatment is from sediment, and the lowest is from periphyton.

This is misleading until it is understood that higher BCFs indicate a much lower concentration in the denominator of the BCF equation.

Table 9: Comparing BCFs for L. plumulosus based on different matrices. Bioconcentration factors were calculated by dividing the 28 d tissue concentrations by the time-weighted average (TWA) concentrations of PFOS or PFOA in the water, sediment, and the 28 d concentration for periphyton. BCF values > 1 indicate possible biomagnification. (NS = No survival).

Species	PFOA BCF		PFOS BCF		Mixture PFOA and PFOS BCF	
	Low PFOA	High PFOA	Low PFOS	High PFOS		
	(7 mg/L Treatment)	(70 mg/L Treatment)	(0.55 mg/L Treatment)	(5.5 mg/L Treatment)	(7 mg/L) PFOA	(0.55 mg/L) PFOS
Water BCF	36	NS	88	46	33	85
Sediment BCF	61	NS	6.4	11.0	64	21
Periphyton BCF	3.19	NS	1.56	1.54	4.53	2.62

With the exception of the fish (*C. variegatus*), the highest tissue concentrations were found in periphyton, at 57 and 190 µg/g from the Low PFOA and High PFOA treatments, respectively (Table 6). Assuming these periphyton values serve as surrogates for all microorganisms (microalgae), it can be inferred that ingestion (of microalgae) as a route of exposure likely plays the most significant role in the bioaccumulation and toxicity of PFOA to *L. plumulosus*.

In the present mesocosm study, TWA derived BCFs for *M. mercenaria* were low in comparison to other bivalves from laboratory-based studies. For example in a 7-d laboratory exposure, Aquilina-Beck et al. (2020) found that oysters (*Crassostrea virginica*) had BCF values

of 50 and 116 after being exposed to 0.3 mg/L and 3 mg/L PFOS, respectively – about 2-3 orders of magnitude higher than *M. mercenaria* BCFs in the present study. Additionally, oysters were able to depurate up to 96% of PFOS from exposure at 0.3 and 3 mg/L after 2 days of depuration in un-dosed seawater which puts the BCF values more in agreement with the current study with *M. mercenaria* at the 28 d timepoint. Prior to the depuration, the PFOS oyster tissue residues consisted of both branch and linear PFOS isomers whereas after the depuration the remaining tissue PFOS residues were largely linear isomers (Aquilina-Beck et al., 2020). In the present study, *M. mercenaria* PFOS tissue isomer percentages after the 28 d exposure were predominantly linear isomers (90-92% at 7-d and 85-88% at 28 d) despite the mix of isomers being more even in the water. Zero day and 28 d water PFOS isomer ratios (linear:branched) were 57:43 and 58:42, respectively. This is an interesting phenomenon that warrants further study.

In another bivalve laboratory-based study, Jeon et al. (2010) calculated more complex BCFs and BAFs based on uptake and depuration rate constants in the Pacific Oyster (*Crassostrea gigas*). In the 56-d study, oysters were subjected to exposure conditions for 28 d and then allowed to depurate for another 28 d. The BCF only took into account exposure and depuration in water whereas the BAF took into account exposure and depuration from water and food, both under varying salinity regimes. These BCFs and BAFs were again 1-3 orders of magnitude greater for PFOS and 1-2 orders of magnitude greater for PFOA than what was observed in the present study for the *M. mercenaria*. Interestingly, Jeon et al. (2010) found that increasing salinity levels led to increasing BAFs for PFOS and PFOA, indicating that increased salinity led to greater bioaccumulation from food. Given that these two cited studies were quite different from one another as well as being markedly different from the present study, *M.*

mercenaria and oysters (both being bivalves) are perhaps not directly comparable in these contexts, but this underscores that further investigation is needed with both species in similar study designs.

C. variegatus exhibited the highest tissue concentrations for PFOS among all fauna at 28 d (Tables 6 and 8). It also had the third-highest concentration for PFOA, following periphyton and *L. plumulosus*. BCF values derived from 28 d TWA water concentrations were 210 and 69 for the Low PFOS and High PFOS treatments, respectively. These values are greater than those observed in short-term laboratory studies such as Thornton (2024) (3.44-41.68) but lower than other studies that report much greater BCFs in the 720-1300 range (Beach et al., 2006; Giesy et al., 2010). These differences between the present mesocosm study and the aforementioned laboratory studies could be due to differences in food supply (as another route of exposure for PFAS) and test duration. *C. variegatus* in the present study had an unrestricted food supply in the mesocosms whereas in controlled laboratory studies a regular food regimen is provided (or no food at all for short-term tests).

When examining *S. alterniflora* tissue concentrations, from above-ground biomass, it was observed that the levels were consistent within the same order of magnitude and range as those found in other organismal tissues. Specifically, PFOS concentrations ranged from 3.5 to 33 ug/g, and PFOA concentrations were between 6.3 and 27 ug/g (refer to Tables 6 and 8 for detailed data). An important assumption of the mass balance calculation was that PFAS uptake in stems could be extrapolated to the below-ground biomass. This may underestimate the amount of PFAS that partitioned into the plants, given that the greater surface area to volume ratio of the roots may have led to greater PFAS uptake than in the stems. Greater uptake by the root systems

could also account for some of the unrecovered fraction of PFAS in the systems, and further study to directly measure PFAS uptake in *S. alterniflora* roots is needed.

BCFs for *S. alterniflora* for both PFOS and PFOA were generally similar, falling within a range of 1.6 to 3.5 across all experimental treatments. These BCF values are comparable to those reported for certain plant species as noted by Li et al. (2021), though they are considered low when juxtaposed with many other aquatic and terrestrial plant species. It is also a common observation that many plants exhibit higher BCFs for PFOS than for PFOA (Li et al., 2021) but the reason for that difference remains undetermined.

The translocation factors—which describe the movement of PFOS and PFOA from roots to stems—also display differences between the two compounds. This disparity in translocation could potentially influence our findings, particularly given the absence of data from root systems in our study. However, it is important to acknowledge the general scarcity of dedicated estuarine plant or mesocosm studies, and the understanding that such behavior can be highly species-dependent. For instance, one particular species of terrestrial grass (*Bromus diandrus*) has been documented to accumulate approximately 3.6 times more PFOA and 3.4 times more PFOS in shoots compared to its roots (Li et al., 2021), highlighting the variability of PFAS distribution within different plant structures. Understanding these partitioning patterns and identifying pathways for PFAS compounds can improve our ability to model their fate in natural systems and guide management strategies to mitigate environmental impacts.

MASS BALANCE

At the start of the experiment (time zero), a discrepancy was observed between the target and measured concentrations of PFOS in the mesocosm tanks. The target water concentration was 5.5 mg/L in the High PFOS treatment, corresponding to a total of 2,420 mg of PFOS

introduced into each 440 L tank. However, initial measurements registered a concentration of only 4 mg/L, which accounts for 1,760 mg of PFOS. This left a difference of 660 mg unaccounted for in the water column, despite high confidence in the initial dosage amount. To locate this missing PFOS, an analysis of the sediment was performed. Samples from the top 1 cm of the sediment trays showed a PFOS concentration of approximately 12 µg/g. By multiplying this concentration by the mass of the sampled sediment layer, it was calculated that this compartment contained approximately 35.7 mg of PFOS. After subtracting this amount from the initial discrepancy, approximately 624 mg of PFOS remained unaccounted for just after initial dosing.

After 28 days, a more comprehensive mass balance was performed showing again that the majority of PFAS partitioned into the water and sediment compartments (Figure 19). Partitioning into biota only constituted about 1.5 % of all partitioning across treatments with the greatest being in the PFOS treatments averaging 2.8 % (SE: 0.69) which was significantly greater than PFOA ($p < 0.0001$) averaging 0.23 % (SE:0.03). Overall, the periphyton and *S. alterniflora* compartments constituted the majority of biotic PFAS partitioning with the *M. mercenaria* and *L. plumulosus* compartments consistently being the least (Figures 20-25). This is most likely driven, in part, by the sizes of those system compartments (Figure 1). The Low PFOS treatments had a different partitioning pattern than the other PFOS treatments (Figure 19) and that was largely driven by the decreased mass of total PFAS found in the water compartment (15% - Figure 20). While the Mixture treatment (Figure 22) received the same PFOS dose as the low PFOS treatment (0.5 mg/L), its partitioning pattern was more similar to the High PFOS treatment (Figure 21). Approximately 33-38% of total PFAS was accumulated in the sediments and 48-49% remained in the water for the High PFOS treatment and mixture. The reasons for

these differences between the Low PFOS treatment and the others remain unknown. PFOA showed a greater percentage in the water column (~ 67-74%) across the PFOA treatments (Figures 23-25) and less in the sediments (~ 7-10%) and biota (~ 0.19-0.29 %) than what was observed in the PFOS treatments. This is in agreement with the PFOA K_d values (< 1) reported in Table 5 indicating that PFOA is more likely to remain in the water rather than accumulate in sediments.

Potential reasons for the unrecovered PFAS in the mass balance could include: adsorption or binding to tank materials such as plastic trays, tubing, oyster shells and associated biofilms; trace losses via volatilization; microbial degradation; differences in uptake into roots and rhizomes of *S. alternoflora* compared to the above ground stems; or differential (patchy) partitioning into the sediment. These results demonstrate the differential behavior of PFOS and PFOA in estuarine systems, with PFOS showing a higher potential for sediment and biota accumulation than PFOA.

CONCLUSIONS

This mesocosm study provides critical insights into the environmental behavior and ecotoxicological effects of PFOS and PFOA in an estuarine environment. While some species, such as *C. variegatus* and *T. obsoleta*, exhibited resilience, others like *P. pugio*, *L. plumulosus*, and *M. mercenaria* demonstrated significant sensitivity to these per- and polyfluoroalkyl substances (PFAS). The observed mortality in *L. plumulosus* at concentrations below current acute EPA criteria for PFOA highlights a potential gap in existing protective measures, emphasizing the need for a re-evaluation of these guidelines, particularly for sensitive benthic invertebrates. Furthermore, the reduction in periphyton chlorophyll-*a* concentration across all treatments indicates a broader impact on ecosystem health, underscoring the subtle but significant ways PFAS can disrupt ecological processes beyond direct mortality.

The study also elucidated key differences in the environmental fate and partitioning of PFOS and PFOA. PFOS generally exhibited higher sediment binding and bioconcentration in organisms compared to PFOA, suggesting distinct pathways for their distribution and accumulation within the estuarine system. The higher PFOA tissue concentrations in *L. plumulosus*, despite its lower overall bioconcentration factors, points to the importance of species-specific physiological and behavioral factors in determining PFAS uptake. The discrepancies in BCFs observed across different species and compared to other studies underscore the complexity of PFAS bioaccumulation, which is influenced by a myriad of factors including exposure routes and duration, trophic dynamics, and organismal characteristics.

Overall, this research contributes valuable data to the understanding of PFAS ecotoxicology and environmental fate in estuarine systems, which are disproportionately understudied. The findings suggest that while existing criteria may offer some protection for

certain species, they may not be fully protective for all organisms, particularly those inhabiting benthic environments. The nuanced responses observed, from species-specific mortality to changes in primary producer productivity, emphasize the need for a holistic approach to assessing the ecological risks posed by these persistent contaminants.

Future research should prioritize the development of chronic criteria for PFOA in marine and estuarine environments, with a specific focus on benthic invertebrates. Further investigation into the mechanisms behind periphyton chlorophyll-*a* reduction in the presence of PFAS, and the specific role of ingestion as an exposure route for bioaccumulation in organisms like *L. plumulosus*, is also crucial. Additional studies are warranted to explore the observed phenomenon of predominant linear PFOS isomers in *M. mercenaria* tissues despite mixed isomer ratios in the water, as well as to delve into the reasons for variability in BCFs across different species and study designs. Finally, a comprehensive understanding of the full mass balance of PFAS in mesocosm systems, including deeper sediment layers and root systems of plants, would provide invaluable insights into their long-term environmental fate and transport.

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