Assessing Per- and Polyfluoroalkyl Substances (PFAS) in Fish Fillet Using Non-Targeted Analyses

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ABSTRACT

Per- and polyfluoroalkyl substances (PFAS) are a class of thousands of man-made chemicals that are persistent and highly stable in the environment. The diverse structures of PFAS give them different chemical properties that influence their solubility in different environmental matrices and biological tissues. PFAS in drinking water have been extensively studied, but information on their presence in fish and other exposure routes is limited. To address this, a non-targeted analysis using liquid chromatography, ion mobility spectrometry, and mass spectrometry (LC-IMS-MS) was performed to evaluate PFAS in fish fillets from in central North Carolina and compare with PFAS data from previously published water. A total of 22 different PFAS were detected in the fillets, including only 4 of the PFAS reported in water. Both more PFAS types and higher concentrations were observed in fish caught near a known PFAS point-source compared to those from a reservoir used for drinking water and recreation. Median fillet PFOS levels were 54 ppb in fish closest to the point source and 14-20 ppb in fish from the reservoir. Thus, future PFAS monitoring should include both targeted and non-targeted analyses of both water and fish to increase understanding of human exposure risks and ecosystem impacts.

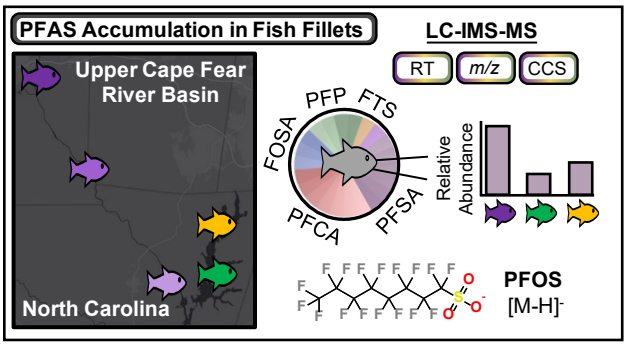
KEYWORDS

Bioaccumulation, *Lepomis*, consumption advisory, mass spectrometry, ion mobility spectrometry

SYNOPSIS

Fish fillet samples were collected from five sites in North Carolina. PFAS were detected in all samples and differences in analytes and abundances were observed at the different sites.

GRAPHICAL ABSTRACT



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INTRODUCTION

Per- and polyfluoroalkyl substance (PFAS) are anthropogenic chemicals that have been produced since the 1940s. PFAS are a diverse class of fluorinated organic chemicals with varying length carbon chains and shared properties including oil and water repellency, stability at high temperatures, and inability to readily break down.[1] Because of these properties, PFAS have been incorporated into nonstick coatings, stain- and water-resistant materials, and firefighting foams.[2] Through the manufacture, use, and disposal of these products, PFAS ultimately end up in the environment.[3] Drinking water and consuming fish and seafood have been identified as primary PFAS exposure sources for people.[3, 4] Further, because different PFAS partition differently between water and tissue, with longer chain PFAS being less soluble in water,[5, 6] water and food are expected to have different PFAS present.[4] Thus, information about PFAS in water, fish, and other matrices is critical for understanding environmental impacts of PFAS and informing meaningful regulatory and advisory limits for human exposure.

Drinking water PFAS levels have been extensively studied, and the U.S. Environmental Protection Agency has proposed enforceable maximum contaminant levels for two long chain PFAS, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS), due to their negative health associations.[7] However, these federal limits only apply to drinking water, and do not account for exposure to PFAS through other routes such as diet. Several states have established their own reference doses for PFAS exposure through fish consumption.[8] For example, in July 2023, the state of North Carolina enacted its first fish consumption advisories for several species of freshwater fish due to high levels of PFOS.[9] Specifically, women of childbearing age and other sensitive groups are advised not to eat any amount of bluegill sunfish, redear sunfish, flathead catfish, largemouth bass, and striped bass. For all other individuals, the recommendation is no

more than one meal per year of any of those species combined. However, these new fish consumption advisories apply only to the middle and lower part of the Cape Fear River, but other areas in North Carolina have not been evaluated. Additionally, most existing state-issued consumption advisories are specific to PFOS and do not account for other PFAS that may be present in fish tissue.[8]

In this study, we assessed PFAS in fillets of commonly caught and consumed panfish collected from five locations in North Carolina during the summer of 2020 (**Figure 1**). Water samples simultaneously collected from these locations contained elevated levels of PFAS.[10] Therefore, to understand if similar PFAS were also present in the fish, we used a non-targeted

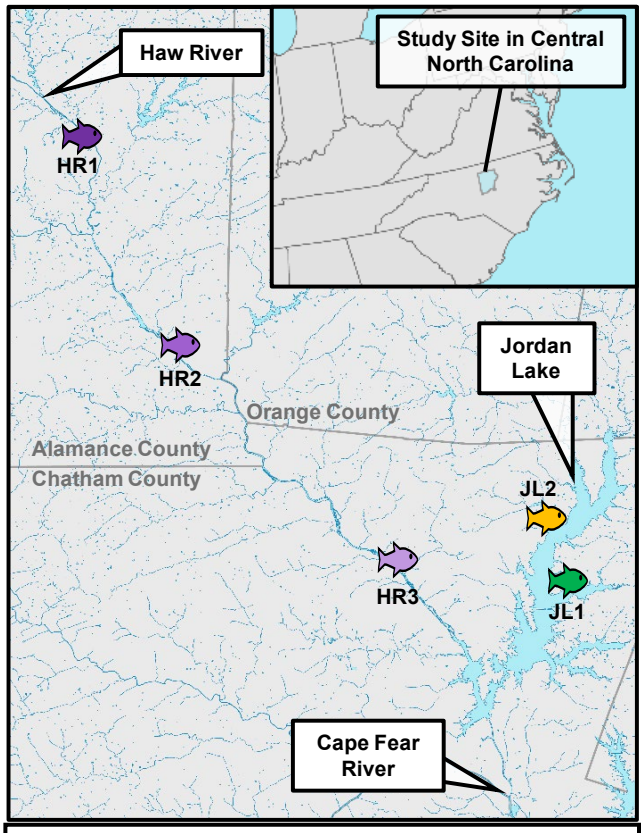

Figure 1. Map of study site showing fish sampling locations. Fish icons show the approximate location of the study site, which spanned three NC counties (Alamance, Orange, and Chatham). Fish were sampled from three sites along the Haw River and two sites within Jordan Lake between May and August 2020.

analysis platform combining liquid chromatography, ion mobility spectrometry, and mass spectrometry (LC-IMS-MS) and a suspect screening data analysis workflow to examine the frequency and abundance of PFAS in the fillets and visualized the spatial differences in the five sites along the watershed.[11-13] Additionally, we explored fish species differences by comparing *Lepomis* spp. sunfish to yellow perch, white perch, and catfish. Finally, we calculated PFOS concentrations in the fish fillets and compared these to levels reported in other areas around the United States.

MATERIALS AND METHODS

Study Area and Sample Collection

All animal procedures were performed with approval from the North Carolina State University Institutional Animal Care and Use Committee, protocol number 19-816-01. Fish were collected between May and August 2020 in North Carolina at three sites along the Haw River (HR1, HR2, HR3) and two sites in Jordan Lake (JL1, JL2). Sampling locations were selected based on proximity to sites with water PFAS data available and angling access. Fish were caught using hook-and-line angling and total length, weight, and sex were recorded. Fillets were removed following accepted decontamination procedures for fish tissue PFAS sampling[14] and approximately 0.1 to 1 g of skin-on fillet from each fish was stored at -20 °C prior to extraction and analysis. The targeted water analyses for 13 PFAS have been previously reported.[10] Fish species sampled are detailed in **Tables S1** and **S2**, and included 38 *Lepomis* spp. sunfish, two white perch (*Morone americana*), four yellow perch (*Perca flavescens*), and three channel catfish (*Ictalurus punctatus*).

Standards and Reagents

A stable isotope-labeled PFAS standard mix (MPFAC-HIF-ES) was obtained from Wellington Laboratories (Guelph, Canada) containing heavy-labeled PFAS at varying concentrations from 250 to 5,000 ng/ml in methanol. The mix was diluted 1:1 in methanol for use as internal standard. Optima LC-MS grade methanol, water, and ammonium acetate were used for extractions and mobile phases (Fisher Scientific). The Standard Reference Material 1947 (SRM1947, Lake Michigan Trout) was also obtained from NIST and used for quality control.

Sample Preparation

A detailed list of consumable items with manufacturer and part number is included in **Table S3**. Fish fillet samples, quality control samples (0.5 g SRM1947), and method blanks (0.5 mL water) were homogenized in a bead mill. Sample wet weights for the fish fillets ranged from 0.1 to 1 g. The entire sample was extracted using the following procedure and the range of sample weights was accounted for in data analysis. The homogenized tissue was spiked with internal standards (5 µL) and extracted in methanol (5 mL) in polypropylene tubes. Samples were sonicated for approximately 1 h and then centrifuged at 4 °C and 10,000 rpm for 5 min. The supernatant was decanted into polypropylene syringes and filtered through 0.2 µm nylon syringe filters into new polypropylene tubes loaded with 10 mg dispersive carbon. Samples were shaken and inverted by hand for 1 min and centrifuged again. The supernatant was transferred into new polypropylene tubes and concentrated to dryness in the speedvac at 45 °C and full vacuum. Samples were reconstituted in 200 µL of 40% methanol, 60% water by volume buffered with 3 mM ammonium acetate, using the methanol-first method described by Weed et al.[15] in which the methanol portion is added to the sample first, vortexed, and then the aqueous portion is added. Reconstituted samples

were transferred to polypropylene LC vials with inserts. Process efficiency was calculated for 24 different PFAS as described in the supplemental methods.[16, 17] Process efficiencies had a median of 24% and ranged from 1.2% (PFBA) to 430% (PFTeDA) (**Figure S1**).

LC-IMS-MS Non-Targeted Analysis and Suspect Screening

Non-targeted sample analysis was performed on an Agilent 6560 IM-QTOF coupled with an Agilent 1290 Infinity UPLC system with consumables and parameters previously described[11] and detailed in **Tables S4-S7**. In brief, samples were injected in randomized order with a double blank and a QC sample injected between every 10 samples to monitor column carryover and instrument performance. The LC separations were carried out on a C18 column in line with a guard column. Mobile phases included: A) water with 5 mM ammonium acetate, and B) 95% methanol:5% water with 5 mM ammonium acetate. Samples were injected at a volume of 2 µl and a gradient (**Table S5**) was applied at a flow rate of 0.4 mL per min. An Agilent JetStream electrospray ionization source operated in negative ionization mode was utilized to ionize the samples. Instrument mass calibration was performed by directly injecting Agilent ESI tune mix solution, and tune mix ions were used to convert measured drift times to collision cross section (CCS) values using a single-field calibration.[11] Ion packets were pulsed through the drift tube using 4-bit multiplexing[18] and data was collected in Agilent .d files. Data processing included the following steps[19]: 1) Data files were demultiplexed using the PNNL Preprocessor (v 4.0)[20] with the signal intensity threshold set to 20 counts and 100% pulse coverage, 2) Agilent IM-MS Browser (v 10.0) was then used for single-field CCS calibration, and 3) Skyline-daily (v 22.2.1.501) was used for suspect screening. The suspect screening workflow uses Skyline capabilities for drift time and retention time filtering through alignment of our in-house library of

100 individual PFAS analytes[21] using LC retention time alignment with internal standard (as applicable), < 10 ppm *m/z* error versus the library, and CCS filtering with a resolving power window of 30, as well as manual validation of PFAS annotations. Analytes not detected in any sample were removed from the Skyline document. PFAS detected in at least one sample are listed in **Table S8**.

Data Analysis

Following PFAS identification in Skyline, the peak areas of detected PFAS analytes were exported into Excel for further analysis. Detection limits were defined as the average plus 3 standard deviations of the peak area of the analytes in the method blank, and samples with peak areas below that level were considered not detected. To account for analyte loss from sample extraction and ion suppression in the ESI source, peak areas of the light PFAS analytes were normalized by dividing the peak area of the heavy internal standard. Exact match internal standards were used where possible, and surrogate standards were assigned as needed. Surrogate standards were selected looking first at structural similarity and then at nearest retention time; details are in **Table S8**. Finally, the light-to-heavy peak area ratio was divided by the wet weight of each fillet sample to obtain the relative abundance of each PFAS detected in the fillets (**Equation 1**).

$$Relative\ Abundance = \frac{Peak\ Area\ (Native\ PFAS)}{Peak\ Area\ (Internal\ Standard)} \div Tissue\ wet\ weight\ (g) \qquad (1)$$

Semi-quantitative estimates of PFAS concentrations were calculated by multiplying the relative abundance (light-to-heavy peak area ratio per g fish tissue, **Equation 1**) by the quantity in nanograms of spiked internal standard to estimate the concentration in ppb (**Equation 2**).

$$[PFAS]_{native} = Relative\ Abundance\ (Native\ PFAS) \times Concentration\ (Internal\ Standard) \qquad (2)$$

The accuracy of these calculations was assessed by comparing PFAS concentrations calculated in the QC samples to values reported on the NIST SRM 1947 certificate. We achieved acceptable accuracy (88% to 101%) and precision (7% RSD) for PFOS in the QC samples. Semi-quantitative concentration estimates were calculated for the other detected analytes with an exact match spiked internal standard; however, the accuracy and precision for these nine analytes were either poor or could not be assessed as no reference values were available (**Table S9**).

Statistical analyses were conducted in R (v.4.2.1). Prior to analysis, PFAS relative abundances were normalized by first adding a pseudo count to ensure there were no zeros, and then applying a $\log_2$ transformation. Principal component analysis (PCA) was performed using the function 'prcomp' and results were visualized using ggplot2. Differences between sampling timepoints were assessed using multivariate analysis of variance (MANOVA) using the function 'manova'. Differences in sampling locations were visualized using a heatmap, which was constructed using the function 'pheatmap'. Relationships between fish length and PFOS concentration were assessed using regression analysis, and significance of the slope was evaluated using the t-test for regression coefficients. Hierarchical clustering was performed on samples using the Euclidean distance metric and average linkage.[22] ToxPi (v 2.3)[23] was used for visualization of relative abundance of PFAS from individual fish or average abundance from different sample groups.

RESULTS AND DISCUSSION

Suspect Screening Analyses

In July 2023, North Carolina's first fish consumption advisory was issued for the middle and lower Cape Fear River after a targeted PFAS study found elevated levels of PFOS in bluegill,

channel catfish, and several other fish species.[9] Typically, targeted studies are used for monitoring PFAS levels, generally quantifying up to a few dozen PFAS analytes with low detection limits, high accuracy, and high precision. While estimates vary of how many chemicals are currently considered PFAS, most public lists include over 5,000 molecules[24] meaning the vast majority of PFAS are not and cannot be monitored in these targeted methods. Thus, non-targeted analysis is a valuable complementary approach as it is not limited to a few dozen different analytes and can inform researchers about what other PFAS may be of concern in the matrix of interest.[25, 26] In this study, we performed a non-targeted analysis of PFAS in 47 individual fillets from 7 species of fish caught at 5 locations, including 3 in the Haw River (HR1, HR2, and HR3), which is downstream from a wastewater treatment plant (a known point source of PFAS), and 2 sites in Jordan Lake (JL1 and JL2), which is in the same watershed but has lower known PFAS water concentrations, to understand PFAS presence in the ecosystem and assess potential human exposure from fish consumption.

Initially, we analyzed fillets from all fish using LC-IMS-MS and then screened all detected features against our library of 100 PFAS. This suspect screening evaluation identified 22 PFAS analytes in the 47 fish fillets, in addition to branched isomers of 5 PFSAs[1] (**Figure 2**, **Table S8**).The maximum number of PFAS analytes detected in one fish was 17, while the minimum was 6. Additionally, 5 of the analytes were detected in every fish (10-12 carbon PFCA and linear and branched 8 and 10 carbon PFSAs) (**Table S10**). Because we cannot confidently identify which specific branched isomers are present in these instances[11], the following discussion counts linear and branched isomers as one analyte. Of the 22 PFAS analytes detected in this study, 10 had an identical heavy labeled internal standard spiked into the samples. Thus, based on the Schymanski scale[27], this allowed Level 1 identifications for these analytes. The remaining analytes were

considered Level 2a identifications due to library matching of retention time, *m/z*, and collision cross section, but absence of a matching internal standard.

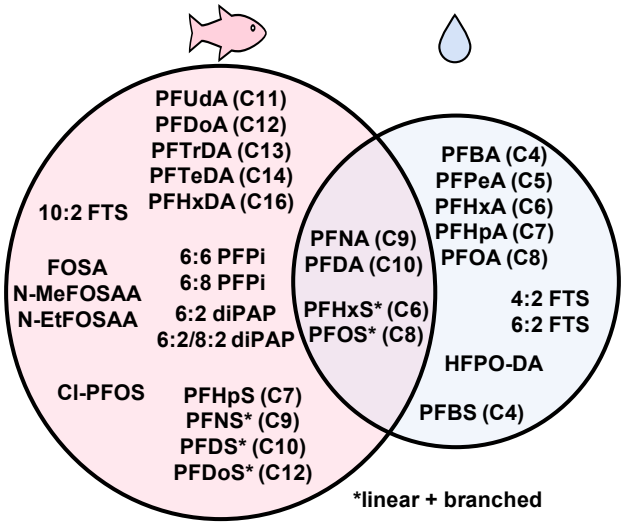

Figure 2. Venn diagram showing the PFAS detected in fish and those targeted and detected in water (results published by Pétré et al., with the number of carbons in the alkyl chain given in parentheses for the PFCAs and PFSAs. Fish and water samples were collected from the same sites at the same time; however, samples were analyzed on different platforms.

In a separate study of PFAS in water from the same sampling sites and dates as our fish study, Pétré et al. targeted and detected 13 different PFAS in their analysis of the Haw River and Jordan Lake.[10] Combining the water and fish fillet results, a total of 31 different PFAS analytes were detected. Of these 31 PFAS, only four were detected in both fish and water (**Figure 2**). These four analytes are all long-chain PFAS[1, 28] which are known to bioaccumulate in tissue.[3, 4, 29] While our PFAS library does contain the other nine PFAS that were detected in the targeted water study, we did not observe them in the fillets. These analytes were all shorter chain PFAS that are known to have shorter biological half-lives, so it is possible that these analytes had not accumulated to detectable levels in the fillets. On the other hand, the water study did not target the other 18 analytes detected in the fish tissue. A key limitation of this comparison is that water and fish analyses were

conducted using separate targeted and non-targeted approaches. The longer chain PFAS most frequently detected in the fish samples were not targeted in the water analysis of the Haw River and Jordan Lake, so it is unknown whether they would have been detectable in the water. Despite these limitations, our study provides evidence that water PFAS levels do not accurately describe fish PFAS accumulation, which is also supported by other researchers.[4, 30, 31]

Spatial PFAS Trends in *Lepomis* Sunfish

To investigate spatial trends of PFAS accumulation in fish fillets for the 5 sites, we evaluated PFAS detection frequency as well as relative abundance (Equation 1) in individual fish samples for the 4 species of *Lepomis* sunfish (n = 38).[32] The other 3 species in our sample set were collected primarily from the JL2 site and will be discussed in the next section. While the sunfish were caught over four months in the summer of 2020, statistical analysis by MANOVA of relative abundances of the 7 PFAS detected in 90% or more of the individual sunfish samples showed no sampling date differences (p-value = 0.249, **Figure S2**). This result was not surprising because these analytes are primarily long chain PFAS known to have long biological half-lives. Therefore, subsequent analysis combined all sunfish from each site regardless of the sampling date. Next, we performed a PCA and a heat map evaluation, which showed that the Haw River samples separated from the Jordan Lake samples, but the individual sites did not separate distinctly from each other (*e.g.*, HR1, HR2, and HR3 clustered together, and JL1 and JL2 clustered together) (**Figures S3 and S4**).

To investigate the drivers of these differences, we first compared detection frequency of each PFAS in individual sunfish from each of the 5 sites. Specifically, the HR1 sunfish had more PFAS analytes detected than the JL1 and JL2 sites (**Figure 3A**) with a median of 13 PFAS analytes

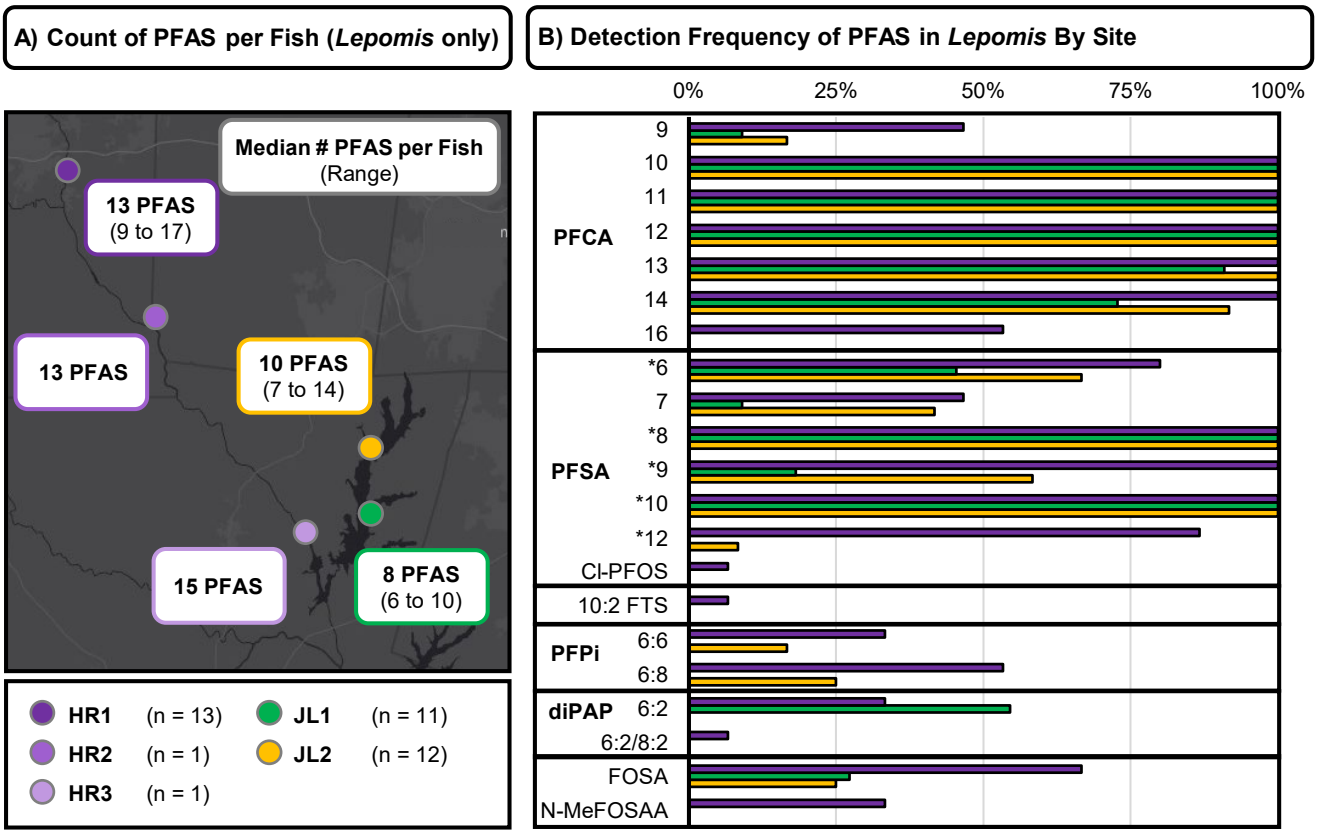

Figure 3. Proportion of *Lepomis* sunfish fillet samples with PFAS detected at each sampling location. **A)** Sunfish (genus *Lepomis*) were caught at five sampling sites between May and August 2020 and analyzed for PFAS by LC-IMS-MS. The number of sunfish from each site is given by n. The median number of PFAS detected in sunfish from each site is shown in bold, with the minimum and maximum shown below. **B)** The proportion of sunfish with each analyte detected was calculated by counting the number of sunfish with the analyte observed and dividing by the total number of sunfish from that site. Because only one *Lepomis* sunfish was captured from HR2 and HR3, all 15 HR fish were combined for reporting the detection frequencies.

at HR1 (range: 9 to 17 in individual fillets, 21 different analytes in total), compared to a median of 8 at JL1 (range: 6 to 10, 13 total) and 10 at JL2 (range: 7 to 14, 17 total) (**Table S10**). Interestingly, seven analytes (the 10 through 14-carbon PFCAs and the 8 and 10-carbon PFSAs) were detected in 90% or more of the fillets regardless of location. Three analytes reported here (Cl-PFOS, 10:2 FTS, and 6:2/8:2 diPAP) were detected in only one fillet, but we opted to include those annotations given our high confidence in these annotations and relatively small sample set. Cl-PFOS and 10:2 FTS were detected in the same fish, a redbreast sunfish from HR1, that had the highest number of PFAS detected out of all the sunfish sampled (17 analytes). 6:2/8:2 diPAP was detected in another redbreast sunfish from HR1, which also had 6:2 diPAP present. The diPAP class of PFAS have been found extensively in toilet paper, and given that the HR1 site is directly

downstream of a wastewater treatment plant, it has a known potential source of this molecule.[33] Additionally, the HR1 fillets had equivalent or greater detection frequencies for nearly every analyte compared to the JL1 and JL2 sunfish (**Figure 3B**). For example, perfluorononanoate sulfonic acid (PFNS, the perfluoroalkyl sulfonic acid with 9 carbons) was observed in 100% of samples from HR1, 18% of JL1 samples, and 58% for JL2.

The relative abundances of PFAS in the sunfish fillets (**Equation 1, Table S11**) were then compared using ToxPi visualizations to compare PFAS analyte abundances at each site.[23] In ToxPi, each analyte is represented by a slice, and the relative abundance of each PFAS is normalized to the maximum abundance of that PFAS detected in a sample, so a full-length slice represents the maximum measured abundance of that specific analyte in the sample set. Initial relative abundance comparisons focused on the 7 PFAS detected in > 90% of the fish. The average relative abundances of each of the 7 most frequently detected PFAS were highest in the Haw River sunfish compared to the Jordan Lake sunfish, and specifically higher at JL2 compared to JL1 (**Figure 4**). While we expected to see higher PFAS levels in the Haw River fish because the water PFAS levels were higher at that site, the Jordan Lake result was unexpected as the corresponding water data for Jordan Lake indicated slightly higher concentrations on average at JL1 versus JL2[10]. One possible explanation for this discrepancy is that most of the long chain PFAS detected most frequently in the fish were not targeted in the water analysis. For example, the 12- and 14-carbon PFCAs were detected in 100% of fish samples but were not targeted in the water. Additionally, several distinguishing features were noted between the JL1 and JL2 sites that may contribute to the different PFAS profiles observed in the fish. For instance, a residential wastewater treatment plant discharges into Morgan Creek, which flows into Jordan Lake near the JL2 site and has been identified as a presumptive source of PFAS contamination for the watershed by Northeastern's

PFAS Project Lab.[34, 35] A wastewater reclamation facility also discharges into New Hope Creek, which flows into the northern portion of Jordan Lake (closer to JL2) and has also been identified as a presumptive PFAS contamination source.[35] Furthermore, this comparison was focused on *Lepomis* spp. fillets because we did not have samples of other fish species from the JL1 or HR1 sites. Thus, it is possible that evaluating different species and different tissue types would show different trends. Additional studies are therefore needed to determine the sources of PFAS into Jordan Lake.

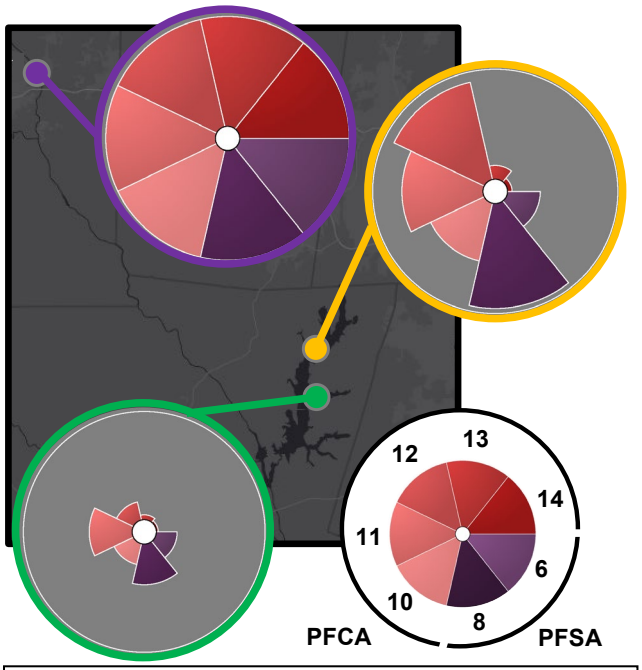

Figure 4. Average relative abundance of the 7 most frequently detected PFAS in *Lepomis* fillets from 3 sites. The ToxPi graphs show the each PFAS analyte as a different slice. A full-length slice indicates the maximum relative abundance of that analyte at any site. Here the HR1 site is shown with a purple outline, while JL1 is green and JL2 yellow.

Comparison of PFAS in Different Species

While most of our fillet samples were *Lepomis* spp. sunfish, three other fish species were also evaluated. From JL2 these included 4 *Perca flavescens* yellow perch, 2 *Morone americana* white perch, and 2 *Ictalurus punctatus* catfish, and from HR2, 1 *Ictalurus punctatus* catfish. When

assessing only the JL2 fish, PCA of the 21 PFAS detected in these 20 fish fillets (also including the sunfish) showed separation by genus (**Figure S5**), indicating there are species differences in PFAS accumulation. To investigate this observation, we used ToxPi visualizations to compare differences in average relative PFAS abundance for each species and sampling site (**Figure 5**). At the JL2 site, we observed similar relative abundance trends for PFCAs and PFSAs in *Lepomis* spp. sunfish, *Morone americana*, and *Perca flavescens* as illustrated by the similar length slices in the ToxPis. The *Ictalurus punctatus* channel catfish, however, had the lowest relative abundance of all PFAS detected in any species and site, as illustrated by the barely visible slices in the ToxPi. In contrast with that finding, the one channel catfish fillet sample from HR2 had the highest relative abundances for almost all PFAS detected compared to all other species and locations as illustrated by the full-length slices for all the PFCAs, several PFSAs, and FOSAs. Additionally, the HR2 catfish contained the second-highest number of PFAS analytes detected (20 different PFAS analytes) and was the only fish fillet in which N-EtFOSAA was detected. While our sample size of channel catfish is limited, these findings indicate that species differences may be location-specific and emphasize the importance of thorough sampling of multiple species and locations.

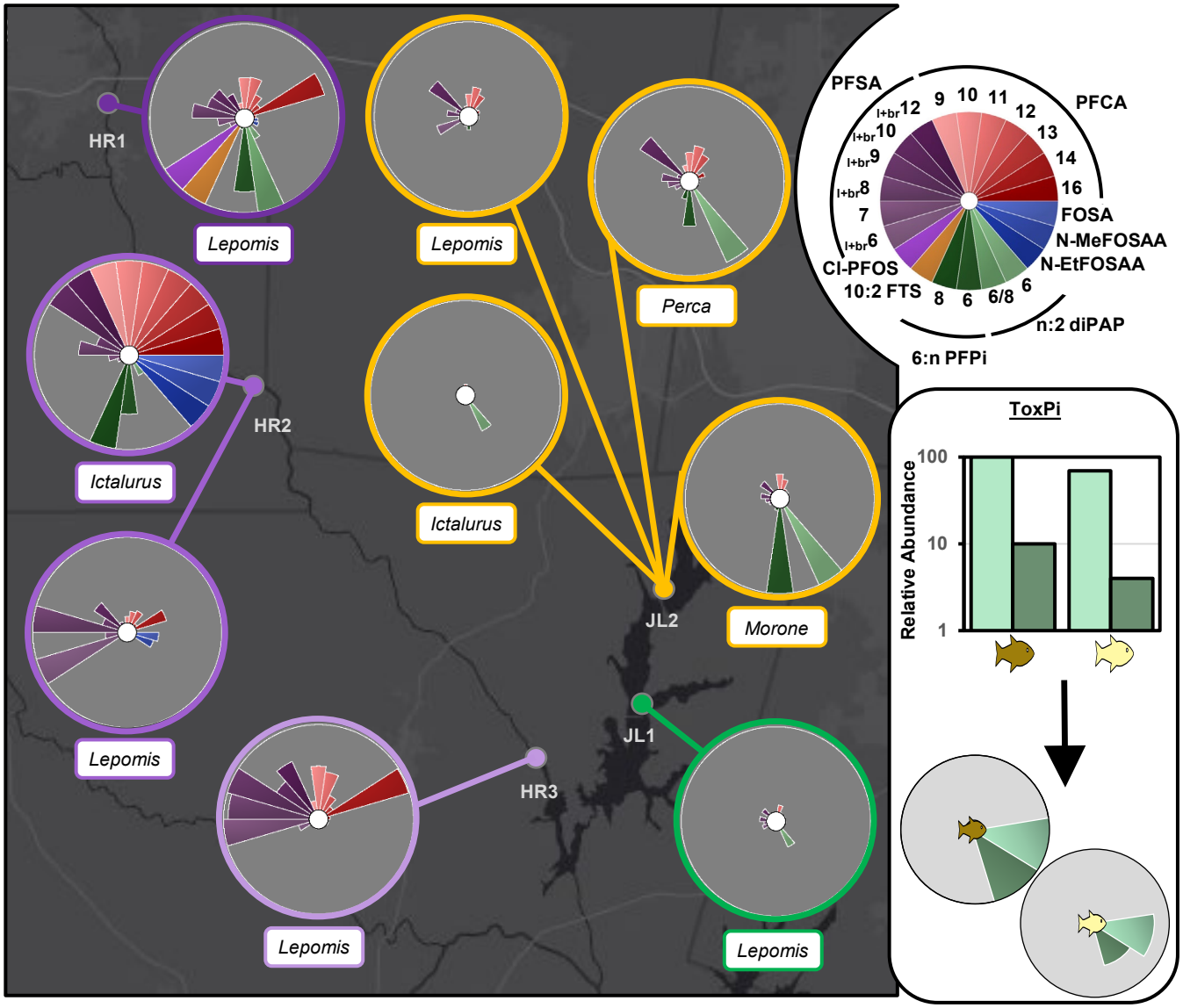

Figure 5. Average relative abundance of the 22 PFAS detected fish fillet at 5 sampling locations visualized using ToxPi. The 4 fish genera included 4 species of *Lepomis* (common sunfish), *Morone americana* (white perch), *Perca flavescens* (yellow perch), and *Ictalurus punctatus* (channel catfish). The ToxPi graphs show the each PFAS analyte as a different slice. Relative abundances of each analyte are normalized to the maximum abundance across the sample set. A full-length slice indicates the maximum relative abundance.

PFOS Concentrations

The purpose of this non-targeted study was to screen for a wider range of PFAS analytes than possible in targeted studies. While non-targeted analyses are typically qualitative, we used a simultaneous discovery and targeted monitoring technique[36] to calculate PFOS concentrations in the fillets using a semi-quantitative approach (**Equation 2**), where the ratio of the heavy spiked

PFAS was utilized to determine the endogenous concentrations. In contrast to semi-quantitation, absolute quantitation is typically performed using targeted methods and requires an external calibration curve for each PFAS measured to ensure accurate measurement of a limited number of PFAS at a time. In non-targeted analysis, it is often not practical or possible to create appropriately matrix-matched calibration curves required for absolute quantitation because presence of analytes in environmental samples is not known in advance. Thus, the semi-quantitation approach using single-point calibration of the spiked internal standard (**Equation 2**) is an efficient method for estimating concentrations of certain PFAS analytes without the need for external calibration curves. When applied to this study, suspect screening results showed that 10 PFAS analytes detected in the fish tissue had an identical internal standard spiked and were candidates for semi-quantitation. Of these 10, we achieved good accuracy and precision for PFOS; however, we could not achieve acceptable accuracy or validate our calculations for the other 9 analytes due to low concentrations. Thus, semi-quantitation results for those 9 are reported in the supplemental information (**Table S12, Table S13, Figure S8**).

Semi-quantitative PFOS levels ranged from 3.8 ppb to 140 ppb for individual fish fillets in the study (**Table S12, Table S13, Figure S6**). Looking at spatial differences between the sunfish, the HR1 sunfish fillets had higher PFOS concentrations (median 54 ppb) than Jordan Lake sunfish (median of 14 ppb at JL1 and 20 ppb at JL2). The differences between the HR samples and the JL samples were statistically significant by one-way ANOVA comparisons; however, the JL1 and JL2 sites were not significantly different from each other. Although the sample size for the non-sunfish species was not large enough for statistical comparisons, the PFOS concentration trends generally aligned with the observed relative abundance trends for all PFAS (**Figure 5**). At the JL2 site, the catfish had lower PFOS levels than the sunfish (median 4.6 ppb in the catfish), while the

yellow perch (median 29 ppb) and white perch (median 18 ppb) had similar PFOS concentrations as the sunfish.

Three covariates, sex, water PFOS concentrations, and fish length, were next evaluated using the *Lepomis* spp. sunfish subset of samples. First, there was not a statistically significant difference in PFOS concentrations in females compared to males at any site individually or combining all sites (**Figure S7**). Next, comparing PFOS concentrations in fish fillet to those reported in water, the average PFOS concentrations in fish were positively correlated with PFOS concentrations in water (**Figure 6A**). Average PFOS concentrations were higher at the HR1 site for both water and fish compared to the Jordan Lake sites, suggesting that when PFOS levels are high in the water they may also be elevated in fish. Finally, regression analysis of PFOS concentration versus sunfish length indicated a positive correlation between fish length and PFOS concentration at JL1 and JL2, but not at HR1 (**Figure 6B**). Because fish length is related to age within a population[37], we expected to see a positive correlation between age and PFOS levels due to the long biological half-life of PFOS in muscle tissue. However, numerous factors unrelated to PFOS exposure, such as pharmacokinetic changes from other chemicals present at HR1 and differences in the riverine compared to lake ecosystems, could explain this lack of relationship observed in the Haw River fish.[38] Additional studies would be needed to investigate this observation further. Importantly, these results indicate that consuming larger sunfish is likely to result in greater PFOS exposure, and at sites with high water PFOS levels, such as HR1, smaller fish may not necessarily have lower levels of PFOS than larger fish. In future studies, both water and fish fillet should be collected and analyzed on the same instrument to better understand these trends.

To our knowledge, no prior PFOS levels have been reported in fish from our precise study locations. A recently-published EPA study of 44 species of freshwater fish sampled between 2013

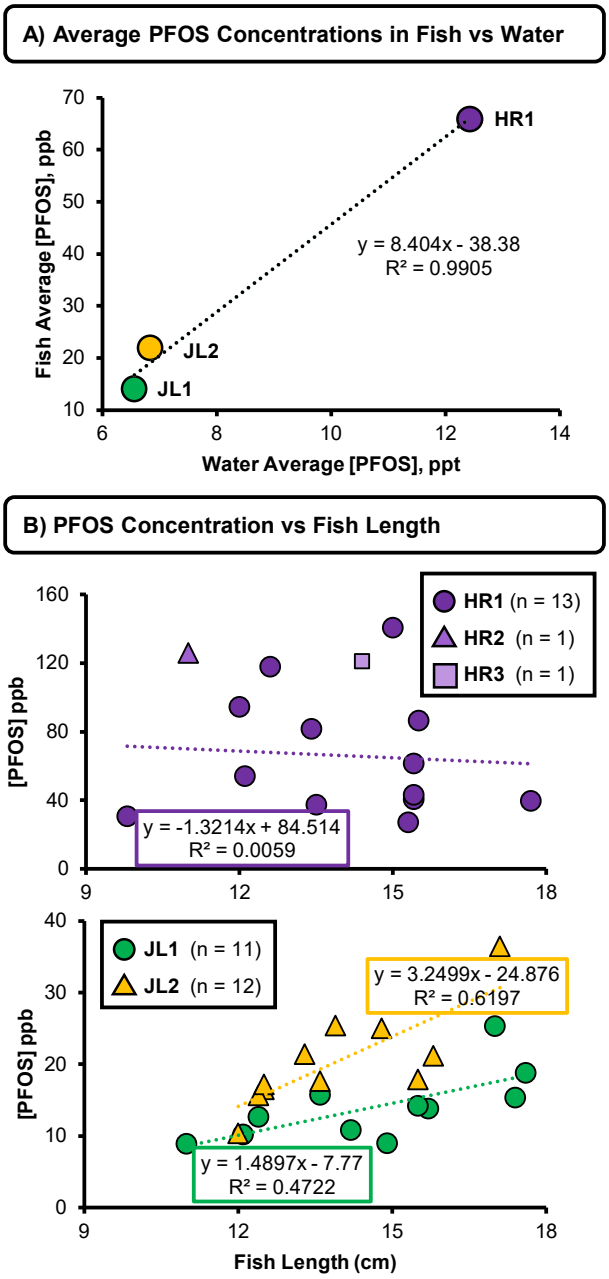

Figure 6. A) Average PFOS concentration in *Lepomis* spp. sunfish fillet versus water at each site. Fish PFOS concentrations from each site were averaged. Water PFOS concentrations from weekly sampling were provided in the supplemental information of Petre et al.'s 2022 study and averages were calculated for each site over the date range of June 2019 through April 2020. B) Regression analysis of Lepomis spp. fillet PFOS concentration versus fish length. Using a t-test for regression coefficients, a significant relationship was observed at JL1 ($p = 0.02$) and JL2 ($p = 0.004$) but not at HR1 ($p = 0.8$). No regression could be performed at HR2 and HR3 due to an n of 1 at those sites.

and 2015[8] reported that the median PFOS level in fish from rivers and streams across the U.S. was 6.60 ppb, with a higher median value of 12.35 ppb in fish from the Great Lakes. The median PFOS concentrations we report here are higher than reported by the EPA study in all cases with the exception of the JL2 catfish. In a 2007 study of North Carolina fish, Delinsky et al. reported a median concentration of 30.3 ppb PFOS (range: 15.9 to 47.5 ppb) in bluegill sunfish collected from the Haw River, south of the inflow to Jordan Lake.[39] The median PFOS concentrations we report in fish from the upper Haw River are higher than those reported downstream in 2007, while the Jordan Lake fish had generally lower median PFOS concentrations but within the observed range from this area in 2007. More recently, the North Carolina Department of Health and Human Services reported average PFOS concentrations of 23.0 ppb in bluegill, 20.4 ppt in redear sunfish, and 2.7 ppb in channel catfish from the middle and lower Cape Fear River in the summer of 2022. Compared to our results from summer 2020, the Cape Fear River fish had lower PFOS concentrations than the Haw River fish, while the PFOS concentrations in the Jordan Lake sunfish and channel catfish were similar to those reported in the Cape Fear River fish. We hope these results encourage continued testing efforts for PFAS in fish from all waters of North Carolina and support the importance of wide geographical testing for PFAS in fish outside of North Carolina as well.

Implications

In this study, non-targeted analysis was performed for fish from the Haw River and Jordan Lake in North Carolina. We identified 22 different PFAS in fish fillets, including only 4 of the 13 PFAS previously identified in water samples collected at the same location and time. These results illustrate that targeted water analyses alone are not an accurate proxy for PFAS levels in fish. We

found that fish at different locations within 30 miles in the same watershed accumulated different types and various levels of PFAS. However, regardless of location, long chain PFCAs and PFSAs were detected in the fillets of every fish sampled. Fillet PFOS concentrations as high as 140 ppb were observed in the Haw River fish and 38 ppb in Jordan Lake fish. These levels are high enough to be concerning and warrant continued monitoring of PFAS. Future targeted studies of fish in this region of North Carolina should consider quantifying these 22 analytes in addition to others of concern to inform fish consumers and regulatory bodies about which types of PFAS are present in local fish. Studies of PFAS in fish in other regions should also consider adopting non-targeted analyses in addition to targeted monitoring to inform meaningful prioritization and risk assessment. In conclusion, our results emphasize the need for thorough sampling of multiple matrices to understand PFAS presence in an ecosystem and highlight the utility of non-targeted analysis worldwide as a complementary technique to inform targeted approaches.

ASSOCIATED CONTENT

Supporting Information

Individual fish collection data (Table S1); Summary of fish genera by site (Table S2); Consumables used in sample preparation (Table S3); Process efficiencies (Figure S1); Consumables used in LC-IMS-MS analysis (Table S4); LC gradient settings (Table S5); ESI source settings (Table S6); IMS-MS settings (Table S7); All PFAS detected and internal standards (Table S8); Accuracy and precision (Table S9); PCA of sampling date (Figure S2); PCA of sampling location (Figure S3); Averages and ranges of PFAS detections (Table S10); Heat map (Figure S4); PCA by genus (Figure S5); Box plot of PFOS by genus and location (Figure S6); Box plot of PFOS by sex (Figure S7); Proportion of ΣPFAS (Figure S8) (PDF)

Relative abundances of all PFAS detected (Table S11); Concentrations of 10 PFAS (Table S12); Summary of semi-quantitation (Table S13) (XLSX)

Panorama

Skyline files with all LC-IMS-MS data are available at:

https://panoramaweb.org/2023-NC-Fish-PFAS-NTA.url

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The authors declare no competing interests.

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