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. Fish consumption has been identified as a key route of PFAS exposure for humans. However, routine fish monitoring targets only a handful of PFAS, and non-targeted analyses have largely only evaluated fish from heavily PFAS-impacted waters. Here, we evaluated PFAS in fish fillets from recreational and drinking water sources in central North Carolina to assess whether PFAS are present in these fillets that would not be detected by conventional targeted methods. We used liquid chromatography, ion mobility spectrometry, and mass spectrometry (LC-IMS-MS) to collect full scan feature data, performed suspect screening using an in-house library of 100 PFAS for high confidence feature identification, searched for additional PFAS features using non-targeted data analyses, and quantified perfluorooctane sulfonic acid (PFOS) in the fillet samples. A total of 36 PFAS were detected in the fish fillets, including 19 that would not be detected using common targeted methods, with a minimum of 6 and a maximum of 22 in individual fish. Median fillet PFOS levels were concerningly high at 11.6 to 42.3 ppb, and no significant correlation between PFOS levels and number of PFAS per fish was observed. Future PFAS monitoring in this region should target more of these 36 PFAS, and other regions not considered heavily PFAS contaminated should consider incorporating non-targeted analyses into ongoing fish monitoring studies.

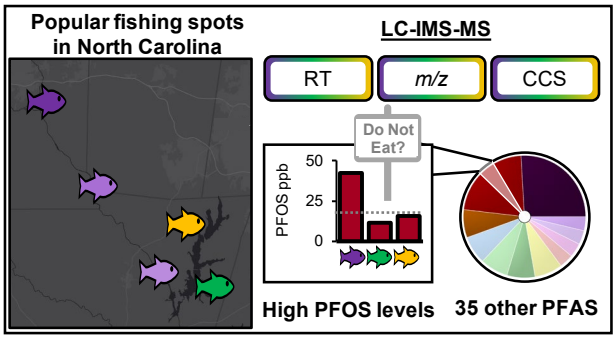
KEYWORDS

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

SYNOPSIS

Concerningly high PFOS levels were detected in fish fillets from recreational and drinking water sources in central North Carolina. Of the 36 total PFAS identified, 19 would not be detected using routine targeted monitoring methods.

GRAPHICAL ABSTRACT



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40 **INTRODUCTION**

41 Per- and polyfluoroalkyl substances (PFAS) are a class of fluorinated anthropogenic
42 chemicals that have been detected in water, soil, and biological tissue worldwide, and PFAS
43 exposure is associated with a suite of adverse health effects. Several recent reviews have detailed
44 how PFAS are manufactured and used,[3, 4] PFAS global spread and exposure,[5, 6] and
45 bioaccumulation and toxicity[7, 8] People are primarily exposed to PFAS through drinking water and
46 food, especially fish consumption.[1, 9] U.S. federal level regulations on safe PFAS levels in drinking
47 water were enacted for the first time in April 2024.[10] No federal limits for fish are in place, though
48 several states have implemented their own standards.[1, 11] Fish consumption advisories are set by
49 comparing measured PFAS concentrations to a reference dose (a daily amount expected to have
50 no adverse effects) to determine how much fish is safe to eat.[2, 12-14] Establishing reference doses
51 requires toxicity testing to determine what amount of a chemical is safe. These studies typically
52 use validated liquid chromatography separations coupled with targeted mass spectrometry (LC-
53 MS) methods to quantify between 12 and 25 different PFAS, typically including perfluoroalkyl
54 carboxylic acids (PFCAs), perfluorosulfonic acids (PFSAs) including PFOS, and occasionally
55 other types of PFAS.[15] However, with over 14,000 known PFAS structures and counting,[16]
56 alternative analytical techniques are essential to understand the full extent of PFAS present in a
57 sample.[15, 17-20]

58 To increase PFAS coverage in fish tissue analyses, several techniques beyond targeted LC-
59 MS have been used. One option is the total oxidizable precursor assay, which is used in conjunction
60 with targeted MS to assess the presence of precursor PFAS such as fluorotelomers (FTs) and
61 perfluoroalkyl sulfonamides (PFASAs). Pickard et al. quantified two precursor PFAS in fish fillets
62 using this technique, and noted challenges in quantification when precursors were present at low

levels.[21] Another option is non-targeted analyses (NTA) using high-resolution mass spectrometry (HRMS). Rather than pre-selecting specific PFAS to target, NTA techniques rely on accurate mass measurements and other characteristics of PFAS such as fragments, CF_2 homologous series, and mass defect to filter features and annotate molecules.[15, 22, 23] NTA may compare feature lists to in-house or publicly-available databases, or propose novel molecule structures when no suitable database match is found. For example, Ren et al. used suspect screening and NTA to detect 15 known and an additional 400 unknown PFAS in freshwater organisms.[24] Confident annotations of unknowns is a key challenge with any NTA, as Level 1 confidence annotations require matching to a standard.[25, 26] Ren et al. noted a challenge with low intensity ions that often had no observable MS/MS fragment ions, and thus the unknown PFAS were identified to a Level 4 confidence. Ion mobility spectrometry (IMS) has been suggested as a promising technology to increase annotation confidence[26, 27] and several studies have demonstrated its utility for PFAS NTA of environmental samples.[28-31] Another drawback to NTA using HRMS is that these analyses are often qualitative, as it is not possible to create calibration curves for molecules with no existing standards. This can be mitigated by performing a targeted analysis on the sample to quantitate a limited number of PFAS, either on a separate instrument (such as a low resolution mass spectrometer) or concurrently with the high resolution data collection.[15, 32] However, sample preparation methods that have been validated for use in targeted analyses may not always be optimal for NTA, which favor minimal sample handling to minimize loss of low-abundance analytes. Further, if sample quantities are limited, it may not always be possible to perform both types of analysis.

Fish NTA studies have largely focused on identifying novel PFAS in fish from waters that are highly PFAS contaminated. Little non-targeted effort has been applied in areas considered to have low PFAS levels, despite evidence that water PFAS levels may not be indicative of fish PFAS

accumulation.[21] Routine monitoring of PFAS in fish tissue is not yet commonplace in the U.S., though some states have adopted monitoring programs which test fish tissue using targeted methods to inform consumption advisories; however, the advisories generally only include PFOS.[1] Therefore, a knowledge gap exists in evaluating whether PFAS that are not commonly targeted are present in fish tissue. Here, 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**). Three sites were in the Haw River, a historically polluted water body under scrutiny for high levels of PFAS contamination.[33] Two sites were in Jordan Lake, a popular recreational state park and the drinking water source for over 700,000 nearby residents. Water samples simultaneously collected from these locations contained detectable levels of 13 different PFAS, with average PFCA and PFSA concentrations as high or higher than those measured in the Cape Fear River.[34] However, as of April 2024, no fish consumption advisories for PFAS exist in either the Haw River (HR) or Jordan Lake (JL).[2] In this study, we analyzed fish fillets on an LC-IMS-HRMS platform and applied target and NTA workflows with the following aims: 1) identify as many PFAS as possible at a high level of confidence; 2) evaluate whether spatial differences in PFAS fingerprints exist between the study sites; and 3) report PFOS concentrations in the fillets to determine whether PFOS levels are a reliable indicator of overall PFAS levels in the fish. Our intent with this non-targeted study was to explore the utility of NTA in fish samples from a lake that is not considered heavily PFAS-impacted, and to inform regulatory testers which PFAS should be targeted in subsequent monitoring efforts in this region.

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 and two sites in Jordan Lake (**Table 1, Figure 1**).

Table 1. Sampling sites and number of fish from each site.

Site code	Site name	n (*Lepomis* spp.)	n (other species)
HR1	East Burlington	13	0
HR2	Saxapahaw	1	1
HR3	Haw Below 64	1	0
JL1	Bell's Landing	11	0
JL2	Farrington Point	12	9

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[41] 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.[34] Fish species sampled are detailed in **Table ST1**, and included 38 *Lepomis* spp. sunfish, two white perch (*Morone americana*), four yellow perch (*Perca flavescens*), and four 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

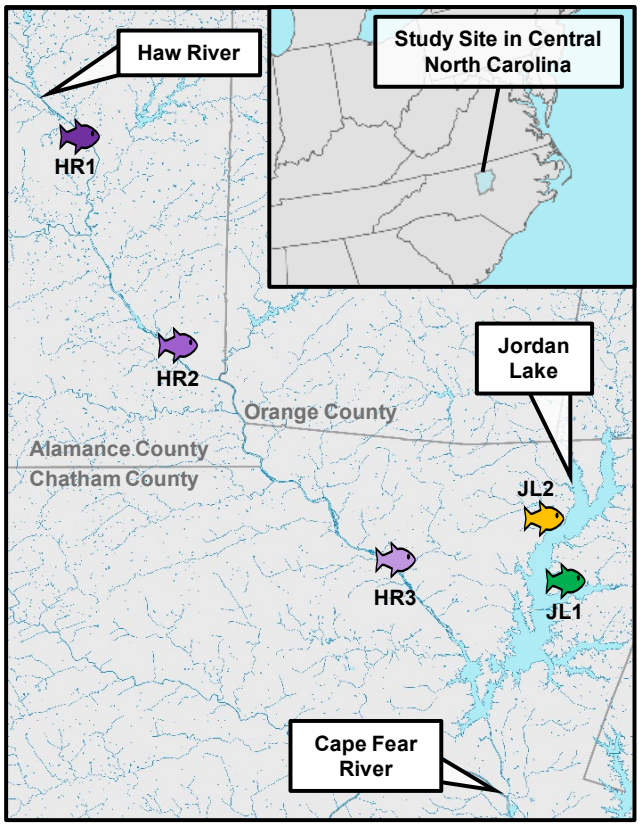

Figure 1. Map of study site showing fish sampling locations. Fish icons show the approximate location of the study site, which spanned three NC counties (Alamance, Orange, and Chatham). Fish were sampled from three sites along the Haw River and two sites within Jordan Lake between May and August 2020.

as internal standard. An unlabeled standard mix (PFAC-MXH) from Wellington and farm-raised tilapia fillet purchased from a local grocery store was used for a matrix-matched calibration curve. 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 (Gaithersburg, MD) and used for quality control.

Sample Preparation

In brief, fish fillet samples (0.1 to 1 g wet weight), quality control samples (0.5 g SRM1947), and method blanks (0.5 mL water) were homogenized, spiked with internal standards, and extracted in methanol (5 mL). Clean up of the supernatant included filtration through 0.2 µm

137 nylon syringe filters, dispersive carbon treatment, and centrifugation. The extract was concentrated
138 to dryness in the speedvac and reconstituted in 200 µL of 40% methanol, 60% water by volume
139 buffered with 3 mM ammonium acetate.[42] Recoveries for 24 PFAS were calculated (**Table ST2**)
140 and were overall quite good, with averages by structural class of 71% for PFCAs, 90% for PFSAs,
141 91% for FTs, and 70% for PFASAs. Poor recovery was noted for HFPO-DA (24%), N-MeFOSA
142 and N-EtFOSA (4%), and N-MeFOSE and N-EtFOSE (0%); difficult recovery of these 5 analytes
143 have been noted elsewhere.[43, 44] Detailed sample preparation steps and method characterization
144 including recovery calculations are included in the **Supplemental Methods**.
145
146 **LC-IMS-MS Data Collection**
147 Non-targeted sample analysis was performed on an Agilent 6560 IM-QTOF coupled with
148 an Agilent 1290 Infinity UPLC system with consumables and parameters previously described[28]
149 and detailed in **Tables S1-S4**. In brief, samples were injected in randomized order with a double
150 blank and a QC sample injected between every 10 samples to monitor column carryover and
151 instrument performance. The LC separations were carried out on a C18 column in line with a guard
152 column. Mobile phases included: A) water with 5 mM ammonium acetate, and B) 95%
153 methanol:5% water with 5 mM ammonium acetate. Samples were injected at a volume of 2 µl and
154 a gradient was applied at a flow rate of 0.4 mL per min. An Agilent JetStream electrospray
155 ionization source operated in negative ionization mode was utilized to ionize the samples.
156 Instrument mass calibration was performed by directly injecting Agilent ESI tune mix solution,
157 and tune mix ions were used to convert measured drift times to collision cross section (CCS) values
158 using a single-field calibration.[28] Ion packets were pulsed through the drift tube using 4-bit
159 multiplexing[47] and full-scan data was collected in Agilent .d files. Data files were demultiplexed

using the PNNL Preprocessor (v 4.0)[48] with the signal intensity threshold set to 20 counts and 100% pulse coverage and then Agilent IM-MS Browser (v 10.0) was used for single-field CCS calibration.

Target Screening and Relative Abundance Comparisons

Skyline-daily (v 22.2.1.501) was used for target screening. The target screening workflow uses drift time and retention time filtering against our in-house library of 100 PFAS analytes previously compiled with reference standards.[29] Identifications required 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 with an exactly matching internal standard were identified at a Level 1a confidence, and all other analytes present in our library were assigned Level 1b.[26, 27]

Following PFAS identification in Skyline, the peak areas of detected PFAS analytes were exported into Excel for further analysis. For analytes detected in method blanks (PFHpA, PFOA, PFOS, and 6:2 diPAP), reporting limits were defined as the average plus 3 standard deviations of the peak area 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; surrogate assignments are shown in **Table ST3**. 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**).

183 $$Relative\ Abundance = \frac{Peak\ Area\ (Native\ PFAS)}{Peak\ Area\ (Internal\ Standard)} \div Tissue\ wet\ weight\ (g) \quad (1)$$

184

185 **PFOS Quantitation**

186 PFOS concentrations were calculated using a matrix-matched nine-point calibration curve
187 to correlate light-to-heavy peak area ratios to PFOS concentrations in the sample extracts. The
188 LOD (defined as the average of the blanks plus 3 standard deviations) was 0.0352 ng/ml in the
189 sample extracts (0.388 ng/g in tissue). Three replicates of NIST SRM 1947 were extracted and
190 injected in triplicate to verify calibration curve accuracy against published reference values for
191 PFOS. The average accuracy was 101% with a 10.2% standard deviation. Samples were originally
192 run without the calibration curve, and then later re-run with the calibration curve; unfortunately,
193 due to concerns with sample degradation during storage, only PFOS could be confidently
194 quantified. Detailed methods on the quantification and verification of data reproducibility are
195 included in the **Supplemental Methods, Figure S1,** and **Table ST4**. Although additional analytes
196 could not be reliably quantified in this study, PFOS is the most relevant PFAS to human exposure
197 via fish consumption given that state consumption advisories exist are typically limited to PFOS
198 only, and we believe reporting these results provides important context for fish PFOS
199 concentrations that have not been previously evaluated. Future NTA studies relevant to human
200 PFAS exposure should include absolute quantification of the most relevant PFAS to continue
201 increasing our understanding of human PFAS exposure routes.

202

203 **Non-Targeted Data Analysis**

204 Agilent MassProfiler (v 10.0) was used to generate a feature list for all fish samples,
205 filtering the demultiplexed data files by ion intensity (≥ 100 counts), RT tolerance (± 0.3 min), DT

206 tolerance (2.0%), mass tolerance (± 10.0 ppm + 2.0 mDa), and abundance compared to method
207 blanks (fold change ≥ 2.0). Fluoromatch-IM (v. 1.2) was then used to search the feature list for
208 additional PFAS not present in our library. Fluoromatch-IM flags CF_2(n) homologous series and
209 allows the user to filter the data based on other PFAS characteristics such as mass defect, negative
210 Kendrick mass defect (KMD), and drift time and retention time ordering. Features determined to
211 be possible PFAS were added to the Skyline document for visualization and manual verification.[49]
212 Additionally, using results from the initial target screening, molecular formulas for PFAS not
213 present in our library but theoretically part of a CF2(n) homologous series with detected analytes
214 were added to the Skyline document. The CCS was measured for features detected in at least one
215 sample and annotations were assigned using the criteria described below, adapted from previously
216 published guidance on confidence reporting for PFAS (which does not include the IMS
217 dimension)[26] and for ion mobility data (which does not include PFAS characteristics like
218 homologous series and mass defect).[27] All identifications Level 3 and higher required < 10 ppm
219 *m/z* error and retention time ordering consistent with in-house library values. Level 1a and 1b
220 required matching to a reference standard as described in the target screening methods. Level 2a
221 required a CCS < 2% in comparison to external database values. Level 2b required a fit to in-house
222 CCS vs *m/z* trendlines and presence of 1 or more analyte in homologous series identified at Level
223 1 confidence. Level 3a and 3b required the same criteria as 2a and 2b, respectively, but contained
224 a structural element that could not be elucidated (e.g. branching position). Analytes annotated
225 using these non-targeted methods were added to the Skyline document for peak picking and
226 relative abundance comparisons as described above. We planned to assign level 5 to features
227 annotated using Fluoromatch-IM that did not match any database CCS and *m/z* entry; however,
228 none were ultimately found so this level was not used.

229

230 **Statistical Analysis**

231 Statistical analyses were conducted in R (v.4.2.1) and GraphPad Prism (v.10.2.2). Prior to
232 analysis, PFAS relative abundances were normalized by first adding a pseudo count to ensure there
233 were no zeros, and then applying a $\log_2$ transformation. Principal component analysis (PCA) was
234 performed using the function 'prcomp' and results were visualized using ggplot2. Differences
235 between sampling timepoints were assessed using multivariate analysis of variance (MANOVA)
236 using the function 'manova'. Differences in sampling locations were visualized using a heatmap,
237 which was constructed using the function 'pheatmap'. Hierarchical clustering was performed on
238 samples and analytes using the Euclidean distance metric and average linkage.[50] The
239 Toxicological Prioritization Index (ToxPi) tool (v 2.3)[51] was used for visualization of relative
240 abundance of PFAS from individual fish or average abundance from different sample groups.
241 Relationships between continuous variables (fish length, number of PFAS detected) and PFOS
242 concentration were assessed using regression analysis, and significance of the slope was evaluated
243 using the t-test for regression coefficients. Relationships between sex, site, and PFOS
244 concentration were assessed using a two-way ANOVA with Šídák's multiple comparisons test.
245 Relationships between site and PFOS concentration were assessed using one-way ANOVA with
246 Tukey's multiple comparisons correction.

247

248 **RESULTS AND DISCUSSION**

249 **High Confidence PFAS Identifications**

250 All 48 fillets were analyzed using LC-IMS-MS to collect full scan feature data which was
251 screened against our library of 100 PFAS, and additional PFAS were annotated based on mass

252 defect, presence in homologous series, LC retention time ordering, IMS drift time ordering, and
253 *m/z* vs CCS trend lines (**Figure S2**). In total, 36 PFAS analytes were identified at high confidence
254 in at least one fish fillet, including 27 PFAS at a Level 1a or 1b confidence, 2 PFAS at Level 2b,
255 6 PFAS at Level 3a, and 1 PFAS at Level 3b (**Tables 2, ST5, ST6**). Because the exact position of
256 branching for the PFSA-br isomers[3] could not be elucidated[28], the following discussion counts
257 linear and branched PFSA isomers as one analyte. The maximum number of PFAS analytes
258 detected in one fish was 22, while the minimum was 6. Notably, 5 analytes (PFNA, PFUdA, PFDA,
259 PFOS, and PFDS) were detected in every fish. Using Mass Profiler, 15,783 LC-IMS-MS features
260 were found. Fluoromatch-IM's algorithm was applied to the feature list (**Table ST7**) and features
261 not matching our filtering criteria for possible PFAS (negative mass defect, negative Kendrick
262 mass defect, retention time ordering, drift time ordering, presence in homologous series) were
263 removed. After removing features previously identified in our target screening workflow, 55
264 features remained, but none were annotated as PFAS. Investigation of these peaks in Skyline
265 revealed that 53 features were smearing or noise (**Figure S3**) while the remaining 2 features were
266 incorrectly ordered with others in the flagged homologous series and did not match any formula
267 listed in the CompTox Chemical Dashboard. While it is possible that deeper investigation could
268 uncover additional and potentially novel PFAS, our primary aim was to investigate what PFAS we
269 could confidently identify in these fillets. Any additional annotations using this workflow could
270 not be higher than Level 5 confidence. As NTA tools improve, this data set can be revisited and
271 mined to determine if additional PFAS can be discovered.

Table 2. All PFAS identified in at least one *Lepomis* fish fillet. PFAS are grouped by structural class. Confidence levels are based on the Schymanski scale [ref] modified by Charbonnet *et al.* [ref] for PFAS use.

Structural class	Acronym	Full name	Confidence	Total detection frequency
PFCA	PFOA [a, b]	perflurooctanoic acid	1a	8%
	PFNA [a, b]	perfluononanoic acid	1a	31%
	PFDA [a, b]	perfluorodecanoic acid	1a	100%
	PFUdA [b]	perfluoroundecanoic acid	1a	100%
	PFDoA [b]	perfluorododecanoic acid	1a	100%
	PFTrDA [b]	perfluorotridecanoic acid	1b	94%
	PFTeDA [b]	perfluorotetradecanoic acid	1a	88%
	PFPeDA	perfluoropentadecanoic acid	2b	46%
	PFHxDA	perfluorohexadecanoic acid	1b	13%
PFSA	PFHxS-br	perfluorohexanesulfonate (branched)	3a	4%
	PFHxS [a, b]	perfluorohexanesulfonate	1a	73%
	PFHpS [b]	perfluoroheptanesulfonate	1b	42%
	PFOS-br	perfluorooctanesulfonate (branched)	3a	100%
	PFOS [a, b]	perfluorooctanesulfonate	1a	100%
	PFNS-br	perfluononanesulfonate (branched)	3a	31%
	PFNS [b]	perfluononanesulfonate	1b	58%
	PFDS-br	perfluorodecanesulfonate (branched)	3a	100%
	PFDS[b]	perfluorodecanesulfonate	1b	100%
	PFUdS-br	perfluoroundecanesulfonate (branched)	3b	6%
	PFUdS	perfluoroundecanesulfonate	2b	10%
	PFDoS-br	perfluorododecanesulfonate (branched)	3a	2%
	PFDoS [b]	perfluorododecanesulfonate	1b	31%
	Cl-PFOS	chloro-perfluorooctanesulfonic acid	1b	10%
FT	8:2 FTS [b]	8:2 fluorotelomer sulfonic acid	1a	2%
	8:2 FTPA	(1H,1H,2H,2H-Heptadecafluorodec-1-yl)phosphonic acid	1b	2%
	10:2 FTS	10:2 fluorotelomer sulfonic acid	1b	2%
PFASA	FBSA	perfluoro-1-butanesulfonamide	1b	44%
	FHxSA	perfluoro-1-hexanesulfonamide	1b	4%
	FOSA [b]	perfluoro-1-octanesulfonamide	1a	48%
	N-MeFOSAA [b]	N-methylperfluoro-1-octanesulfonamideoacetic acid	1a	13%
	N-EtFOSAA [b]	N-ethylperfluoro-1-octanesulfonamideoacetic acid	1a	2%
PFP	6:6 PFPi	6:6 Perfluorophosphinic acid	1b	27%
	6:8 PFPi	6:8 Perfluorophosphinic acid	1b	35%
	6:2 diPAP	bis(1H,1H,2H,2H-perfluorooctyl)phosphate	1b	10%
	6:2/8:2 diPAP	6:2/8:2 Fluorotelomer phosphate diester	1b	4%
	8:2 diPAP	bis(1H,1H,2H,2H-perfluorodecyl)phosphate	1b	2%

[a]: previously detected in water
[b]: targeted in quantitative method EPA 1633
-br: branched isomer

279

280 PFAS have previously been reported present in Haw River and Jordan Lake water, and a
281 recent study targeted and detected 13 different PFAS in water samples taken from the exact same
282 sampling locations and time points as the fish samples.[34] Of the 13 PFAS previously reported in
283 water samples, 5 (PFOA, PFNA, PFDA, PFHxS, and PFOS) were also detected in our fish
284 samples. These 5 analytes are all long-chain PFAS[3, 52] which are known to bioaccumulate in
285 tissue.[7, 18, 53] However, 8 other PFAS (PFBA, PFPeA, PFHxA, PFHpA, PFBS, 4:2 FTS, 6:2 FTS,
286 and HFPO-DA ("GenX")) were detected in the targeted water study but not in the fillets, despite
287 all 8 of these being present in our in-house library. These analytes were all shorter chain PFAS
288 that are known to have shorter biological half-lives, so it is possible that these analytes had not
289 accumulated to detectable levels in the fillets. A key limitation of this comparison is that water and
290 fish analyses were conducted using separate targeted and non-targeted approaches. The longer
291 chain PFAS most frequently detected in the fish samples were not targeted in the water analysis
292 of the Haw River and Jordan Lake, so it is unknown whether they would have been detectable in
293 the water. Future studies aiming to understand differences in water and fish tissue PFAS presence
294 should analyze both sample types on both platforms. Despite these limitations, our study provides
295 evidence that water PFAS levels do not accurately describe fish PFAS accumulation, which is also
296 supported by other researchers.[18, 21, 54]

297

298 **Spatial PFAS Trends**

299 To evaluate differences in PFAS accumulation in fish fillets at the different sampling sites,
300 detection frequencies and relative abundances of the PFAS identified at Level 3 or higher were
301 compared. Of the fish species collected, the majority (36 samples) were *Lepomis spp.* sunfish from

302 sites HR1, JL1, and JL2. Due to the low number of samples from the other sites and species,
303 subsequent data visualizations and statistical analyses include only the PFAS detected in at least
304 one *Lepomis spp.* sample to minimize confounding effects from biological variation. Results from
305 sites HR2 and HR3 and the other fish species are included in the SI. The sunfish were caught over
306 four months in the summer of 2020. A statistical analysis by MANOVA of relative abundances of
307 the 7 PFAS detected in 90% or more of the individual sunfish samples showed no sampling date
308 differences (p-value = 0.249, **Figure S4**). This result was not surprising because these analytes are
309 primarily long chain PFAS known to have long biological half-lives. Therefore, subsequent
310 analysis combined all sunfish from each site regardless of the sampling date.

311 Unsurprisingly, the HR1 sunfish had more PFAS analytes detected than the JL1 and JL2
312 sites (**Figure 2**), with 25 PFAS detected at HR1, 15 PFAS at JL1, and 20 PFAS at JL2. While
313 some of these PFAS were detected in only 1 fillet, we have opted to include them in this discussion
314 due to the high confidence that the IMS dimension affords these identifications. The average
315 number of PFAS detected per fish followed the same spatial trend, with 15.4 PFAS per fillet on
316 average at HR1 (range: 10 to 22), 8.4 PFAS per fillet at JL1 (range: 6 to 11), and 10.3 PFAS per
317 fillet at JL2 (range: 7 to 17). Targeted methods for PFAS in fish fillet monitor for a predefined list
318 of *m/z* values, for example, EPA 1633 tests for 40 PFAS. Of the 40 PFAS on the 1633 target list,
319 17 were detected in the fish fillets. The remaining 13 PFAS identified in the fish would not be
320 detected using common targeted methods. The HR1 site is immediately downstream of a
321 wastewater treatment plant, which is a known point source of PFAS into the river, and so we
322 expected to find more PFAS using NTA at HR1 compared to JL1 or JL2. Nevertheless, PFAS
323 were detected using NTA at both JL1 and JL2. For example, 100% of sunfish fillets from HR1 and
324 17% from JL2 had detectable levels of PFPeDA. PFPeDA is a PFCA with 15 carbons which is not

325 targeted in any validated regulatory method and has rarely been reported in literature, possibly due
326 to how challenging it is to obtain a standard for this molecule. Additionally, more than 40% of
327 sunfish fillets at all 3 sites had detectable FBSA, a short chain sulfonamide PFAS which is not
328 commonly targeted in fish or water methods despite standards being available.

329 The relative abundances of PFAS in the sunfish fillets (**Equation 1, Table ST4**) were
330 compared using ToxPi to visualize PFAS analyte abundances at each site.[51] In ToxPi, each analyte
331 is represented by a slice, and the relative abundance of each PFAS is normalized to the maximum
332 abundance of that PFAS detected in a sample, so a full-length slice represents the maximum
333 measured abundance of that specific analyte in the sample set. The width of each slice is

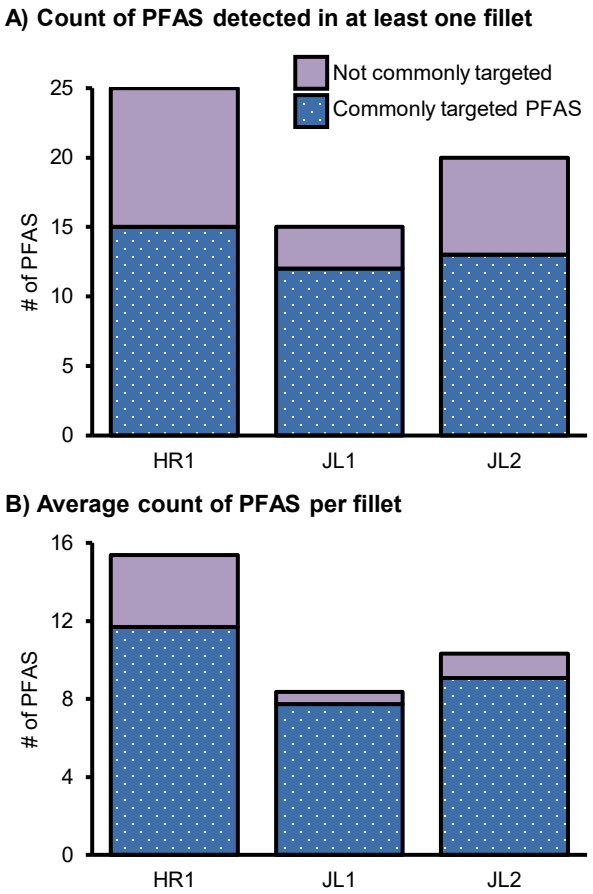

Figure 2. Number of PFAS detected **A)** in at least one *Lepomis* fish fillet and **B)** on average in fillets from the three sampling sites. The dotted dark blue bars are PFAS that are commonly targeted in validated methods such as EPA 1633. The solid light purple bars are PFAS that were uniquely detected using non-targeted analysis.

proportional to the number of analytes grouped within the slice; here, they are grouped by structural class and whether the molecule is commonly targeted. In general, sunfish from HR1 had higher relative abundances of all detected PFAS, as expected. The relative abundances of the commonly targeted PFAS were generally much lower in fillets from JL1 and JL2. Principal component analysis and heatmap clustering showed that HR fillets separated from JL fillets due to the higher abundances of the PFAS detected with > 70% frequency (the C10-C14 PFCAs and the C6, C8, and C10 PFSAs) in the HR fish (**Figure S5, S6**), though separation of the two JL sites was not observed. However, some notable observations were made in individual fillets (**Figure 3**). One fillet from JL1 had the highest relative abundance of 6:2 diPAP and 8:2 diPAP of all fish tested, despite having some of the lowest reported PFOS concentrations. Phosphate-containing PFAS are not commonly targeted despite standards being available, and one recent study suggests that their levels are grossly underestimated in most targeted groundwater studies.[20] PFUdS, the 11-carbon PFSA, is not

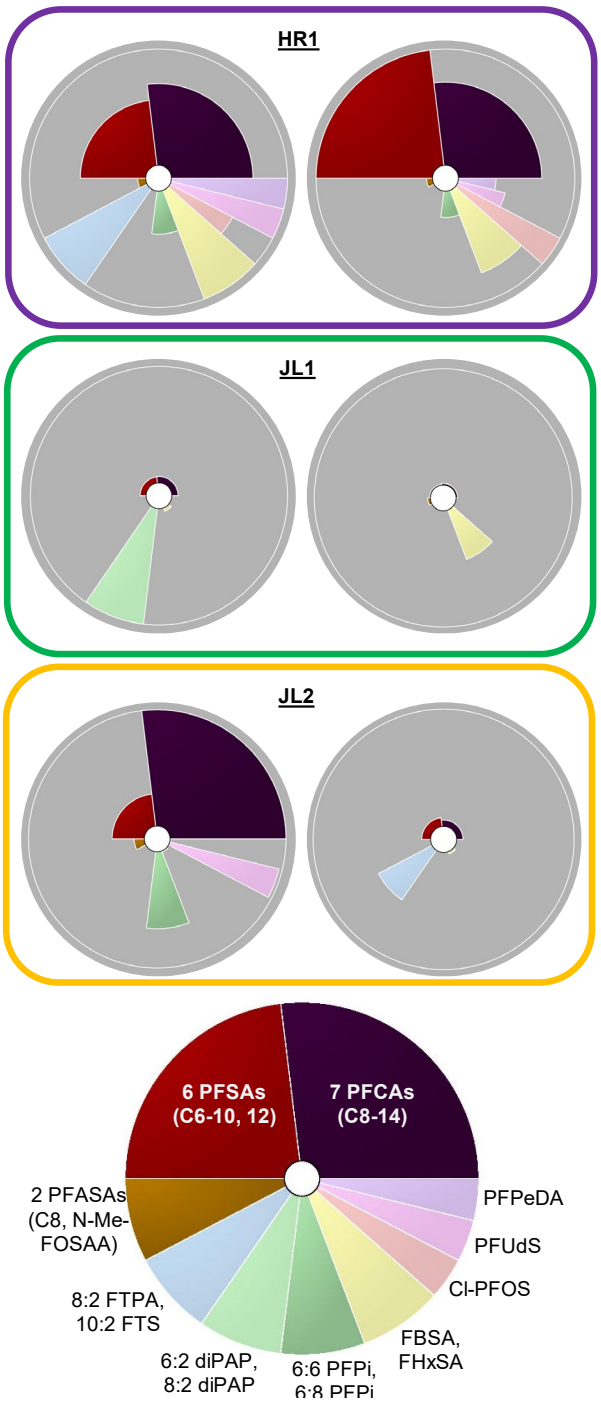

Figure 3. ToxPi visualizations showing relative PFAS abundances in two individual *Lepomis* fillets from each site. The commonly targeted PFAS are shown in dark colors while the PFAS not commonly targeted are shown in pastels. Structurally similar PFAS are combined into one slice and each slice is weighted based on the number of PFAS it represents. The length of each slice indicates the abundance of that PFAS relative to its abundance in all other samples so that a full-length slice represents the maximum abundance of that molecule.

commonly targeted and to our knowledge has not previously been reported detected in fish. Linear and branched isomers of PFUdS were detected in 23% of sunfish from HR1 as well as in one fish from JL2. This JL2 fish had a relative abundance of PFUdS nearly as high as the highest fish from HR1, despite having comparatively lower abundances of the other PFSAs. Toxicity information is not available for most of these compounds, so it is not clear how much risk to health consumption of these fish could pose.

PFOS Concentrations

PFOS concentrations in the *Lepomis spp.* sunfish fillets ranged from 7.5 to 124 ppb, with the highest at HR1 (median 42.3 ppb), followed by JL2 (15.6 ppb) and JL1 (11.6 ppb) (**Figure 4, Table ST4)**. The differences between the HR samples and the JL samples were statistically significant by one-way ANOVA comparisons using Tukey's multiple comparisons test ($F(2, 33) = 18.57$, adjusted p-value < 0.0001). The JL1 and JL2 sites were not significantly different from each other (adjusted p-value = 0.700). Sex and fish length were evaluated as potential covariates for PFOS concentrations (**Figures S7, S8**). No sex differences were observed at JL1 or JL2, and half of the fish from HR1 were not sexed; therefore, fish from each site were grouped regardless of sex. There was a positive correlation between fish length and

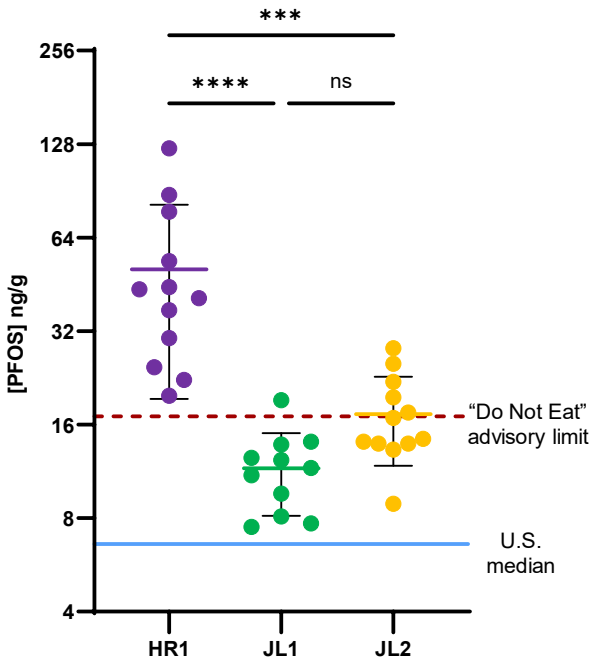

Figure 4. Concentration of PFOS measured in fillets from each site. The blue horizontal line indicates the national median levels for freshwater fish across the U.S.[1] The dashed red line at 17.2 ppb corresponds to a "Do Not Eat" level of PFOS based on the only fish consumption advisory currently in effect in North Carolina, which applies to the lower Cape Fear River only.[2]

380 PFOS concentration at JL1 and JL2, but not at HR1. Because fish length is related to age within a
381 population[62], we expected to see a positive correlation between length and PFOS levels due to the
382 long biological half-life of PFOS in muscle tissue. However, numerous factors unrelated to PFOS
383 exposure, such as pharmacokinetic changes from other chemicals present at HR1 and differences
384 in the riverine compared to lake ecosystems, could explain this lack of relationship observed in the
385 HR1 fish.[63] These results suggest that consuming larger sunfish could result in greater PFOS
386 exposure; however, at sites with high water PFOS levels, such as HR1, smaller fish may not
387 necessarily have lower levels of PFOS than larger fish. Additional studies are needed to investigate
388 this observation further.

389 To our knowledge, these are the first PFOS concentrations reported in fish from Jordan
390 Lake and the upper Haw River. A recent EPA study of 44 species of freshwater fish sampled
391 between 2013 and 2015[1] reported that the median PFOS level in fish from rivers and streams across
392 the U.S. was 6.60 ppb. The median PFOS concentrations we report here in the *Lepomis spp.* sunfish
393 fillets are higher than reported by the EPA study in all cases. In the geographically closest study
394 we could find, a 2007 study of North Carolina fish, Delinsky et al. reported a median concentration
395 of 30.3 ppb PFOS (range: 15.9 to 47.5 ppb) in bluegill sunfish collected from the Haw River, south
396 of the inflow to Jordan Lake.[64] The median PFOS concentrations we report in fish from the upper
397 Haw River are higher than those reported downstream in 2007, while the Jordan Lake fish had
398 generally lower median PFOS concentrations that were nevertheless within the observed range
399 from this area in 2007. More recently, the North Carolina Department of Health and Human
400 Services reported average PFOS concentrations of 23.0 ppb in bluegill from the middle and lower
401 Cape Fear River in the summer of 2022, which is high enough for a "do not eat" advisory which
402 is triggered at levels exceeding 17.2 ppb. The PFOS concentrations we measured in Jordan Lake

403 fillets were comparable to the advisory triggering levels in the Cape Fear, and levels in the Haw
404 River fillets were more than twice that high. While the Cape Fear River has garnered a lot of
405 attention due to GenX contamination from Chemours and the Haw River is the subject of ongoing
406 litigation around PFAS pollution, Jordan Lake is not considered a highly contaminated area and
407 has not been as highly scrutinized. Our results provide evidence that fish in this watershed have
408 concerningly high levels of PFOS, and urgent attention is needed in this area.

409 Currently in North Carolina, PFOS is the only PFAS applicable for fish consumption
410 advisories. However, we found a total of 36 different PFAS, including PFOS, in fish fillets from
411 Jordan Lake and the Haw River. To evaluate whether PFOS concentrations in fish fillets are
412 associated with greater numbers of different PFAS detected, regression analysis was performed
413 between PFOS concentrations and number of PFAS detected in sunfish fillets from each site.
414 Surprisingly, PFOS concentration did not generally appear to correlate with increasing number of
415 PFAS detected (**Figure 5**). The goodness of fit as measured by R^2 was low at all three sites, and a
416 statistically significant correlation was observed at JL2 ($p = 0.03$) but not at HR1 ($p = 0.50$) or JL1
417 ($p = 0.62$). These results indicate that there is not a consistent relationship between PFOS
418 concentration and number of different PFAS in sunfish fillets. Notably, the sunfish with the lowest
419 PFOS concentration at JL1 (7.50 ppb) had the highest number of PFAS detected at that site (11).
420 This fish had some of the highest FBSA and FHxSA levels of all the sunfish fillets**.** At HR1, the
421 sunfish with the lowest PFOS concentration (19.8 ppb) had the third-highest number of PFAS
422 detected (19), including the long chain PFCAs PFPeDA and PFHxDA. All the long-chain PFCAs
423 and PFSAs are thought to be harmful and some of them are more bioaccumulative than PFOS. In
424 North Carolina as well as in most other states in the U.S., however, water and fish are rarely tested
425 for these long-chain PFAS. Our results suggest that they could be present at harmful levels in fish

426 even when PFOS levels are considered safe. It is possible that the same trends would not be
427 observed at lower PFOS concentrations, which we could not evaluate here because our sample set
428 did not include any fish with low enough PFOS levels to be safely consumed more than one time
429 per year based on the reference dose applied in the Cape Fear River.

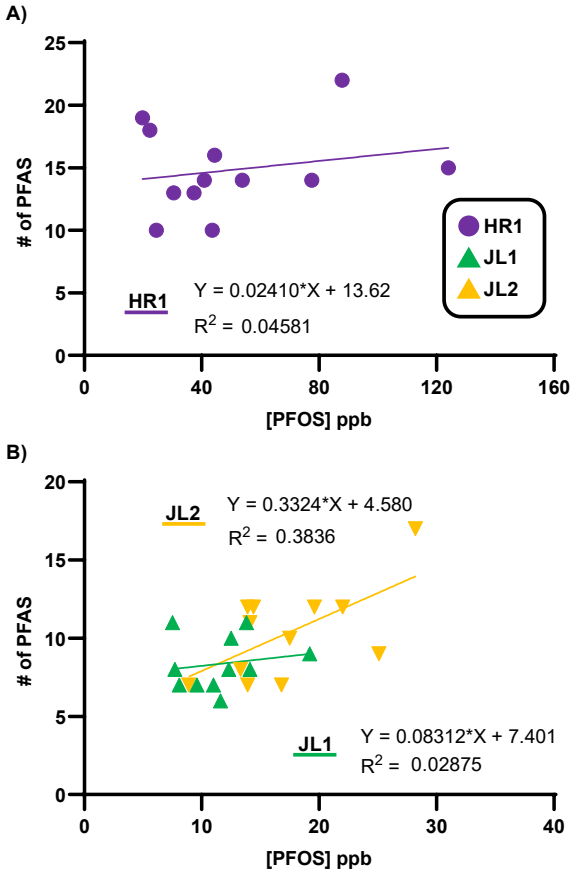

Figure 5. Concentration of PFOS measured versus number of PFAS detected in individual *Lepomis* fillets from **A)** the Haw River site and **B)** the Jordan Lake sites. A general trend of increasing [PFOS] with number of PFAS detected was observed. Regression analysis indicated a statistically significant association at JL2 ($p = 0.03$) but not at JL1 or HR1.

430

431 **Implications**

432 In this study, NTA was performed to find PFAS in fish from the Haw River and Jordan
433 Lake in North Carolina, a previously unstudied water body upstream of the Cape Fear River (which

has received considerable attention for its PFAS contamination). In total, 36 different PFAS were identified in fish fillets at a high level of confidence made possible by multidimensional LC-IMS-MS separations. Of these 36 PFAS, 5 have been previously reported in the water from these sites. Only 17 of these 36 PFAS are targeted in the validated EPA method for PFAS in biological samples, EPA 1633. The remaining 19 PFAS are not commonly targeted in fish or water studies, and therefore little information is available on their toxicities and environmental presence. While these 19 PFAS were not quantified in this study, standards are available for most of them and future fish monitoring studies in this region should include calibration curves for as many of these PFAS as feasible. Our unknown discovery workflow flagged 55 additional features as possible PFAS; however, manual inspection of the peaks determined that they were not likely PFAS and could not be annotated. Incorporating more CCS values into existing databases will help increase the number of PFAS that may be confidently identified and subsequently tracked and monitored in future studies.

PFOS concentrations in all fillets exceeded the "no more than one meal per year" consumption advisory limit which is triggered at 1.4 ppb in the lower Cape Fear River, with the lowest at 7.50 ppb and the highest at 124 ppb. To our knowledge, no PFAS fish monitoring is in place in North Carolina outside of the Cape Fear River. The fish in our study were collected in 2020, so it is possible that PFAS levels today are different from those we report here. Given that conventional wastewater treatment methods do not remove PFAS and that very little federal or state-level PFAS regulations are in place, we urge government and regulatory agencies to implement a fish PFAS monitoring program for this region so the millions of annual visitors to Jordan Lake State Park are informed of any potential risk of PFAS exposure they might accept from consuming fish from this lake. While our study area was limited to a small geographic region

in North Carolina, we believe our results provide clear evidence that fish PFAS monitoring studies should include NTA even in areas not considered heavily PFAS-impacted.

ASSOCIATED CONTENT

Supporting Information

Fish fillet sample preparation methods; Consumables used in LC-IMS-MS analysis (Table S1); LC gradient settings (Table S2); Electrospray ionization source settings (Table S3); IMS-MS settings (Table S4); Absolute and semi-quantification methods; Reproducibility of PFOS calculations (Figure S1); Accuracy and precision of PFAS quantitation (Table S5); CCS vs *m/z* trendline example (Figure S2); Example of possible PFAS ruled out (Figure S3); PCA of sampling date (Figure S4); PCA of sampling location (Figure S5); Heat map by location (Figure S6); Sex differences in PFOS concentration (Figure S7); Fish length and PFOS concentration (Figure S8) (PDF)

Supplemental Tables

Fish sample information (ST1); Efficiency and recovery (ST2); PFAS identifications (ST3); PFOS quantitation (ST4); Normalized areas (ST5); QC normalized areas (ST6); FM-IM results (ST7) (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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REFERENCES

(1) Barbo, N.; Stoiber, T.; Naidenko, O. V.; Andrews, D. Q. Locally caught freshwater fish across the United States are likely a significant source of exposure to PFOS and other perfluorinated compounds. *Environmental Research* **2023**, *220*, 115165. DOI: https://doi.org/10.1016/j.envres.2022.115165.

(2) Health, N. C. D. o. P. *Fish Consumption Advisories - Current Advisories for N.C.* https://epi.dph.ncdhhs.gov/oee/fish/advisories.html (accessed 2023 6/30/2023).

(3) Buck, R. C.; Franklin, J.; Berger, U.; Conder, J. M.; Cousins, I. T.; De Voogt, P.; Jensen, A. A.; Kannan, K.; Mabury, S. A.; Van Leeuwen, S. P. Perfluoroalkyl and polyfluoroalkyl substances in the environment: Terminology, classification, and origins. *Integrated Environmental Assessment and Management* **2011**, *7* (4), 513-541. DOI: 10.1002/ieam.258.

(4) Glüge, J.; Scheringer, M.; Cousins, I. T.; Dewitt, J. C.; Goldenman, G.; Herzke, D.; Lohmann, R.; Ng, C. A.; Trier, X.; Wang, Z. An overview of the uses of per- and polyfluoroalkyl substances (PFAS). *Environmental Science: Processes & Impacts* **2020**, *22* (12), 2345-2373. DOI: 10.1039/d0em00291g.

(5) Cousins, I. T.; Johansson, J. H.; Salter, M. E.; Sha, B.; Scheringer, M. Outside the Safe Operating Space of a New Planetary Boundary for Per- and Polyfluoroalkyl Substances (PFAS). *Environmental Science & Technology* **2022**, *56* (16), 11172-11179. DOI: 10.1021/acs.est.2c02765.

(6) Evich, M. G.; Davis, M. J. B.; McCord, J. P.; Acrey, B.; Awkerman, J. A.; Knappe, D. R. U.; Lindstrom, A. B.; Speth, T. F.; Tebes-Stevens, C.; Strynar, M. J.; et al. Per- and polyfluoroalkyl substances in the environment. *Science 375* (6580), eabg9065. DOI: 10.1126/science.abg9065 (acccessed 2023/03/08).

(7) De Silva, A. O.; Armitage, J. M.; Bruton, T. A.; Dassuncao, C.; Heiger-Bernays, W.; Hu, X. C.; Kärrman, A.; Kelly, B.; Ng, C.; Robuck, A.; et al. PFAS Exposure Pathways for Humans and Wildlife: A Synthesis of Current Knowledge and Key Gaps in Understanding. *Environmental Toxicology and Chemistry* **2021**, *40* (3), 631-657. DOI: 10.1002/etc.4935.

(8) Fenton, S. E.; Ducatman, A.; Boobis, A.; Dewitt, J. C.; Lau, C.; Ng, C.; Smith, J. S.; Roberts, S. M. Per- and Polyfluoroalkyl Substance Toxicity and Human Health Review: Current State of Knowledge and Strategies for Informing Future Research. *Environmental Toxicology and Chemistry* **2021**, *40* (3), 606-630. DOI: 10.1002/etc.4890.

(9) Augustsson, A.; Lennqvist, T.; Osbeck, C. M. G.; Tibblin, P.; Glynn, A.; Nguyen, M. A.; Westberg, E.; Vestergren, R. Consumption of freshwater fish: A variable but significant risk factor for PFOS exposure. *Environmental Research* **2021**, *192*, 110284. DOI: https://doi.org/10.1016/j.envres.2020.110284.

(10) EPA, U. National Primary Drinking Water Regulation. 2024.

(11) Brennan, N. M.; Evans, A. T.; Fritz, M. K.; Peak, S. A.; Von Holst, H. E. Trends in the Regulation of Per- and Polyfluoroalkyl Substances (PFAS): A Scoping Review. *International Journal of Environmental Research and Public Health* **2021**, *18* (20), 10900. DOI: 10.3390/ijerph182010900.

(12) EPA, U. Reference Dose (RfD): Description and Use in Health Risk Assessments. IRIS, Ed.; 1993.

(13) EPA, U. *Risk Management for Per- and Polyfluoroalkyl Substances (PFAS) under TSCA*. 2023. https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/risk-management-and-polyfluoroalkyl-substances-pfas (accessed 2023 6/26/2023).

(14) Cordner, A.; De La Rosa, V. Y.; Schaider, L. A.; Rudel, R. A.; Richter, L.; Brown, P. Guideline levels for PFOA and PFOS in drinking water: the role of scientific uncertainty, risk assessment decisions, and social factors. *Journal of Exposure Science & Environmental Epidemiology* **2019**, *29* (2), 157-171. DOI: 10.1038/s41370-018-0099-9.

(15) EPA, U. *PFAS Analytical Methods Development and Sampling Research*. 2024. https://www.epa.gov/water-research/pfas-analytical-methods-development-and-sampling-research (accessed 2024 04/11/2024).
(16) EPA, U. S. *CompTox Chemicals Dashboard*. 2022. https://comptox.epa.gov/dashboard/ (accessed 2023 3/9/2023).
(17) Wang, Z.; Dewitt, J. C.; Higgins, C. P.; Cousins, I. T. A Never-Ending Story of Per- and Polyfluoroalkyl Substances (PFASs)? *Environmental Science & Technology* **2017**, *51* (5), 2508-2518. DOI: 10.1021/acs.est.6b04806.
(18) Sunderland, E. M.; Hu, X. C.; Dassuncao, C.; Tokranov, A. K.; Wagner, C. C.; Allen, J. G. A review of the pathways of human exposure to poly- and perfluoroalkyl substances (PFASs) and present understanding of health effects. *Journal of Exposure Science & Environmental Epidemiology* **2019**, *29* (2), 131-147. DOI: 10.1038/s41370-018-0094-1.
(19) Ruan, T.; Jiang, G. Analytical methodology for identification of novel per- and polyfluoroalkyl substances in the environment. *TrAC Trends in Analytical Chemistry* **2017**, *95*, 122-131. DOI: https://doi.org/10.1016/j.trac.2017.07.024.
(20) Ackerman Grunfeld, D.; Gilbert, D.; Hou, J.; Jones, A. M.; Lee, M. J.; Kibbey, T. C. G.; O'Carroll, D. M. Underestimated burden of per- and polyfluoroalkyl substances in global surface waters and groundwaters. *Nature Geoscience* **2024**. DOI: 10.1038/s41561-024-01402-8.
(21) Pickard, H. M.; Ruyle, B. J.; Thackray, C. P.; Chovancova, A.; Dassuncao, C.; Becanova, J.; Vojta, S.; Lohmann, R.; Sunderland, E. M. PFAS and Precursor Bioaccumulation in Freshwater Recreational Fish: Implications for Fish Advisories. *Environmental Science & Technology* **2022**, *56* (22), 15573-15583. DOI: 10.1021/acs.est.2c03734.
(22) Phillips, A. L.; Peter, K. T.; Sobus, J. R.; Fisher, C. M.; Manzano, C. A.; McEachran, A. D.; Williams, A. J.; Knolhoff, A. M.; Ulrich, E. M. Standardizing non-targeted analysis reporting to advance exposure science and environmental epidemiology. *Journal of Exposure Science & Environmental Epidemiology* **2023**, *33* (4), 501-504. DOI: 10.1038/s41370-022-00490-1.
(23) Koelmel, J. P.; Paige, M. K.; Aristizabal-Henao, J. J.; Robey, N. M.; Nason, S. L.; Stelben, P. J.; Li, Y.; Kroeger, N. M.; Napolitano, M. P.; Savvaides, T.; et al. Toward Comprehensive Per- and Polyfluoroalkyl Substances Annotation Using FluoroMatch Software and Intelligent High-Resolution Tandem Mass Spectrometry Acquisition. *Analytical Chemistry* **2020**, *92* (16), 11186-11194. DOI: 10.1021/acs.analchem.0c01591.
(24) Ren, J.; Fernando, S.; Hopke, P. K.; Holsen, T. M.; Crimmins, B. S. Suspect Screening and Nontargeted Analysis of Per- and Polyfluoroalkyl Substances in a Lake Ontario Food Web. *Environmental Science & Technology* **2022**, *56* (24), 17626-17634. DOI: 10.1021/acs.est.2c04321.
(25) Schymanski, E. L.; Jeon, J.; Gulde, R.; Fenner, K.; Ruff, M.; Singer, H. P.; Hollender, J. Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence. *Environmental Science & Technology* **2014**, *48* (4), 2097-2098. DOI: 10.1021/es5002105.
(26) Charbonnet, J. A.; McDonough, C. A.; Xiao, F.; Schwichtenberg, T.; Cao, D.; Kaserzon, S.; Thomas, K. V.; Dewapriya, P.; Place, B. J.; Schymanski, E. L.; et al. Communicating Confidence of Per- and Polyfluoroalkyl Substance Identification via High-Resolution Mass Spectrometry. *Environmental Science & Technology Letters* **2022**, *9* (6), 473-481. DOI: 10.1021/acs.estlett.2c00206.
(27) Celma, A.; Sancho, J. V.; Schymanski, E. L.; Fabregat-Safont, D.; Ibáñez, M.; Goshawk, J.; Barknowitz, G.; Hernández, F.; Bijlsma, L. Improving Target and Suspect Screening High-Resolution Mass Spectrometry Workflows in Environmental Analysis by Ion Mobility Separation. *Environmental Science & Technology* **2020**, *54* (23), 15120-15131. DOI: 10.1021/acs.est.0c05713.

(28) Dodds, J. N.; Hopkins, Z. R.; Knappe, D. R. U.; Baker, E. S. Rapid Characterization of Per- and Polyfluoroalkyl Substances (PFAS) by Ion Mobility Spectrometry–Mass Spectrometry (IMS-MS). *Analytical Chemistry* **2020**, *92* (6), 4427-4435. DOI: 10.1021/acs.analchem.9b05364.
(29) Foster, M.; Rainey, M.; Watson, C.; Dodds, J. N.; Kirkwood, K. I.; Fernández, F. M.; Baker, E. S. Uncovering PFAS and Other Xenobiotics in the Dark Metabolome Using Ion Mobility Spectrometry, Mass Defect Analysis, and Machine Learning. *Environmental Science & Technology* **2022**, *56* (12), 9133-9143. DOI: 10.1021/acs.est.2c00201.
(30) Kirkwood, K. I.; Fleming, J.; Nguyen, H.; Reif, D. M.; Baker, E. S.; Belcher, S. M. Utilizing Pine Needles to Temporally and Spatially Profile Per- and Polyfluoroalkyl Substances (PFAS). *Environmental Science & Technology* **2022**, *56* (6), 3441-3451. DOI: 10.1021/acs.est.1c06483.
(31) Díaz-Galiano, F. J.; Murcia-Morales, M.; Monteau, F.; Le Bizec, B.; Dervilly, G. Collision cross-section as a universal molecular descriptor in the analysis of PFAS and use of ion mobility spectrum filtering for improved analytical sensitivities. *Analytica Chimica Acta* **2023**, *1251*, 341026. DOI: https://doi.org/10.1016/j.aca.2023.341026.
(32) BP4NTA. *Methods: Study Design*. 2024. https://nontargetedanalysis.org/reference-content/methods/study-design/ (accessed 2024 04/11/2024).
(33) Assembly, H. R. Industrial Contaminants Factsheet. 2024.
(34) Pétré, M. A.; Salk, K. R.; Stapleton, H. M.; Ferguson, P. L.; Tait, G.; Obenour, D. R.; Knappe, D. R. U.; Genereux, D. P. Per- and polyfluoroalkyl substances (PFAS) in river discharge: Modeling loads upstream and downstream of a PFAS manufacturing plant in the Cape Fear watershed, North Carolina. *Science of The Total Environment* **2022**, *831*, 154763. DOI: https://doi.org/10.1016/j.scitotenv.2022.154763.
(35) Goodrow, S. M.; Ruppel, B.; Lippincott, R. L.; Post, G. B.; Procopio, N. A. Investigation of levels of perfluoroalkyl substances in surface water, sediment and fish tissue in New Jersey, USA. *Science of The Total Environment* **2020**, *729*, 138839. DOI: https://doi.org/10.1016/j.scitotenv.2020.138839.
(36) Lewis, A. J.; Yun, X.; Spooner, D. E.; Kurz, M. J.; McKenzie, E. R.; Sales, C. M. Exposure pathways and bioaccumulation of per- and polyfluoroalkyl substances in freshwater aquatic ecosystems: Key considerations. *Science of The Total Environment* **2022**, *822*, 153561. DOI: https://doi.org/10.1016/j.scitotenv.2022.153561.
(37) EPA, U. Technical Overview Webinar Presentation: Proposed PFAS National Primary Drinking Water Regulation. Water, O. o., Ed.; 2023.
(38) Services, N. C. D. o. H. a. H. NCDHHS Recommends Limiting Fish Consumption from the Middle and Lower Cape Fear River Due to Contamination With “Forever Chemicals”. 2023.
(39) Luo, Y.-S.; Aly, N. A.; McCord, J.; Strynar, M. J.; Chiu, W. A.; Dodds, J. N.; Baker, E. S.; Rusyn, I. Rapid Characterization of Emerging Per- and Polyfluoroalkyl Substances in Aqueous Film-Forming Foams Using Ion Mobility Spectrometry–Mass Spectrometry. *Environmental Science & Technology* **2020**, *54* (23), 15024-15034. DOI: 10.1021/acs.est.0c04798.
(40) Aly, N. A.; Luo, Y.-S.; Liu, Y.; Casillas, G.; McDonald, T. J.; Kaihatu, J. M.; Jun, M.; Ellis, N.; Gossett, S.; Dodds, J. N.; et al. Temporal and spatial analysis of per and polyfluoroalkyl substances in surface waters of Houston ship channel following a large-scale industrial fire incident. *Environmental Pollution* **2020**, *265*, 115009. DOI: 10.1016/j.envpol.2020.115009.
(41) Quality, M. D. o. E. Fish Tissue PFAS Sampling Guidance. 2019.
(42) Weed, R. A.; Boatman, A. K.; Enders, J. R. Recovery of per- and polyfluoroalkyl substances after solvent evaporation. *Environmental Science: Processes & Impacts* **2022**, *24* (12), 2263-2271. DOI: 10.1039/d2em00269h.
(43) Water, U. S. E. O. o. Draft Method 1633: Analysis of Per- and Polyfluoroalkyl Sunstances (PFAS) in Aqueous, Solid, Biosolids, and Tissue Samples by LC-MS/MS. 2021.
(44) Carrizo, J. C.; Munoz, G.; Vo Duy, S.; Liu, M.; Houde, M.; Amé, M. V.; Liu, J.; Sauvé, S. PFAS in fish from AFFF-impacted environments: Analytical method development and field

application at a Canadian international civilian airport. *Science of The Total Environment* **2023**, *879*, 163103. DOI: https://doi.org/10.1016/j.scitotenv.2023.163103.
(45) Taylor, P. J. Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry. *Clinical Biochemistry* **2005**, *38* (4), 328-334. DOI: https://doi.org/10.1016/j.clinbiochem.2004.11.007.
(46) Matuszewski, B. K.; Constanzer, M. L.; Chavez-Eng, C. M. Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC−MS/MS. *Analytical Chemistry* **2003**, *75* (13), 3019-3030. DOI: 10.1021/ac020361s.
(47) Ibrahim, Y. M.; Baker, E. S.; Danielson, W. F.; Norheim, R. V.; Prior, D. C.; Anderson, G. A.; Belov, M. E.; Smith, R. D. Development of a new ion mobility time-of-flight mass spectrometer. *International Journal of Mass Spectrometry* **2015**, *377*, 655-662. DOI: 10.1016/j.ijms.2014.07.034.
(48) Bilbao, A.; Gibbons, B. C.; Stow, S. M.; Kyle, J. E.; Bloodsworth, K. J.; Payne, S. H.; Smith, R. D.; Ibrahim, Y. M.; Baker, E. S.; Fjeldsted, J. C. A Preprocessing Tool for Enhanced Ion Mobility–Mass Spectrometry-Based Omics Workflows. *Journal of Proteome Research* **2022**, *21* (3), 798-807. DOI: 10.1021/acs.jproteome.1c00425.
(49) Kirkwood, K. I.; Pratt, B. S.; Shulman, N.; Tamura, K.; MacCoss, M. J.; MacLean, B. X.; Baker, E. S. Utilizing Skyline to analyze lipidomics data containing liquid chromatography, ion mobility spectrometry and mass spectrometry dimensions. *Nature Protocols* **2022**, *17* (11), 2415-2430. DOI: 10.1038/s41596-022-00714-6.
(50) *Pretty Heatmaps*; CRAN, 2019. https://cran.r-project.org/web/packages/pheatmap/pheatmap.pdf (accessed.
(51) Marvel, S. W.; To, K.; Grimm, F. A.; Wright, F. A.; Rusyn, I.; Reif, D. M. ToxPi Graphical User Interface 2.0: Dynamic exploration, visualization, and sharing of integrated data models. *BMC Bioinformatics* **2018**, *19* (1). DOI: 10.1186/s12859-018-2089-2.
(52) ITRC. Naming Conventions for Per- and Polyfluoroalkyl Substances. 2022.
(53) Burkhard, L. P. Evaluation of Published Bioconcentration Factor (BCF) and Bioaccumulation Factor (BAF) Data for Per- and Polyfluoroalkyl Substances Across Aquatic Species. *Environmental Toxicology and Chemistry* **2021**, *40* (6), 1530-1543. DOI: 10.1002/etc.5010.
(54) Sun, J. M.; Kelly, B. C.; Gobas, F. A. P. C.; Sunderland, E. M. A food web bioaccumulation model for the accumulation of per- and polyfluoroalkyl substances (PFAS) in fish: how important is renal elimination? *Environmental Science: Processes & Impacts* **2022**, *24* (8), 1152-1164. DOI: 10.1039/d2em00047d.
(55) Richard, C. G. G. P. B. M. A. W. A. Structure-based categorization of Per- & Polyfluoroalkyl substances in the DSSTox database and EPA's ToxCast inventory using Markush representations. *American Chemical Society Annual Meeting* **2019**. DOI: 10.23645/epacomptox.8022443.
(56) Ren, J.; Fernando, S.; Hopke, P. K.; Holsen, T. M.; Crimmins, B. S. Suspect Screening and Nontargeted Analysis of Per- and Polyfluoroalkyl Substances in a Lake Ontario Food Web. *Environmental Science & Technology* **2022**, *56* (24), 17626-17634. DOI: 10.1021/acs.est.2c04321.
(57) Jia, S.; Marques Dos Santos, M.; Li, C.; Snyder, S. A. Recent advances in mass spectrometry analytical techniques for per- and polyfluoroalkyl substances (PFAS). *Analytical and Bioanalytical Chemistry* **2022**, *414* (9), 2795-2807. DOI: 10.1007/s00216-022-03905-y.
(58) Katherine E. VanderKooy, C. F. R. a. R. W. H. Trophic Relationships of Three Sunfishes (Lepomis spp.) in an Estuarine Bayou. *Estuaries* **2000**, *23* (5), 621-632.
(59) Thompson, J. T.; Chen, B.; Bowden, J. A.; Townsend, T. G. Per- and Polyfluoroalkyl Substances in Toilet Paper and the Impact on Wastewater Systems. *Environmental Science & Technology Letters* **2023**, *10* (3), 234-239. DOI: 10.1021/acs.estlett.3c00094.
(60) OWASA. *Wastewater*. https://www.owasa.org/wastewater/ (accessed 2023 6/13/23).

(61) Lab, N. s. P. P. PFAS Sites and Community Resources. https://experience.arcgis.com/experience/12412ab41b3141598e0bb48523a7c940/page/Page-1/?views=Important-Information%2CPresumptive-Contamination (accessed 6/28/2023).
(62) Gulland, J. A.; Rosenberg, A. A. A review of length-based approaches to assessing fish stocks. **1992**.
(63) Åkerblom, S.; Negm, N.; Wu, P.; Bishop, K.; Ahrens, L. Variation and accumulation patterns of poly- and perfluoroalkyl substances (PFAS) in European perch (Perca fluviatilis) across a gradient of pristine Swedish lakes. *Science of The Total Environment* **2017**, *599-600*, 1685-1692. DOI: https://doi.org/10.1016/j.scitotenv.2017.05.032.
(64) Delinsky, A. D.; Strynar, M. J.; Nakayama, S. F.; Varns, J. L.; Ye, X.; McCann, P. J.; Lindstrom, A. B. Determination of ten perfluorinated compounds in bluegill sunfish (Lepomis macrochirus) fillets. *Environmental Research* **2009**, *109* (8), 975-984. DOI: https://doi.org/10.1016/j.envres.2009.08.013.
(65) Burnum-Johnson, K. E.; Nie, S.; Casey, C. P.; Monroe, M. E.; Orton, D. J.; Ibrahim, Y. M.; Gritsenko, M. A.; Clauss, T. R. W.; Shukla, A. K.; Moore, R. J.; et al. Simultaneous Proteomic Discovery and Targeted Monitoring using Liquid Chromatography, Ion Mobility Spectrometry, and Mass Spectrometry *. *Molecular & Cellular Proteomics* **2016**, *15* (12), 3694-3705. DOI: 10.1074/mcp.M116.061143 (acccessed 2023/06/30).