Assessing Bivalves as Biomonitors of Per- And Polyfluoroalkyl Substances (PFAS) in Coastal Environments

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Abstract: Per- and polyfluoroalkyl substances (PFAS) are widely used chemicals that enter coastal ecosystems through various pathways. Despite the ecological and economic significance of coastal environments, monitoring efforts of PFAS in these regions are limited. Bivalves have been used as biomonitors for many pollutants, but their effectiveness in reflecting environmental PFAS contamination and the mechanisms of PFAS bioaccumulation are poorly understood. This study examined the impact of biological, chemical, and ecological variables on PFAS bioaccumulation in two bivalve species (i.e., Eastern oyster and Atlantic ribbed mussel) and developed a statistical model to predict PFAS content in wild bivalves. Overall, the summed PFAS concentration in bivalves closely mirrors that in water. We observed higher bioaccumulation factors for some perfluoroalkyl sulfonamides and branched PFAS isomers than terminal PFAS of equivalent chain length. The isomer distribution and precursor-to-terminal compound ratios provide compelling evidence that the biotransformation of PFAS precursors likely drives these elevated factors. Additionally, the bioaccumulation factors of PFAS decrease with increasing organism size and age, suggesting that smaller and younger bivalves have a greater bioaccumulation potential and are more susceptible to PFAS contamination. These findings provide critical information guiding the use of bivalves as biomonitors to evaluate PFAS contamination in aquatic environments.

Keywords: Bioaccumulation; Biomonitoring; Biotransformation; Coastal ecosystem; Contamination; Mussel; Oyster; Statistical model.

Synopsis: This study evaluates bivalves as biomonitors and identifies key factors influencing their PFAS bioaccumulation, offering guidance for integrating bivalves into biomonitoring programs and future PFAS contamination research in aquatic environments.

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are a large group of chemicals that have been widely manufactured and used in commercial products and aqueous film-forming firefighting foams.[1-3] They can enter coastal ecosystems via contaminated water, particulate phases, or atmospheric deposition, with the ocean as a final sink.[4] Coastal ecosystems are economically and ecologically important, providing hubs for biodiversity and human activities. Several studies have shown elevated PFAS levels in water and biota in coastal regions that are downstream of industrial sites,[5] waste treatment facilities,[6] airports,[7] and military bases.[8] Globally, comprehensive monitoring data describing PFAS levels in coastal water and biota remain limited despite the clear presence of PFAS across estuaries and bays,[9-11] coastal margins,[12] and remote ocean locations.[13]

Biomonitoring efforts using wildlife offer a direct means of assessing the extent of contamination and its potential impacts on ecological health. Bivalves are useful biomonitors of the coastal environment due to their wide distribution, simple exposure pathway (i.e., filter-feeding), and sessile nature which ties their contamination profiles to their local ecosystems.[14] Different from environmental sampling via grab samples or passive samplers, bivalves can be used to understand integrated environmental exposure to PFAS over time, reflecting bioavailable contaminants in their environment. Moreover, bivalves are hardy and place-based, coping with variable and dynamic conditions occurring in coastal ecosystems that make passive sampling or field work campaigns challenging. As low trophic level species, bivalves indicate the contaminant burden at the base of food webs, offering critical information on pollutants that can be transferred to higher trophic level organisms and humans. Many studies have used bivalves as

biomonitors for a variety of contaminants (e.g., heavy metals [15], polycyclic aromatic hydrocarbons[16], and microplastics[17]).

Prior studies have demonstrated that bivalves can accumulate PFAS in both natural environments[18-20] and laboratory settings.[21, 22] The PFAS burden in wild oysters is affected by local PFAS sources (e.g., discharge from fluoropolymer manufacture vs. plastics manufacturing) and differ significantly across bivalve species grown in the same area, likely due to different physiology and feeding behaviors.[23] The mechanisms of PFAS bioaccumulation in bivalves seem different from that of fish.[24] Bivalves generally have lower PFAS concentrations and different compositions than those of fish.[18-21] While many studies reported that perfluorooctanesulfonic acid (PFOS) is the predominant PFAS compound in fish,[19, 25] bivalves often exhibit higher levels of the precursor like perfluoroalkane sulfonamide (FOSA) than PFOS.[20, 26-28] Authors attributed the difference to bivalves' lower capacity for biotransformation compared to fish, but the biotransformation capabilities of bivalves remain poorly studied.

A limited number of studies have reported that bioaccumulation potential of even the same PFAS compounds in bivalves varies by orders of magnitude, influenced by geographic location as well as inter- and intraspecies differences.[26, 29, 30] However, the mechanisms behind this variation remain unclear. Environmental variables, such as salinity, can also influence PFAS accumulation in bivalves. For example, Jeon et al. (2010) suggest that higher salinity enhances dietary uptake of PFAS by increasing adsorption to food particles and elevating ingestion rates.[22] In contrast to fish, there is no consensus on the relationship between the bioaccumulation factor of a given PFAS and its chemical structure (e.g., fluorinated carbon chain length) in shellfish.[24] More research is needed to elucidate the factors affecting PFAS bioaccumulation in bivalves.

Effective biomonitoring of PFAS contamination in coastal ecosystems is crucial for informing management of coastal waters and mitigation strategies to safeguard both environmental integrity and public health. The main objective of this study is to quantitatively assess the suitability of bivalves as PFAS biomonitors in coastal environments and gain a mechanistic understanding of PFAS accumulation in bivalves. Specifically, we investigated how biological, chemical, and ecological variables affect PFAS bioaccumulation in bivalves. We also constructed a comprehensive statistical model predicting PFAS content in wild bivalves. The results highlight current gaps in biomonitoring programs, ecotoxicology assessments, and fish consumption advisories.

2. Methods

2.1 Study sites and organisms

We used Delaware Bay, the third-largest estuary in the United States, as our study site. Delaware Bay connects the Delaware River to the North Atlantic Ocean, with inputs from several tributary rivers and the Chesapeake-Delaware canal (Figure S1). It is a long shallow estuary that varies greatly biologically and physiochemically (e.g., salinity, temperature) over a spatial scale,[31] thus creating natural experiments for us to investigate how natural variability in the ecosystem can vary PFAS bioaccumulation. Industries in the region have resulted in frequent inputs of pollutants to the bay and prior research has found PFAS in the water and organisms of Delaware Bay, including in plankton,[32] fish,[33] dolphins,[34] and osprey.[35]

Two bivalve species, the Eastern oyster (*Crassostrea virginica*) and the Atlantic ribbed mussel (*Geukensia demissa*), were selected for this study due to their wide distribution across the east coast of United States and tolerance of a broad range of salinities and temperatures. These

two species have similar habitats,[36, 37] filtering rates,[38, 39] and consume particles of similar sizes (> 4 or 5 μm).[40, 41] Though the two species are of a similar trophic level, the Eastern oyster and ribbed mussel have some apparent physiological differences. These oysters grow faster and have shorter lifespan than mussels in the Delaware Bay (typical life span of Eastern oysters: less than 6 years; ribbed mussels: 15 to 20 years).[42, 43] Additionally, these two bivalves species have different particle sorting mechanisms.[44] While both species are able to select certain algal species for ingestion, [40] mussels are more adept at assimilating non-algal food sources, like cellulose and bacteria.[45]

2.2 Sample collection

We collected paired samples of surface water and bivalves during low tide from eight publicly accessible shoreline locations along a gradient of salinity and temperature (salinity range: 10.0-26.2 ppt, temperature range: 8.8-17.9°C) in 2021-2022 (Figure S1). At each site, we collected 10-13 organisms of each species if available. Living oysters were present at all other sites but site #7, while limited amounts of mussels were present at site 1 (n=6) and site 3 (n=1). Water temperature and salinity were measured on site using a salinity meter (Orapxi S-100). Table S1 provides a full list of site information, including locations, sampling dates, water temperature and salinity.

Water samples were collected approximately 10 cm below the surface using Nalgene brand high-density polyethylene (HDPE) bottles. HDPE bottles (500 mL) were pre-cleaned with Milli-Q water and HPLC grade methanol in lab and rinsed in the field 3 times with site water before filling. A water field blank was collected during each sampling effort by opening a 500 mL HDPE bottle filled with Milli-Q water in the field during sample collection. Although

multiple water grab samples were collected per site, only one sample per site, analyzed within 90 days of collection, is included in data analysis. Samples analyzed approximately a year later exhibited shifts in PFAS profiles, particularly a loss of precursor and volatile compounds, likely due to the precursor decomposition, despite storage at -20°C. Therefore, we consider the data from samples analyzed within 90 days to be the most representative of the true PFAS profile at these sites.

Bivalves were collected using gloved hands, with oyster knives to pry individual organisms from substrate. Bivalves collected were of a range of sizes, randomly sampled and placed into methanol-cleaned Ziploc bags. All water and bivalve samples were stored on ice in the field, and then stored at -20°C until dissection. In the laboratory, we recorded organism size and weight characteristics (in-shell weight, shell length, width, and depth). All tissue was removed from the shell, weighed, and recorded as body weight. We estimated the age of the mussels using a traditional aging method, counting annual growth rings in the prismatic layer of the shell.[46] Growth rings are difficult to read in oysters due to their rugged shell surface so we estimated their age using shell length and cumulative growth curves previously derived for this species from Delaware bay (SI Section 1.2).[42] To avoid PFAS cross-contamination, we followed the general PFAS sampling guidance established by relevant environmental agencies.[47] Additional details regarding site selection, sample collection, and sample storage can be found in SI Section 1.1.

2.3 Sample extraction

Water samples (~500 ml) were extracted using weak anion exchange solid phase extraction (WAX-SPE) cartridges, modified from established methods (SI Section 1.3).[48-50] As

intertidal water often contains a large amount of easily re-suspended sand particles, we pre-screened water samples with 5µm Nitex nylon mesh and adjusted pH to 6.5±0.5 with 1% formic acid before extraction. Water samples were weighed and spiked with 100 µl of 200x diluted Wellington Mass-Labelled Extraction Standard Solution (Wellington Laboratories; Guelph, ON, Canada). Each sample set included one Milli-Q procedural blank and one sample spiked with 2 ng of native PFAS mixture standard solution (Wellington PFAC30-PAR) prior to extraction. Oasis WAX SPE cartridges (6 mL, 150 mg sorbent) were conditioned with 5 ml of 0.1% ammonium hydroxide (NH_4OH) in methanol, 5 ml of methanol, and 5 ml of Milli-Q water before the sample was loaded onto the cartridge at a flow rate of 1 drop per second. We washed the loaded cartridges with 5 ml of 25 mM sodium acetate buffer and eluted the concentrated PFAS with 5 ml methanol and 5 ml of 0.1% ammonium hydroxide in methanol. The elute was then concentrated to 1 ml using a nitrogen evaporator (Organomation N-Evap 112 at 55°C) and stored at 4°C until instrumental analysis.

For each bivalve sample, we homogenized the soft tissue and extracted PFAS from about 1 gram aliquot using acetonitrile and ultrasonication, followed by dispersive Envi-Carb cleanup, modified based on prior studies (SI Section 1.4).[51, 52] Briefly, we extracted the tissue by adding 5 mL acetonitrile and sonicating for 10 min, then collected supernatant after centrifugation. We repeated the process and then combined the supernatants. We added 30 µl of ethylene glycol to the combined supernatant to prevent volatilization, then concentrated the extract to near dryness using nitrogen evaporation before reconstituting to 1 ml with methanol. For cleanup, we added ~100 mg Envi-carb carbon adsorbent and 50 µl of glacial acetic acid to the reconstituted extract in a 2-ml vial and centrifuge to collect the supernatant as the final sample for analysis. All samples were spiked with extracted internal standards (EIS) prior to extraction and non-extracted

internal standards (NIS) prior to injection. Detailed description of sample preparation and extraction as well as chemical reagents and materials are provided in SI Section 1.3-1.5.

2.4 Instrumental analysis

Sample extracts were analyzed for 30 targeted PFAS (18 PFAAs, 3 perfluoroalkane sulfonamides (FASA), 2 sulfonamidoacetic acids (FASAA), 7 other PFAS; Table S2) using a Waters Acquity ultra-performance liquid chromatography system coupled to a Waters Xevo TQ-XS tandem mass spectrometer (UPLC-MS/MS) with a UniSpray source, operating in negative ion mode. Targeted PFAS were quantified using EIS and NIS, following EPA Method 1633.[53] Linear and branched isomers were quantified separately for compounds with available isomer-specific standards. Further details about instrumental conditions, chromatography, and quantification are available in SI Section 1.6.

Previous studies have identified matrix interferents that may appear as PFAS where they are not present. These interferents can significantly affect the accuracy of PFAS measurements and subsequent data interpretation.[54] This issue is especially evident for PFBA and PFPeA, two low molecular weight perfluorinated carboxylic acids that each have only one major MS/MS transition and thus lack additional qualitative transitions needed for verification on UPLC-MS/MS.[55] We used suspect screening for a subset of bivalve samples to confirm the presence of compounds with known interferents (i.e., PFBA, PFPeA, PFHxS, and PFOS) using a Thermo Fisher Orbitrap Exploris 120, Thermo Fisher, Waltham, MA (UHPLC-HRMS). Details of the analysis are provided in SI Section 1.7 HRMS Suspect Screening.

2.5 Quality Assurance/Quality Control

Each batch of 10 samples included a procedural blank, a duplicate sample spiked with native standard, and a replicate of in-house reference standard material. Method detection limits (MDLs) for water samples (range: 0.007-0.532 ng/L; Table S3) and biological samples (range: 0.003-0.095 ng/g; Table S3) were calculated based on the average concentration at which sample signal-to-noise ratio was 10, multiplied by sample dilution factor, following established methods.[20, 25, 56] Method trueness of biological samples was assessed using NIST SRM 1947 reference samples (Lake Michigan Fish Tissue; n=6) and results are comparable with NIST certified values and other studies (Table S4).

Overall method accuracy, assessed as recovery of native PFAS spiked into samples, was on average 105±23% for bivalves (n=19) and 91±25% for water (n=8) (Table S5). We ran in-house reference materials (bivalve tissues) and water replicates across multiple sample sets to determine precision during instrumental analysis. The analytical uncertainty of replicates, calculated as coefficient of variance (1 SD divided by mean), is within 28%, 25%, and 28% for water (n=8), mussel (n=8), and oyster samples (n=8), respectively (Table S6). Procedural blanks were monitored for contamination, with virtually all blanks having concentrations below MDL (Table S7). 6:2 FTS was measured but excluded from further analysis due to its elevated levels in biota procedural blanks, which suggest potential contamination in laboratory. Additional details of quality assurance and quality control can be found in SI section 1.8.

2.6 Statistical analysis

Statistical analyses were performed using R version 4.3.1.[57] For sample concentrations below MDL, we imputed MDL/√2 in calculations of summed PFAS concentration, isomer ratios, and for plotting purposes. PFAS with infrequent detection in both species (<50%) were excluded

224 from further statistical analysis (9 of 27 compounds; see Table S3). We assessed the correlation
225 between measured PFAS concentrations in water and in biota for individual PFAS and PFAS
226 groups (i.e., PFCAs, PFSAs, FASAs, Others; see categorization in Figure 1).

227 To calculate field-based bioaccumulation factor (BAF; L kg^{-1}) of individual PFAS
228 compounds for each sample, we divided the concentration of PFAS in bivalve tissue (ng PFAS
229 per kg wet tissue) by the surface water concentration (ng PFAS per L water) at the corresponding
230 site (Eq. 1).

231 $$BAF = {}^{C_{tissue}}/_{C_{water}} \quad [1]$$

232 For long-chain PFAAs that were not detectable in water but are known to have high
233 bioaccumulation potential,[25, 58] we estimated the lower limit of BAFs using the measured tissue
234 concentration and the MDL of the water concentration, and included them in statistical analysis.
235 If bivalve tissue concentration of a given sample was below the MDL for biological samples,
236 their BAFs were treated as nondetects and were censored in further analysis. Censored maximum
237 likelihood estimation mean BAF of each compound for oysters and mussels are reported in Table
238 S9.

239 The field-based BAF estimates of PFAS can vary due to several factors related to study
240 design.[29] The removal of large particles from our water samples prior to extraction may lead to
241 lower concentrations compared to analyses of whole water. As a result, our field-based BAF
242 estimates may be biased high relative to those from studies that utilize whole water samples with
243 considerable particulate matters. Nonetheless, these data remain valid for comparing PFAS
244 accumulation dynamics in bivalves within the work and investigating factors contributing to their
245 BAFs.

We conducted censored data analysis using the Nondetects and Data Analysis (NADA) package in R to generate descriptive statistics of BAFs.[59, 60] We computed the mean and standard deviation of BAF for each compound for both species using the *cenmle* function, which uses Maximum Likelihood Estimation (MLE) to impute censored data based on the distribution of observed data. We conducted multivariate linear regression to examine factors that affect BAF using the *cencorreg* function (Eq. 2), which fits a censored parametric regression model using MLE to impute censored data. The model includes ecological, water quality, and physicochemical variables that have potential to affect PFAS bioaccumulation. All BAFs were log transformed to satisfy regression assumptions. Example R code is provided in SI Section 1.9.

$$Log_{10}BAF = \beta_0 + (\beta_1 \times species) + (\beta_2 \times tissue\ weight) + (\beta_3 \times N_{fluorinated\ carbons}) + (\beta_4 \times PFAS\ functional\ group) + (\beta_5 \times temperature) + (\beta_6 \times salinity) \quad [2]$$

3. Results and Discussion

3.1 Distinct PFAS patterns in water, oysters, and mussels

All targeted PFAS compounds were detected at some level across samples, and all water and bivalve samples contained detectable levels of C7-C11 perfluoroalkyl carboxylic acids (PFCAs), as well as FOSA (Table S3). Perfluoroalkyl sulfonamides (FASAs, i.e., FBSA, FHxSA, and FOSA) were detected in a majority of samples. Short-chain PFCAs and perfluoroalkyl sulfonic acid (PFSAs) were more commonly found in water, while long-chain PFCAs and PFSA (> 8 carbons) were more frequently detected in bivalves. The Σ_{29}PFAS in water (range:14.1 to 57.4 ng/L) are comparable to levels previously reported in coastal waters that are downstream of a PFAS point-source, such as military bases or wastewater treatment plants, or close to airports and industrial production of textiles and paper.[11, 26, 48, 61]

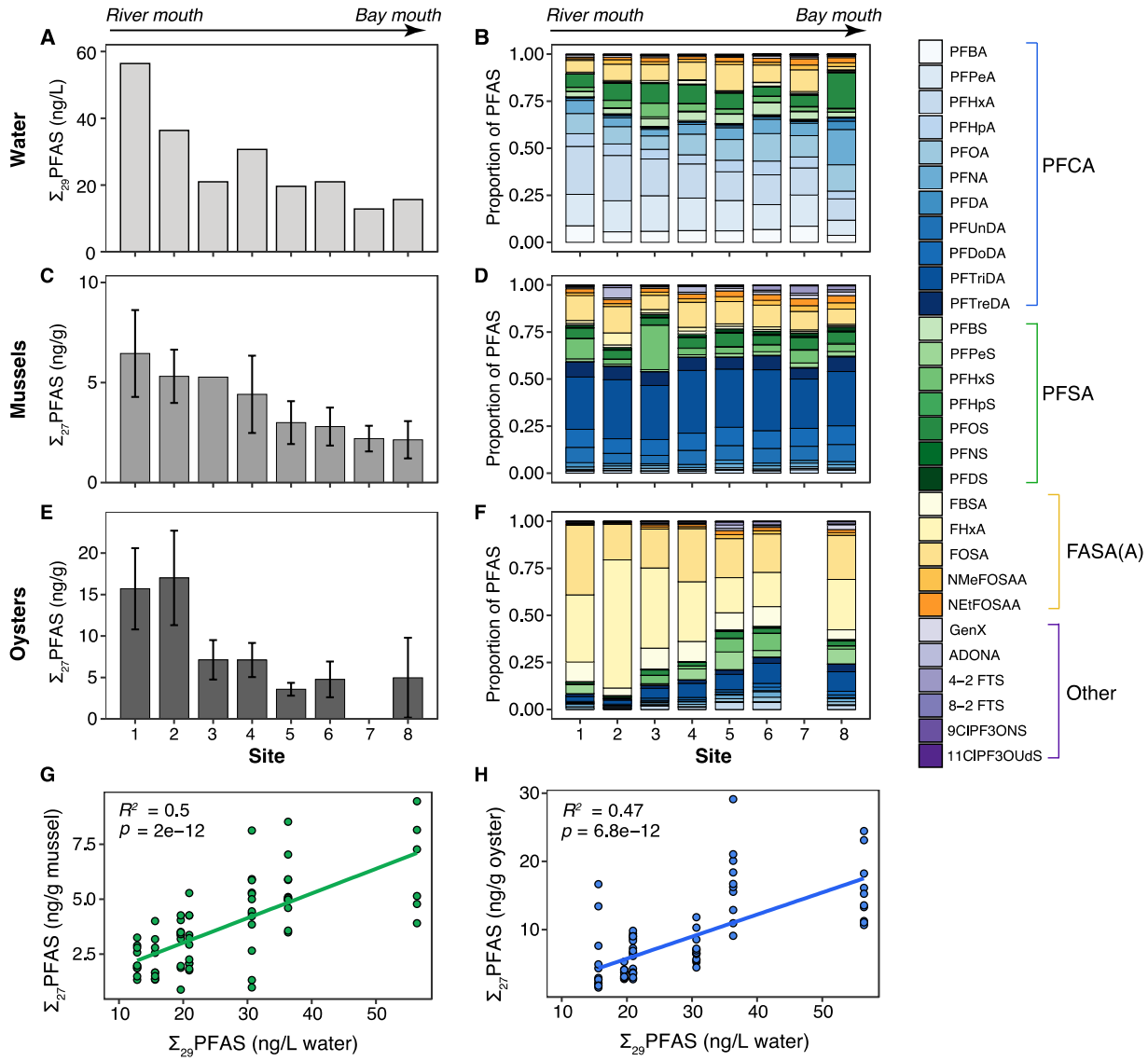

Figure 1. PFAS concentrations and composition in surface water and bivalves (Atlantic ribbed mussels and Eastern oysters) from eight Delaware Bay sites. Panel A, C, and E show the summed PFAS concentrations in surface water, Atlantic ribbed mussels, and Eastern oysters, respectively and panels B, D, and F present the corresponding PFAS composition. Panels G and H show correlations between summed PFAS concentrations in mussels and oysters versus surface water. Perfluoroalkyl carboxylic acids (PFCA), perfluoroalkyl sulfonic acid (PFSA), and perfluoroalkane sulfonamide (FASA) and sulfonamidoacetic acids (FASAA) are in shades of blue, green, and yellow, respectively, and all other compounds are in shade of purple.

We observe clear decreasing trends in summed PFAS concentration (hereafter referred to as ΣPFAS) from river to bay mouth in water, mussels, and oysters (Figure 1 A, C, E), suggesting the Delaware River is the major PFAS source for Delaware Bay. Bivalves from the same

locations exhibit distinct PFAS accumulation patterns by species. Oysters have higher Σ_{27}PFAS levels than that of ribbed mussels, predominantly driven by the high levels of FASAs in oysters. The contribution of FASAs decreases from 85% of Σ_{27}PFAS in upper bay to 55% in lower bay (Figure 1F). In contrast, measured PFAS burden in mussels is primarily composed of long-chain PFCAs and their PFAS composition profiles are generally consistent across sites (Figure 1D).

The distinct PFAS patterns observed between oysters and mussels grown in the same environment may stem from the differences in their dietary composition. While both bivalve species consume particles of similar sizes and primarily rely on algal food sources, non-algal materials such as bacteria, cellulose, and plant-derived detritus are more important for ribbed mussels compared to Eastern oysters.[45] We postulate the differences in diet are at least partially responsible for the observed variation in PFAS profiles between these two species. Further research comparing PFAS profiles across different types of particulate organic matters can improve our understanding of dietary influence on PFAS accumulation in bivalves.

Table 1. Results from censored multivariate regression models analyzing factors associated with log-transformed bioaccumulation factors (BAFs) in bivalves for all measured PFAS compounds (model 1), with separate models for PFCAs, PFSAs, and FASAs/FASAAs (model 2-4). The significant explanatory variables are in bold (p-value <0.05)

Response Variable	Explanatory Variables	β-coefficient	SE	p-value
Model 1. Log_{10} BAF of all PFAS	Intercept	0.347	0.120	0.004
Rescaled likelihood R^2: 0.306	Species			
	Mussel	Referent		
	Oyster	0.064	0.036	0.077
	Body weight	**-0.011**	**0.003**	**0.001**
	F Carbons	**0.231**	**0.007**	**<0.001**
	Compound Category			
	PFCA	**Referent**		
	PFSA	**-0.135**	**0.058**	**0.021**
	FASA and FASAA	**0.616**	**0.043**	**<0.001**
	Other	**1.742**	**0.093**	**<0.001**
	Salinity	0.001	0.007	0.804
	Temperature	0.004	0.007	0.516

Model 2. Log_{10} BAF of PFCAs	Intercept	-0.223	0.137	0.104
Rescaled likelihood R^2: 0.558	Species			
	Mussel	Referent		
	Oyster	**-0.128**	**0.044**	**0.004**
	Body weight	**-0.014**	**0.004**	**<0.001**
	F Carbons	**0.290**	**0.007**	**<0.001**
	Salinity	0.007	0.005	0.177
	Temperature	0.010	0.008	0.185
Model 3. Log_{10} BAF of PFSAs	Intercept	-2.265	0.169	<0.001
Rescaled likelihood R^2: 0.813	Species			
	Mussel	Referent		
	Oyster	**-0.320**	**0.032**	**0.025**
	Body weight	**-0.020**	**0.003**	**<0.001**
	F Carbons	**0.5225**	**0.016**	**<0.001**
	Salinity	0.002	0.004	0.630
	Temperature	**0.015**	**0.006**	**0.015**
Model 4. Log_{10} BAF of FASAs and FASAAs	Intercept	4.140	0.216	<0.001
Rescaled likelihood R^2: 0.309	Species			
	Mussel	Referent		
	Oyster	**0.599**	**0.062**	**<0.001**
	Body weight	**-0.013**	**0.006**	**0.031**
	F Carbons	**-0.238**	**0.019**	**<0.001**
	Salinity	-0.011	0.007	0.111
	Temperature	0.002	0.029	0.863

3.2 Factors influencing PFAS Bioaccumulation in Bivalves

We built censored multivariable regression models to examine factors influencing bioaccumulation potential of PFAS in bivalves (Table 1). Log_{10} BAF of all measured PFAS compounds significantly differs across PFAS categories (i.e., PFCAs, PFSAs, and FASAs/FASAAs) (Table 1 – Model 1). Accounting for all other factors, FASA and FASAA compounds are significantly more bioaccumulative than PFSAs and PFCAs, consistent with prior research showing that FASA/FASAAs tend to have the higher Log_{10} BAF than terminal PFAS with same fluorinated carbon chain length.[25, 62] The Log_{10} BAFs of PFSAs are significantly lower than those of PFCAs. We caution against drawing conclusions from this result, as only 3 PFSAs were frequently detected in our bivalves and biotransformation may

312 influence the findings, as further discussed in Section 3.3. We also investigated the impact of
313 each individual factor on Log_{10} BAF within a given PFAS category (Table 1 – Models 2 to 4),
314 except for compounds labeled "other", as most PFAS compounds in this category were below
315 MDL.

316 Log_{10} BAFs of PFCAs and PFSAs significantly increase with fluorinated carbon chain
317 length (Table 1 – Model 2 and 3). The positive association between Log_{10} BAFs of PFAS and its
318 chain length has been frequently reported in fish,[25, 63, 64] likely due to the higher protein binding
319 affinities,[64-66] increased partitioning to phospholipids,[67] and decreased renal elimination of long-
320 chain PFAAs.[67, 68] Conformational changes of very long-chain PFAS molecules limit protein
321 binding,[68] which explains the observed BAF plateau at C12 (i.e., PFTriDA) in our bivalves
322 (Figure 2). Similar to recent observations of elevated BAFs of FASAs in fish,[20, 25, 62] the
323 FASAs/FASAAs in bivalves have significantly higher BAFs than the PFAAs of equivalent chain
324 length, accounting for all other factors in Model 1. The greater bioaccumulation potential of
325 FASAs may result from the higher capacity for neutral FASAs to readily bind to phospholipids
326 and permeate cell membranes than anionic PFAAs[69] in pH-neutral environments like Delaware
327 Bay.[70] Few studies have systematically examined PFAS bioaccumulation in bivalves. Our
328 findings suggest similarities in mechanisms of PFAA bioaccumulation between bivalves and
329 fish.

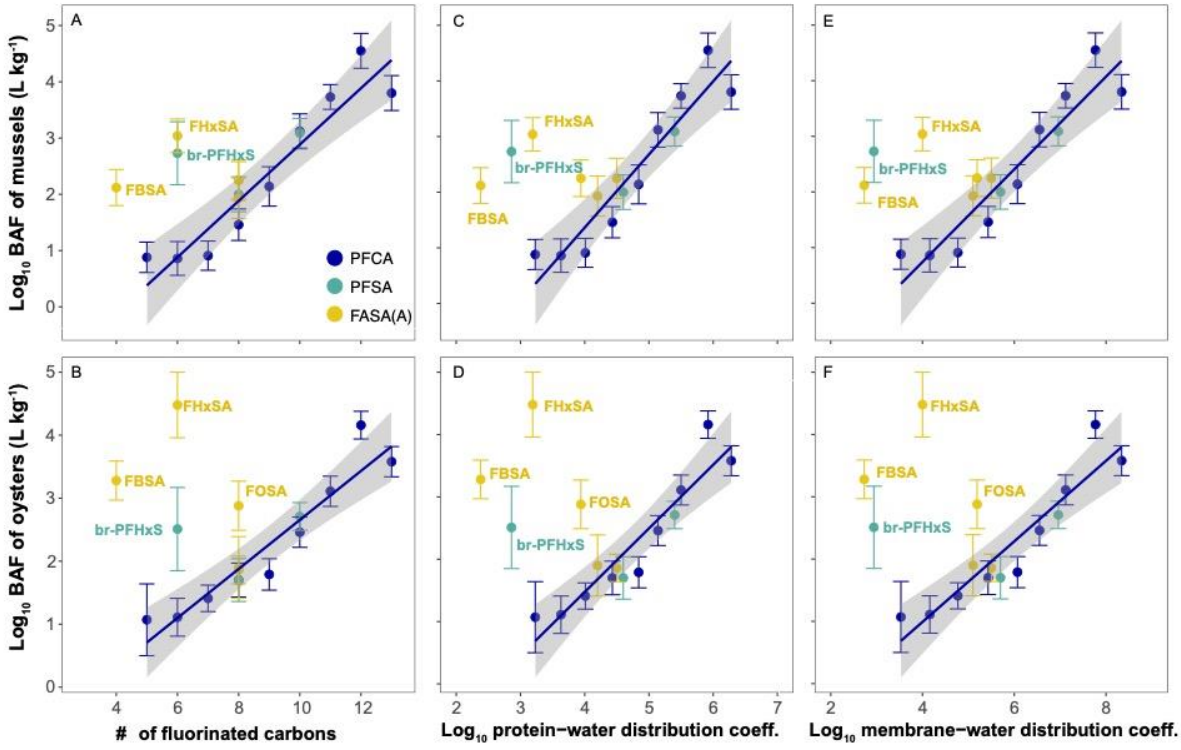

Figure 2. Log transformed field-measured bioaccumulation factors of individual PFAS compounds in mussels and oysters (Log_{10} BAF) with respect to fluorinated carbon chain length, protein-water distribution coefficient, and membrane-water distribution coefficient. Each point represents a censored maximum likelihood estimation mean and error bars represent standard deviation. Perfluoroalkyl carboxylic acids (PFCA), perfluoroalkyl sulfonic acid (PFSA), and perfluoroalkane sulfonamides (FASA) and sulfonamidoacetic acids (FASAA) are in shades of blue, green, and yellow, respectively, and the regression line are plotted using all PFCA points. Perfluorobutane sulfonamide (FBSA), perfluorohexane sulfonamide (FHxSA), branched perfluorohexane sulfonic acid (br-PFHxS) consistently appear as outliers in all plots. See Table S10 for derivation of protein-water distribution coefficient and membrane-water distribution coefficient.

Previous research has shown weak positive relationship between BAF of FASAs and chain length in fish and invertebrates.[58, 62] In contrast, our regression results indicate that the Log_{10} BAFs of FASAs/FASAAs are negatively associated with their fluorinated carbon chain length (Table 1 – Model 4). Additionally, Figure 2A and B suggest no clear relationship among five FASAs/FASAAs compounds, but Log_{10} BAFs of FBSA and FHxSA are unusually high relative to other PFAAs with equivalent chain lengths. As PFAS tend to bind to proteins and

phospholipids in biota,[67, 71] we also examined the relationship between BAF and membrane−water (D_{mw}) and protein−water distribution coefficients (D_{pw}) (Fig. 2C-F, SI 1.9), two key physicochemical parameters used to describe bioconcentration behaviors of individual PFAS compound based on liposome−water partition coefficient and chemical speciation (neutral vs. charged) (See Table S10).[1, 26, 72] The consistent outlier behavior of FBSA and FHxSA in Figure 2 suggests that factors beyond their physicochemical properties contribute to their elevated BAFs. We propose that biotransformation is an important factor, as further discussed in Section 3.3.

In all four regression models, Log_{10} BAF consistently and significantly decreases with body weight of bivalves (Table 1). For highly bioaccumulative PFAS (i.e., BAF >5000),[46] mussel tissues exhibit a consistent decrease in the BAF of PFAS as body weight and age increase (Figure 3). The smaller and younger mussels have the highest BAFs, indicating young mussels possess greater bioaccumulation potential compared to older mussels. Previous studies on fish suggest that smaller individuals tend to accumulate higher levels of PFAS, likely due to their elevated gill respiration rates leading to increased PFAS exposure.[73-75] Similarly, smaller bivalves also have higher filtration efficiency (i.e., size-normalized filtration rates) than the larger ones,[76, 77] which could lead to higher PFAS uptake rate and explain the higher BAFs of PFAS in these individuals. These results highlight that younger organisms may be more vulnerable to PFAS contamination due to physiological differences. Direct measurements of filtration rate, or indirect indicators such as body size and age, can be important predictors of PFAS bioaccumulation in mussels and fish. The relationship between BAF and body weight or age is less evident in oysters (Figure S2). This is likely due to species-specific and life stage-

related differences in PFAS bioaccumulation mechanisms, including variations in biotransformation capacity (see details in Section 3.3).

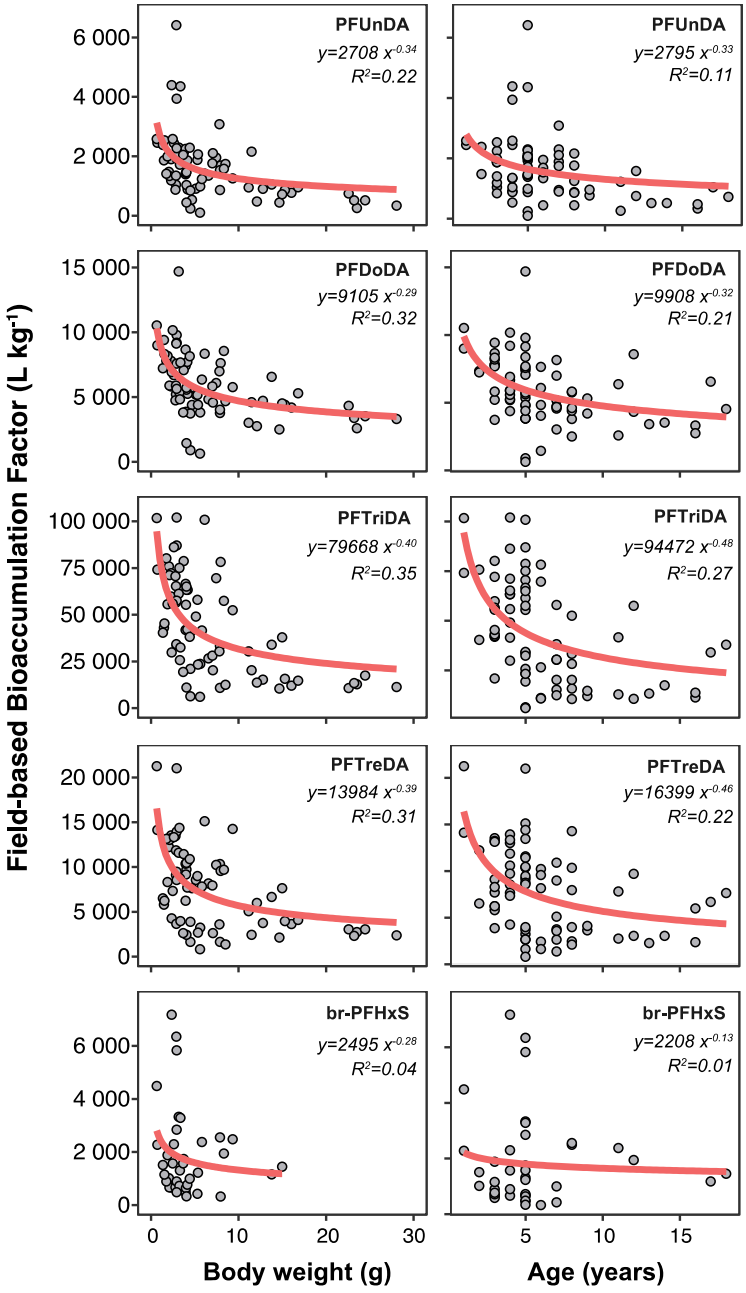

Figure 3. Bioaccumulation factor (BAF) of highly bioaccumulative PFAS compounds (BAF >5000) in ribbed mussels with respect to their wet body weight (left) and age (right). The red line in each plot represents the fitted power-law function, with the equation and R^2 value indicated.

After accounting for other factors in the models, we find no significant effects of salinity on the BAF of PFAS (Table 1). Previous studies have yielded inconsistent findings on how salinity affects PFAS bioaccumulation in bivalves. Laboratory experiments suggest that increased salinity may create a "salting-out" effect, enhancing the binding of PFAS to particulates, which leads to greater dietary uptake of long-chain PFAAs by fish and shellfish.[22, 26, 78-80] However, evidence supporting this trend in natural bivalves is limited. Similar to our study, Wang et al., (2022) reported no significant effect of salinity on PFAS bioaccumulation in wild oysters.[26] We attribute the lack of salinity effects in field studies to the competing factors, such as changes in both PFAS availability and the physiology of bivalves along salinity gradient. Bivalves in lower salinity waters exhibit decreased filtration rates,[81] which results in lower contaminant uptake.[22] Conversely, in the upper bay of this study, low-salinity water receives considerable PFAS inputs from the Delaware River, leading to the higher PFAS levels in water (Figure 1A). Consequently, oysters and mussels in the upper bay are exposed to greater PFAS concentrations compared to those in lower bays with higher salinity. These competing influences may obscure the effects of salinity on PFAS accumulation in wild bivalves. Further research that considers field conditions is necessary to reconcile discrepancies between laboratory and field observations.

The regression model results show that inconsistent effects of water temperature on the BAFs, with significance only observed for the BAF of PFSAs (Table 1, Model 3). Few studies investigated the effects of temperature on PFAS bioaccumulation. Wang et al., (2022) recently suggested that temperature is not a significant predictor of PFAS concentrations in wild oysters.[26] It is important to note that the bivalves in this study can thrive across a wide range of temperatures (Atlantic ribbed mussel: 0 to 36°C,[82] Eastern oyster: 0 to 42°C[83]). The limited

temperature variability across the bay (9 to 18°C) may prevent the detection of temperature effects on PFAS bioaccumulation in these species.[26, 82-84] Future studies could explore a broader temperature range, including seasonal variations, to better understand the temperature effect on PFAS bioaccumulation.

3.3 PFAS biotransformation in bivalves

Prior studies have identified possible biotransformation products in bivalves (e.g., elevated levels of FOSA).[20, 27] In this study, we examined evidence for potential biotransformation through additional data analysis. First, we investigated the isomer distribution (linear vs. branched) of some PFAAs measured in this study. Both linear and branched PFOS isomers are present in water, but only linear PFOS is detectable in bivalves. The isomer pattern of PFOS in bivalves is consistent with previously reported predominantly linear PFSA isomers (>70% linear) in aquatic organisms.[85-88] Both theorical and experimental evidence indicates that direct uptake of terminal PFAAs leads to enrichment of linear isomers of PFAA in biological tissues,[64, 88, 89] likely due to the higher protein binding affinities of linear isomers[90-92] and preferential elimination of branched isomers in biota.[64] Therefore, the direct uptake of PFOS from water and food can explain the enrichment and dominance of linear PFOS in bivalves of this study.

Contrary to PFOS, PFHxS exhibits an opposite trend when comparing water and bivalves. Linear PFHxS is the predominant isomer in water across all sites (average ± 1 SD: 84±2%), in contrast to its contribution in mussels (28±14%) and oysters (36±11%) (Figure S3). The direct uptake of PFHxS from the surrounding environment would lead to an enrichment of linear isomers as above mentioned. Therefore, additional process(es) are responsible for the

observed enrichment of branched PFHxS in bivalve tissues. Laboratory experiments previously showed that fish exposed to PFAS precursors (e.g., FOSA, diSPAP) preferentially metabolize branched precursors, leading to biotransformation products with enriched branched isomers in their tissues.[93-95] Given the presence of precursor and replacement PFAS in the bay water and bivalves, including FHxSA– a known (intermediate) precursor for PFHxS,[96, 97] we infer that the enrichment of branched PFHxS in bivalves likely results from precursor biotransformation.

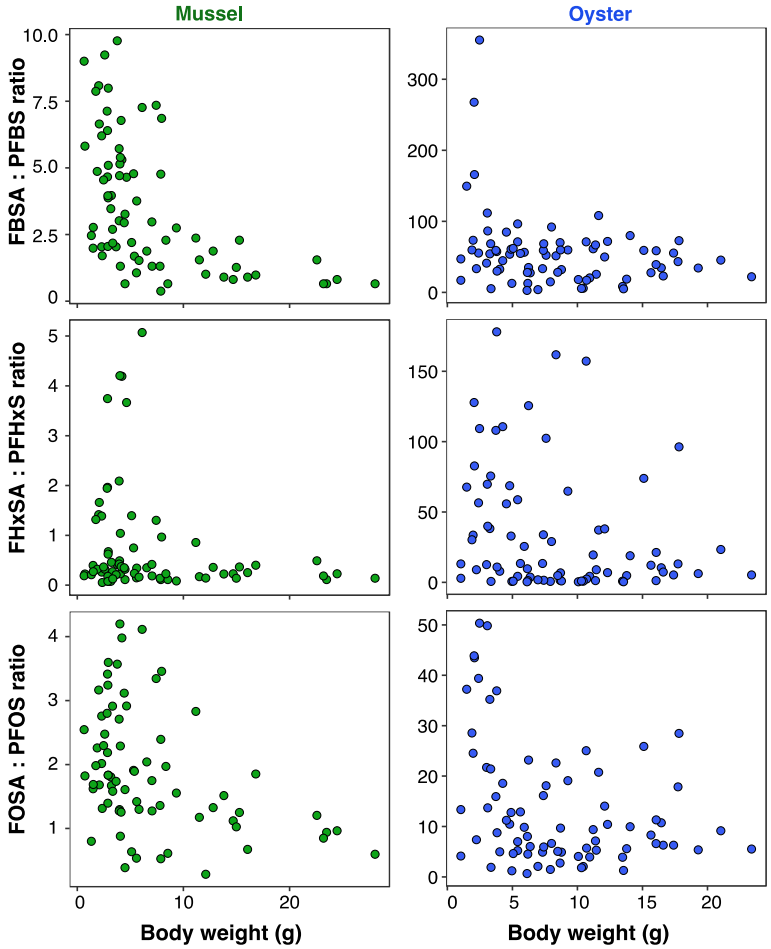

Figure 4. The ratios of three perfluoroalkane sulfonamide compounds and their corresponding presumed terminal PFSA with respect to wet body weight of Atlantic ribbed mussels (left, in green) and Eastern oysters (right, in blue).

Prior studies suggest concentration ratios between FASAs and their corresponding presumed terminal PFSA (i.e., FBSA/PFBS, FHxSA/PFHxS, FOSA/PFOS) can serve as

indicators of biotransformation of PFAS, with a higher ratio suggesting greater representation of precursors thus less biotransformation.[20, 74, 87, 98] Several studies suggest that biotransformation capacity varies among species in a food web and also changes with fish body size.[74, 98] We find both older/larger oysters and mussels exhibit a lower FASA/PFSA ratio (Figure 4), signaling a greater biotransformation capacity in these organisms. In addition, we observe that the FASA to PFSA ratios in oysters are up to 50 times greater than those in mussels (Table S11), indicating that oysters undergo significantly less PFAS biotransformation. This may be related to the fact that the oysters (3.6 ± 1.2 years) in this study are, on average, much younger than the mussels (6.3 ± 3.7) (see SI Section 1.2). To our knowledge, this is the first study documenting changes in biotransformation capacity across bivalve species and their lifespan. We speculate these changes may be related to the variations in lipid and protein content or metabolic process across species and life stages, highlighting the need for future research to elucidate the underlying mechanisms.

The observed isomer distribution and FASA to PFSA ratios provide supporting evidence that biotransformation occurs in these bivalves. Prior studies suggest biotransformation can lead to an overestimation of the bioaccumulation potential of certain intermediate and terminal PFAS.[25, 99] We postulate that biotransformation of precursors contributes to the elevated BAFs of branched PFHxS, FBSA, and FHxSA observed in our bivalves (Figure 2). In other words, these terminal or intermediate PFAS do not solely reflect the direct accumulation of these compounds but rather result from the conversion of precursor compounds through biotransformation. Accurately characterizing PFAS bioaccumulation requires understanding biotransformation pathways and identifying potential precursors. Future efforts to quantify the occurrence and extent of biotransformation leading to the analyzed PFAS will enhance the accuracy of bioaccumulation potential estimates.

3.4 Implications for Environmental Biomonitoring

Mussels and oysters offer valuable insights into the integrated accumulation of PFAS in natural environments. Our findings offer practical recommendations for incorporating bivalves into existing biomonitoring programs (e.g., Mussel Watch) and guidance for future research on PFAS contamination in aquatic environments. For instance, the summed concentration of PFAS in bivalves closely mirrors the observed pattern in water (Fig. 1 G and H), but this correlation is not consistently evident across individual PFAS or cumulative concentrations of PFAS groups (Fig. S4 and 5). This discrepancy may arise from biotransformation processes that alter the PFAS profiles between the water source and the bivalve receptors. Therefore, we recommend using the summed PFAS concentration in bivalves as a more reliable indicator for assessing PFAS contamination in water.

This study reveals that differences in feeding strategies, physiology, and life stage can influence both inter- and intraspecies variability in PFAS accumulation in bivalves, but these factors have frequently been overlooked in earlier research on PFAS bioaccumulation. Larger and more mature bivalves likely have a great biotransformation capacity and can provide crucial data on terminal PFAS concentrations, whereas younger individuals are better suited for studying precursor compounds and biotransformation intermediates. To fully understand PFAS exposure and bioaccumulation in aquatic organisms, future efforts should encompass a range of sizes and ages of study organisms and consider the diverse life histories across species.

Delaware Bay exhibits a high prevalence of FASAs in both water and bivalves. FASAs are generally more accumulative than their PFAAs counterparts in bivalves and fish.[58, 62, 100] The oysters in this study are of a similar age to commercially harvested oysters in the U.S. market

(typically 2 to 3 years old)[101] and exhibit high levels of FASAs. Most FASAs are currently not included in standard targeted methods for PFAS analysis.[53] As a result, appreciable levels of FASAs in seafood may not be detected by laboratories following these methods. Many FASAs and their precursors are known to undergo biotransformation to toxic PFAAs in biota, including the human body.[102, 103] Hence, to protect public health and enhance our understanding of the toxicity and biotransformation processes of PFAS, it is imperative to incorporate FASAs into standardized targeted analytical methods used for testing seafood.

While several studies have suggested the potential of bivalves as biomonitors for PFAS, this is the first comprehensive evaluation of two cohabiting bivalve species, generating a mechanistic understanding of the factors influencing inter- and intra-species PFAS accumulation. This knowledge is likely applicable to other bivalve species and regions and is essential for comparing PFAS contamination using bivalves as biomonitors across different environments. Future research exploring additional bivalve species or the same species in different environmental contexts will strengthen our understanding of the robustness and applicability of bivalves as PFAS biomonitors across diverse environments.

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authors declare no competing financial interests. The views expressed in this article are those of the author(s) and do not necessarily represent the views or the policies of the US Environmental Protection Agency. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the US Government.

Supporting Information

Explanatory texts related to methods; data tables of quality assurance/quality control of PFAS analysis, bioaccumulation factor, and FASA to PFSA ratios; and figures of sampling location, bioaccumulation factor, isomers distribution, and water and bivalves PFAS correlation (PDF); PFAS concentrations, biometric, and water quality data (XLSX).

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