Novel PFAS in Alligator Blood Discovered with Non-Targeted Ion Mobility-Mass Spectrometry

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ABSTRACT

Per- and polyfluoroalkyl substances (PFAS) are a large and growing class of chemicals gaining global attention due to their persistence, mobility, and toxicity. Given the diverse chemical properties of PFAS and their varying distributions in water and tissue, monitoring of different matrices is critical to determine their presence and accumulation. Here, we used a platform combining liquid chromatography, ion mobility spectrometry, and high-resolution mass spectrometry (LC-IMS-HRMS) for non-targeted analysis (NTA) to detect and identify PFAS in alligator plasma from North Carolina (5 years, 2018-2022) and Florida (2021 only). Structures for 12 PFAS were elucidated, including 2 novel structures, and an additional 34 known PFAS were detected. Three of these compounds were previously unreported in environmental media. More PFAS were detected in NC alligators than FL and no novel PFAS were detected in FL gators. Quantitative analysis of 21 of the known PFAS revealed that plasma concentrations did not change over the 5 year study, indicating that exposure is ongoing.

KEYWORDS

per- and polyfluoroalkyl substances; biomonitoring; sentinel species

SYNOPSIS

Non-targeted analysis (NTA) with LC-IMS-HRMS enabled discovery of novel PFAS in alligator plasma, with concerning implications for human exposure.

36 # INTRODUCTION

37 Clean drinking water free of unwanted and harmful chemicals is a basic human right. Per-
38 and polyfluoroalkyl substances (PFAS) have gained widespread attention because of their
39 unparalleled persistence, mobility, toxicity, and presence in humans, wildlife, and ecosystems
40 globally around the world. Some individual PFAS have been well studied and regulation is
41 increasing; however, PFAS are a large and growing class of chemicals, and the presence and
42 health impacts of most of them are not yet understood. Detecting unknown PFAS is therefore a
43 critical part of determining their impact on human and ecosystem health. To date, an incredible
44 amount of research efforts have focused on drinking water and surface water using both targeted
45 and non-targeted analyses.[1-5] Targeted analyses with mass spectrometry use analytical reference
46 standards to quantify known PFAS in water and biological matrices, but are inherently limited to
47 only a few dozen PFAS. When standards are not available or investigations into new PFAS are
48 desired, non-targeted analyses (NTA) are used. NTA efforts in water and biological tissue have
49 revealed that different PFAS are detected in different matrices[6-8], underscoring the importance of
50 testing both the exposure source and the exposed organism.[6, 9] However, NTA of complex
51 biological matrices is challenging. Obtaining biological samples often requires time- and resource-
52 intensive collection processes. Moreover, these samples have many other molecules present,
53 such as proteins, lipids, and metabolites, resulting in feature lists dominated by highly abundant
54 biological molecules with no relevance to the analytes of interest. Work to improve and increase
55 NTA analyses in biological samples is of the utmost importance for contaminant studies, including
56 PFAS.

57 The lower Cape Fear River (CFR) in North Carolina serves as a primary drinking water
58 source for local human and animal residents and is famously located downstream of a
59 fluorochemical manufacturer that produces Nafion using perfluoroether compounds. Previous
60 non-targeted studies in this region discovered perfluoroethers in seabirds[6] in addition to other

61 unknown fluorochemicals in water[5]. Targeted, quantitative studies in the CFR have also
62 consistently noted high PFAS levels in water[1, 5, 10], fish and other wildlife[6, 11], human serum[4, 12],
63 and domestic sentinel species including horses and dogs.[13] In their targeted study of alligators
64 sampled in 2018-2019, Guillette *et al.* found associations between increased serum PFAS levels
65 and several immune health endpoints, including increased autoantibody presence and field
66 observations of unhealed wounds. Reanalysis of the same alligator serum samples by Bangma
67 *et al.* showed the presence of additional perfluoroethers for which standards had not been
68 available in the original targeted analysis.[9] Alligators are valuable sentinels for human exposure
69 to harmful chemicals due to their long life span and position at the top of the food chain, both of
70 which result in high levels of exposure to aquatic bioaccumulative pollutants. Additionally, their
71 complex and robust immune systems make them sensitive predictors for exposure to
72 immunotoxic chemical pollutants such as PFAS. The study authors concluded that the presence
73 of these compounds in alligators is cause for concern about human exposure, and that the
74 detection of additional PFAS with no standards available means that reported PFAS levels
75 underestimate the total amount of PFAS present. Their results clearly demonstrate a need for
76 ongoing biological monitoring of PFAS levels in people and animals from this region.

77 In this study, we contribute 5 years of alligator PFAS NTA data (2018 to 2022) to the
78 growing body of PFAS research on CFR water and wildlife. To contrast with the CFR, we included
79 alligators from Lake Waccamaw (LW), a nearby reference site located within the Lumber River
80 basin in North Carolina, as well as alligators from St. John's River, Florida (FL), a geographically
81 farther away site with no known PFAS industry nearby.[14] The primary aim of this study was to
82 identify as many PFAS as possible in alligator plasma with a focus on novel and uncommon
83 compounds that could go undetected in NTA of water. We additionally quantified up to 26 known
84 PFAS to evaluate geographic and temporal exposure trends. The combination of quantitative data
85 and information on novel PFAS will be of great interest to researchers and local residents because

these results uncover previously unreported PFAS that have accumulated to detectable levels in a sensitive sentinel species.

RESULTS

Non-Targeted PFAS Analysis of Alligator Plasma

Plasma samples were collected from alligators in three watersheds over the 5 year study (2018-2022): the Cape Fear River downstream of a fluorochemical manufacturer in North Carolina (CFR, n = 98); Lake Waccamaw, a nearby reference site in the Lumber River basin with no known PFAS point sources (LW, n = 74); and a region in Florida with no known fluorochemical manufacturing (FL, n = 26, 2021 only) (**Figure 1, Tables S1-S2**). Within the CFR, four distinct locations were sampled: Greenfield Lake (2018-2022), Orton Pond (2019 only), Oak Island (2019 only), and Bald Head Island (2019 and 2022). Our NTA platform combining liquid chromatography, ion mobility spectrometry, collision induced dissociation, and high-resolution mass spectrometry (LC-IMS-CID-HRMS) was used to evaluate the extracted plasma samples. The multiple separations possible with this NTA platform enable the evaluation of known and new PFAS with high identification confidence. In this study, we first searched for PFAS in each alligator plasma sample by collecting precursor-only (or MS1-only) full scan data to maximize feature

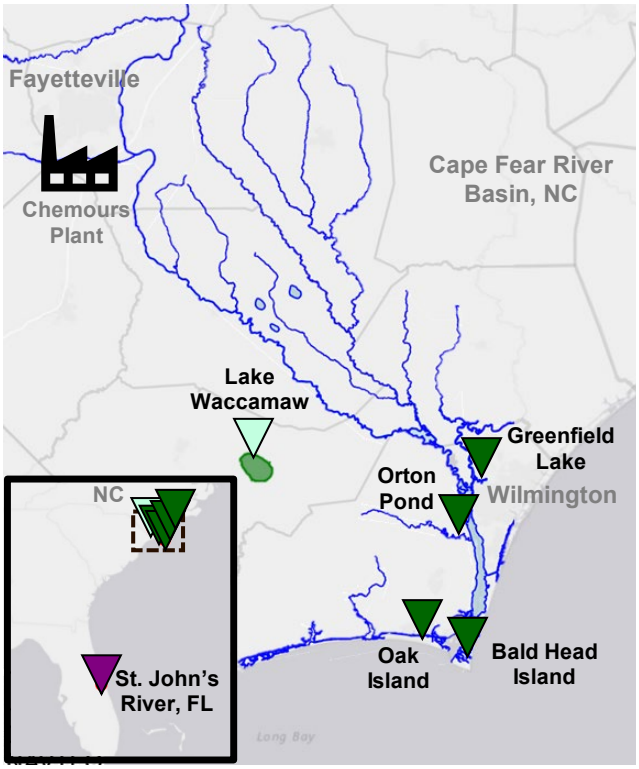

Figure 1. Map of alligator sampling sites in relation to the Cape Fear River Basin, NC and the upstream Chemours fluorochemical manufacturing facility.

111 abundance. Next, we collected fragmentation (or MS2) data using size-dependent all-ions
112 fragmentation (AIF) to elucidate unknown structures.[3, 15-17] The data was then analyzed using
113 complementary approaches combining a library screening workflow to find known PFAS (**Table**
114 **S3**) and a discovery workflow to uncover additional PFAS not present in the library.

115 All together we identified 46 PFAS (**Table 1, Tables S4-S5**) in the alligator plasma. Library
116 screening detected 34 known PFAS (at least 45 different molecules when counting structural
117 isomers), of which 21 were quantified (see ***Spatial and Temporal Trends*** section).

118

Table 1. All PFAS detected in at least one alligator sample.

PFAS detected				Detection frequency (%)		
PFAS class	**Molecule Name**	**Formula**	**Quantified**	**CFR**	**LW**	**FL**
H-substituted polyfluorinated triether acids	Compound 628	C10H2F20O6S	no	87.8	9.6	-
	Compound 592	C11H2F20O5	no	32.7	1.4	-
	Compound 448	C8H2F16O3	no	21.4	1.4	-
PFAESA	PFHpESA	C9HF19O4S	no	55.1	1.4	-
	PFHxESA	C8HF17O4S	no	77.6	6.8	-
PFCA	co-br-PFUdA	C11HF21O2	no	62.2	1.4	-
			no			
U-PFSA	U-PFOS	C8F15HO3S	no	69.4	9.6	-
Fluorinated insecticides	Fipronil sulfone	C12H4Cl2F6N4O2S	no	92.9	42.5	26.9
			no			
PFESA	Unknown PolyFESA C	C7H2F14O5S	no	2.0	-	-
	PolyFESA D		no	20.4	1.4	-
C3F7O fragment	PFECAa	unknown	no	8.2	-	-
	PFECAb	unknown	no	87.8	12.3	-
	PFECAc	unknown	no	14.3	1.4	-
Unknown	Unknown323	unknown	no	100.0	100.0	100.0
	Unknown314	unknown	no	46.9	24.7	-
	Unknown281	unknown	no	72.4	76.7	50.0
	Unknown229	unknown	no	36.7	11.0	3.8
	Unknown207	unknown	no	49.0	24.7	42.3
PFSA	PFDoS l+br	C12HF25O3S	yes (l only)	14.3	-	-
	PFUdS l+br	C11HF23O3S	no	18.4	-	-
	PFDS l+br	C10HF21O3S	yes (l only)	100.0	94.5	100.0
	PFNS l+br	C9HF19SO3	yes (l only)	92.9	26.0	7.7
	PFOS l+br	C8HF17O3S	yes (l only)	100.0	100.0	100.0
	PFHpS	C7HF15O3S	yes	100.0	94.5	92.3
	PFHxS l+br	C6HF13O3S	yes (l only)	100.0	100.0	100.0
	PFPeS	C5HF11O3S	yes	19.4	1.4	-
	PFBS	C4HF9O3S	yes	13.3	21.9	3.8
	PFEtS	C2HF5O3S	no	-	-	3.8
PFCA	PFHxDA	C16HF31O2	no	54.1	35.6	15.4
	PFPeDA	C15HF29O2	no	59.2	28.8	-
	PFTeDA	C14HF27O2	yes	91.8	41.1	-
	PFTrDA	C13HF25O2	yes	100.0	97.3	26.9
	PFDoA	C12HF23O2	yes	100.0	97.3	65.4
	PFUdA	C11HF21O2	yes	100.0	100.0	100.0
	PFDA	C10HF19O2	yes	100.0	100.0	100.0
	PFNA	C9HF17O2	yes	81.6	68.5	92.3
	PFOA	C8HF15O2	yes	17.3	16.4	15.4
	PFHpA	C7HF13O2	yes	3.1	4.1	3.8
	PFHxA	C6HF11O2	yes	9.2	9.6	11.5
FTS	10:2 FTS	C12H5F21SO3	no	34.7	-	-
	8:2 FTS	C10H5F17SO3	yes	39.8	1.4	-
	6:2 FTS	C8H5F13SO3	yes	1.0	-	-
PFASA	N-MeFOSAA	C11H6F17NO4S	yes	3.1	-	-
	FOSA	C8H2F17NO2S	yes	72.4	23.3	19.2
	FHxSA	C6H2F13SO2N	no	10.2	-	-
PFESA	NB2 isomer A	C7H2F14O5S	no	89.8	4.1	-
	NB2 isomer B	C7H2F14O5S	no	95.9	30.1	-
	NB6	C6H2F12O4S	no	31.6	1.4	-
PFP	8:2 diPAP	C20H9F34O4P	no	4.1	-	-
	6:2 diPAP	C16H9F26O4P	no	1.0	1.4	-
	8:8 PFPi	C16HF34O2P	no	10.2	-	-
	6:8 PFPi	C14HF30O2P	no	32.7	-	-
	6:6 PFPi	C12HF26O2P	no	22.4	-	-
PolyFCA	8:2 FTUCA	C10H2F16O2	no	3.1	1.4	-
Cl-PFSA	Cl-PFOS	C8HClF16O3S	no	3.1	-	-

119 The discovery workflow uncovered an additional 22 features of interest which were then manually
120 investigated. Of these 22 features, we elucidated candidate structures for 12 PFAS (2 of which
121 had 2 ion types detected), found fragmentation evidence for 3 PFAS with no precursor signal
122 detected, and the remaining 5 features of interest could not be identified further. Broadly, the 12
123 candidate structures included 2 novel PFAS (H-substituted polyfluorinated triether acids) and 1
124 in-source fragment of these, 2 perfluoroalkyl ether sulfonic acids (PFAESAs) known from patent
125 literature but not previously reported in this region, a novel isomer of PFUdA, 2 PFAS previously
126 detected in fish (PFUdS and PFPeDA)[18], 1 fluorinated insecticide (fipronil sulfone), 2 new isomers
127 of Nafion Byproduct 2 (NB2), and an unsaturated PFOS (U-PFOS, or PFOS with a double bond)
128 isomers, which we count as 1 structure here due to our inability to determine different double bond
129 positions. The following sections describe each structural elucidation in more detail.

H-substituted polyfluorinated triether acids

We report here for the first time structures for two H-substituted polyfluorinated triether acid PFAS (**Figure 2, Table 1**), referred to subsequently as Compound 628 (*m/z* 628.92) and Compound 592 (*m/z* 592.95). These PFAS consist of a branched fluorinated alkyl chain with one hydrogen substitution for a fluorine and three ether linkages with either a carboxylic acid or sulfonic acid headgroup. LC chromatograms for both features show several overlapping peaks at each *m/z*, indicating unresolved structural isomers or conformers. Our discovery workflow also flagged Compound 448 (*m/z* 448.9676), which was previously reported by Donelson *et al*. as an MS2 fragment of the multiheaded polyfluorinated ether Compound 10.[3] We did not detect Compound 10 or any of the multiheaded polyfluorinated ethers previously reported by Donelson *et al*. in the alligators. Our candidate structure for Compound 592 is desulfonated Compound 10 and our candidate for Compound 628 is decarboxylated Compound 10. Compound 448 was present in low energy frames along with Compound 592, followed the same LC peak shape, and had the same drift time. Together these observations suggest that it is a post-source decay product of Compound 592 rather than a separate feature. While fragmentation data was difficult to discern because these compounds elute closely, do not fully resolve in the drift dimension, and had generally low signal in the AIF method, predominant MS2 fragments at *m/z* 282.9822 and

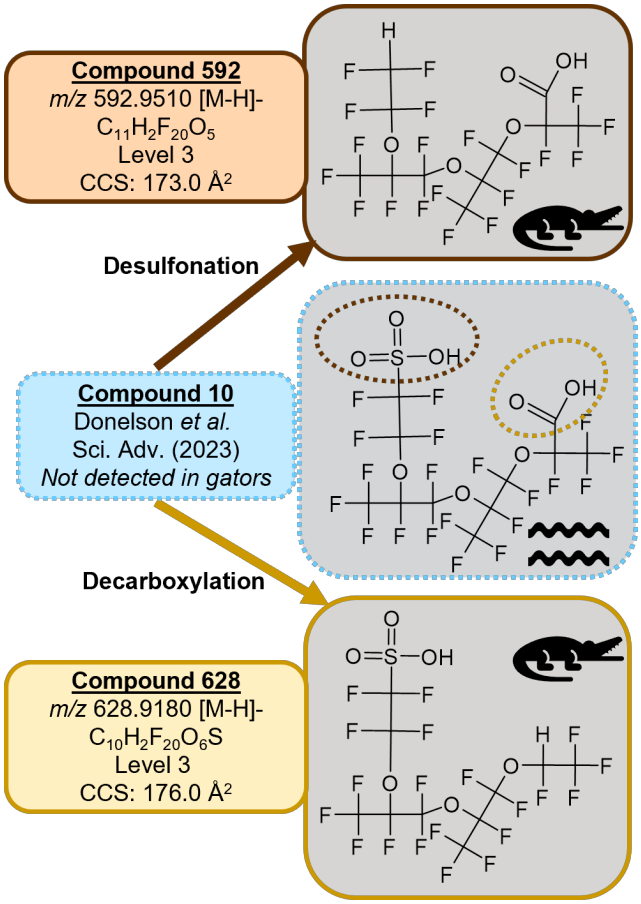

Figure 2. Candidate structures for the novel triether PFAS (Compounds 592 and 628) detected in alligators. Compound 10 was previously discovered in water from the CFR but was not detected in the alligators.

262.9760 were seen in all LC peaks, indicating a loss of HF from adjacent CFH-CF_2 or CFH-CF_3 moieties for both features. Some of the fragments reported for Compound 10 were also seen in the alligator features; for example, Compound 592 had a distinct *m/z* 162.9824 peak ($C_3F_5O_2$), and both features had an *m/z* 212.9792 peak ($C_4F_7O_2$). All together this evidence suggests multiple unresolvable isomers and fragments and diagnostic evidence allowed us to propose tentative candidate structures for Compounds 592 and 628 at a Level 3 confidence[19] (**Figures S1-S3**).

Initially we hypothesized that Compounds 592 and 628 were metabolic transformation products of Compound 10. To test this, we obtained data files from Donelson *et al.* and screened CFR water samples for Compounds 592 and 628. Interestingly, both compounds were detected in the water samples, suggesting that Compounds 592 and 628 are either separate manufacturing byproducts from the Chemours facility, or possibly environmental or microbial metabolic transformation products of Compound 10. Little information is available on biological metabolism of PFAS.[20] Additional studies screening for all three compounds would be informative.

Long Chain Perfluoroalkyl Ether Sulfonic Acids (PFAESAs)

Two long chain perfluoroalkyl ether sulfonic acids (PFAESAs), PFHxESA and PFHpESA, were detected (**Figure 3, Table 1**). These PFAS were reported only one time previously in biobanked European fish liver in a non-targeted study[21] and have never been detected in the CFR. Donelson *et al*. reported a homologous series of this subclass in passive sampling devices deployed in the CFR[3], including PFEESA (a known component of Nafion production[2]), PFPrESA, PFBESA, and PFPeESA, but we did not detect these smaller molecules in the alligator plasma. Data files were screened for a potential larger addition to the series with 8 carbons, PFOESA, but no features with the correct *m/z* were detected. Multiple LC peaks were observed for both molecules, consistent with the peak shapes previously reported in fish liver[21], Trendlines using RT and CCS values from Donelson *et al.* had good fits for the most abundant PFHxESA and PFHpESA peaks, which further supported our tentative identifications. Donelson *et al.* saw fragments diagnostic of an $SO_3(CF_2)_2O$ group linked to a fluorinated alkyl tail expanding by CF_2 units; however, we observed fragmentation

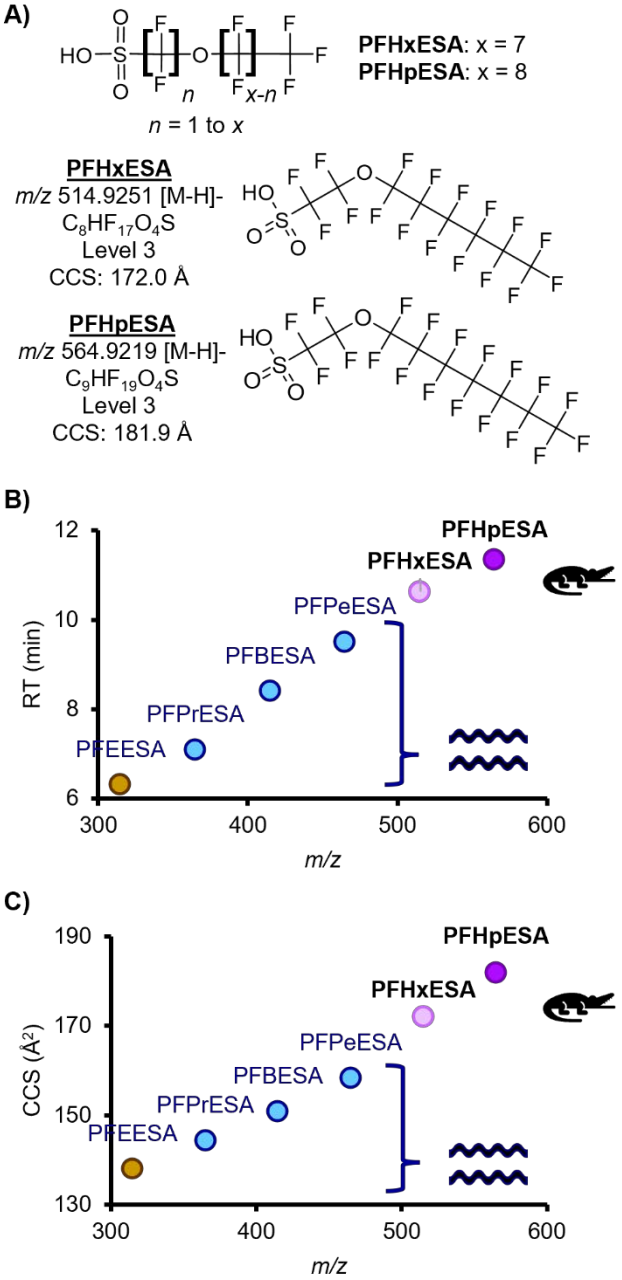

Figure 3. **A)** Example structure and tentative candidate structures for the two PFAESAs detected in alligators using NTA. Homologous series trendlines were created with values reported by Donelson *et al.* for smaller molecules in the PFAESA subclass detected in CFR water. **B)** RT vs *m/z*. **C)** CCS vs *m/z*.

196 patterns that suggested the presence of multiple structural isomers for PFHxESA and PFHpESA.
197 The earlier RT peaks for PFHpESA also had smaller CCS values, which suggests more compact
198 structural conformations that are also more polar, possibly due to branching of the fluorinated
199 alkyl tail. However, for PFHxESA, the earlier RT peak had a larger CCS value, which suggests a
200 more extended conformation that is more polar than the later peak, possibly due to an ether
201 linkage closer to the CF_3 end of the chain. Signal intensity was generally low in AIF injections,
202 limiting our ability to propose a singular structure for each peak. However, the combination of
203 diagnostic fragmentation evidence and CCS and RT predictions using homologous series
204 trendlines supports presence of multiple structural isomers and allows us to identify PFHxESA
205 and PFHpESA at Level 3 confidence[19] (**Figures S4-S6**).

206 ***Novel Perfluoroundecanoic Acid Isomer, co-br-PFUdA***

207 A structural isomer of PFUdA, the 11-carbon carboxylic acid, was detected (**Figure 4,**
208 **Table 1**). Because the proposed tentative candidate structure is branched (br) and has a non-
209 terminal CO_2 group, we refer to this structure as co-br-PFUdA. Fragments including C_2F_5, C_3F_7,
210 C_4F_9, C_6F_{13}, and C_7F_{15} confirmed that this feature is a structural isomer of PFUdA. Additionally,
211 both M-H- and M-H-CO_2- adducts were observed for co-br-PFUdA, including a second drift peak
212 of the M-H-CO_2- drift aligned with the M-H-, indicating post-source decay and characteristic of
213 PFCAs on our platform. Compared with our in-house library values for l-PFUdA, the measured
214 RT of co-br-PFUdA was slightly earlier (0.2 min) and the CCS value for co-br-PFUdA M-H- was
215 slightly smaller (by 0.9%), consistent with the difference between other branched PFAAs and their
216 linear counterparts. However, the M-H-CO_2- CCS was actually 0.9% larger than that of l-PFUdA,
217 indicating a more extended conformation when the CO_2 group pops off and providing evidence
218 that the CO_2 group is in the middle of the chain rather than on the end. A PubChem formula search
219 returned only one entry that satisfied our evidence for a CO_2 group at a non-terminal position with
220 a branched CF_2 chain, which we propose as our most likely candidate structure. We can imagine
221 several other structural possibilities for this molecule with different branching and CO_2 locations

that our evidence could support, so this identification is a Level 3 confidence (**Figures S7-S8**)[19]. Branched isomers of carboxylic and sulfonic acid PFAS are commonly detected in lower abundance than their linear counterparts; however, this co-br-PFUdA detection is notable because its abundance was often as high or higher than the linear form, l-PFUdA, which was also detected in 100% of alligators from all sites and years.

Other PFAS Identified Using NTA

Several other PFAS for which no standards are available were identified in our NTA workflow. We detected 3 polyfluorinated ether sulfonic acids (PolyFESAs), including 2 known isomers of Nafion Byproduct 2 (NB2 A and B, Levels 2 and 1, **Figures S9-S11**) and 1 tentative structural NB2 isomer candidate (PolyFESA D) which has not previously been reported in environmental samples (Level 3, **Figure S13**). One additional possible PolyFESA (Unknown PolyFESA C) was originally annotated as a fourth NB2 isomer but insufficient evidence was available to confirm a tentative structure (Level 5, **Figure S12**).

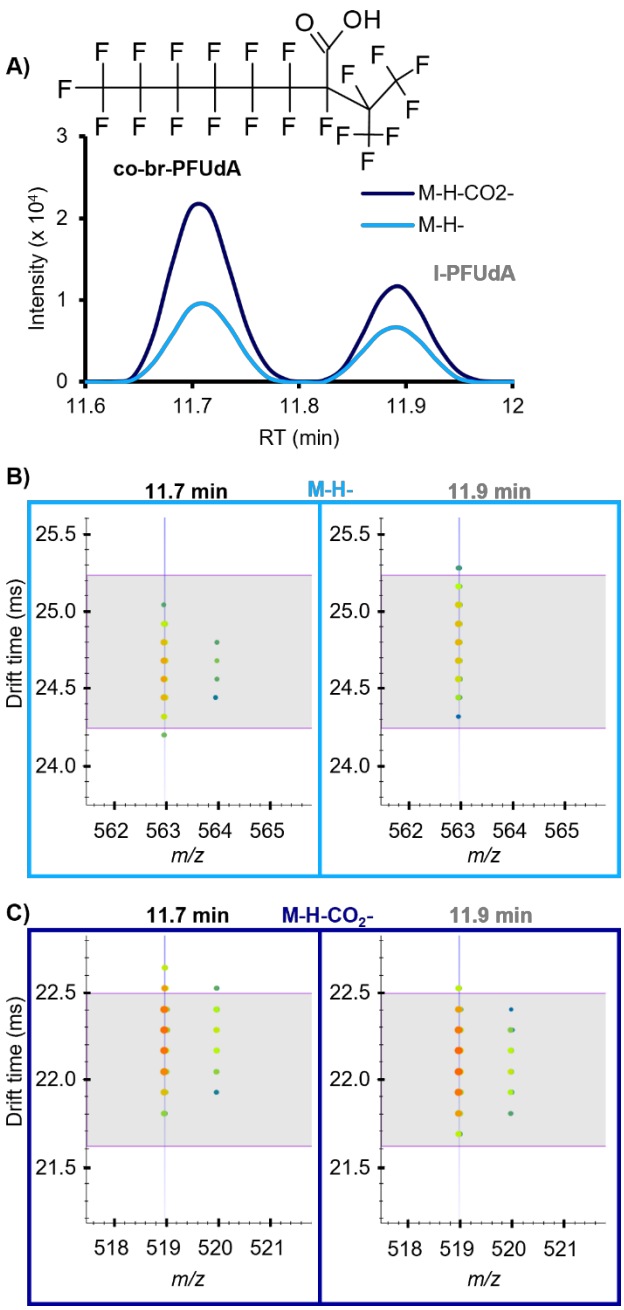

Figure 4. **A)** Candidate structure for the PFUdA isomer (co-br-PFUdA) and EIC illustrating the peak intensity of linear PFUdA (l-PFUdA) in comparison with the isomer. Nested drift spectra for both isomers are shown for both ion types detected, and horizonal lines indicate the expected drift time based on the library CCS value for l-PFUdA. **B)** The M-H- ions show a slightly smaller CCS for co-br-PFUdA, supporting a more compact structure. **C)** The M-H-CO_2- ions show a slightly larger CCS for cobr-PFUdA, supporting a more extended structure when the CO_2 headgroup is lost.

248 Additionally, we detected at least 4 other PFAS including: fipronil sulfone, a fluorinated insecticide
249 considered to be a PFAS due to the presence of two CF_3 groups (Level 2, **Figures S14-S15**);
250 multiple unresolvable isomers of U-PFOS, which contain a double bond (Level 3 confidence,
251 **Figures S16-S17**); the 15-carbon PFCA, PFPeDA (Level 2, **Figure S18**); the 11-carbon PFSA,
252 PFUdS (Level 2, **Figure S19**). Fragmentation evidence suggested the presence of additional
253 undetected perfluoroether carboxylic acids, but no precursor signal was detected so they could
254 not be investigated further (**Figure S20**). Multiple U-PFOS isomers[3] and fipronil sulfone[5] were
255 previously reported in water from the CFR. PFPeDA and PFUdS have not previously been
256 reported in the CFR. To our knowledge, no reports of these PFAS in human residents have been
257 made; however, their presence in alligator plasma are reason for concern.

258 ***Spatial and Temporal Trends***

259 While the primary focus of this work was NTA, we were also interested in evaluating how
260 plasma concentrations of known PFAS are changing over time in these alligator populations and
261 gathering data for comparison to other populations and future studies. Prior to sample analysis,
262 we generated calibration curves using authenticated standards for 26 commonly targeted PFAS
263 to allow absolute quantification (**Table S6**). Of the quantifiable PFAS, 21 of them were detected
264 in the alligators (**Tables S7-S8**). Standards do exist for the other 13 PFAS in our library that were
265 detected and not quantified, and future work on alligators in this area should consider including
266 them in calibration curves. Unsurprisingly, CFR alligators had the highest PFAS concentrations
267 for the 21 quantified PFAS (denoted as Σ21) with an average of 249.1 ng/ml (median: 225.2,

range: 17.3 to 806.5 ng/ml) (**Figure 5**). The LW alligators were almost 6x lower with an average

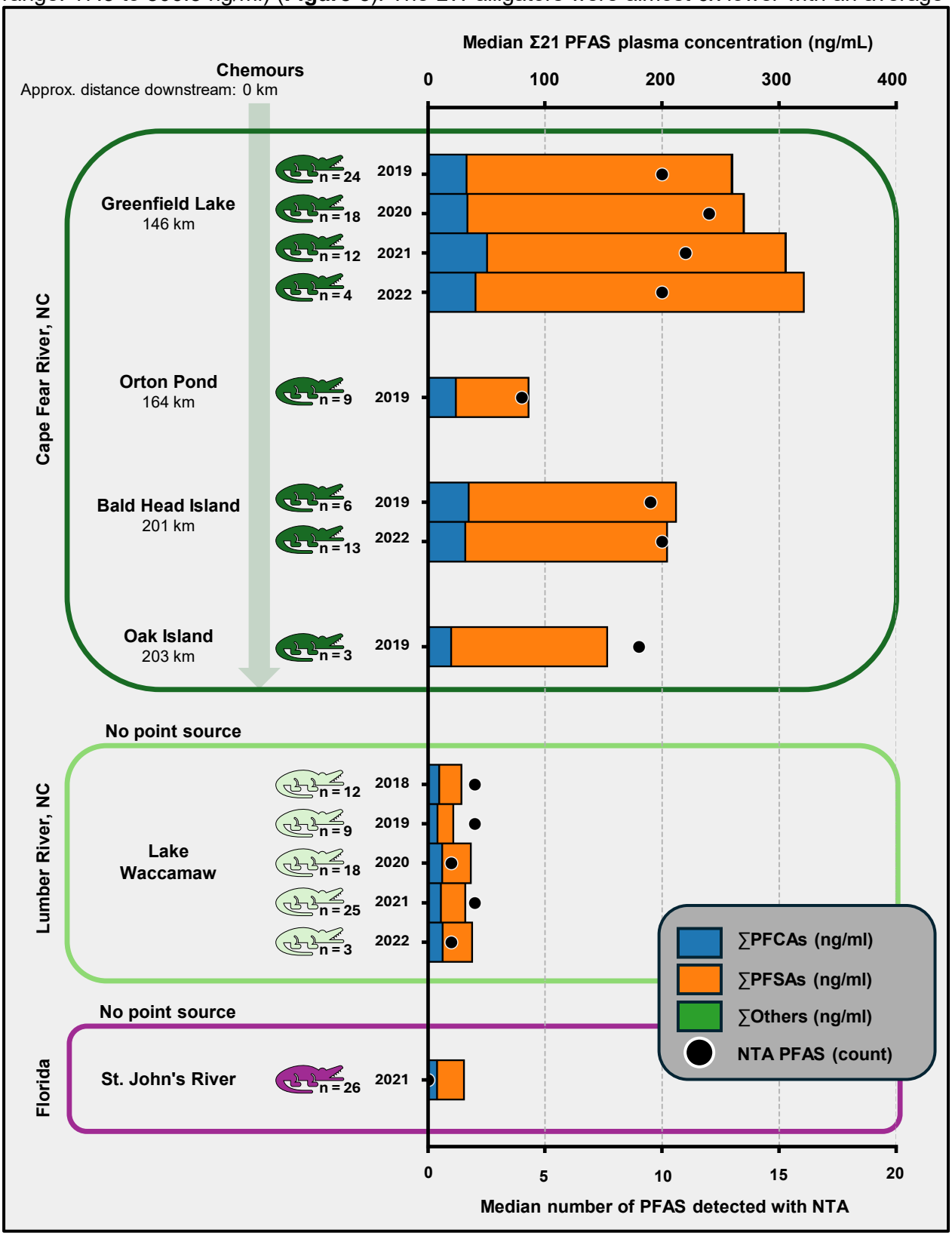

Figure 5. Median summed plasma concentrations by site and year. The Σ21 quantified PFAS are depicted in the bar graph (top x-axis), with the composition by subclass indicated by the colors of each bar section. Only the sites and years with n ≥3 gators sampled are shown. The black dot indicates the number of PFAS uniquely identified using NTA (bottom x-axis), which are not included in the Σ21 PFAS concentrations.

269 of 42.1 ng/ml Σ21 PFAS (median: 33.1, range: 10.8 to 303.5 ng/ml) and FL alligators were lower
270 still with an average of 31.8 ng/ml Σ21 PFAS (median: 30.7, range: 10.1 to 50.2 ng/ml). The
271 composition of the Σ21 PFAS was primarily perfluoroalkyl carboxylic acids (PFCAs) and sulfonic
272 acids (PFSAs) with median 14.8 % PFCAs and 84.9 % PFSAs in the CFR, 34.7% PFCAs and
273 65.3% PFSAs in LW, and 25.6% PFCAs and 74.3% PFSAs in FL. These results aligned with
274 previous data and were expected due to the Chemours discharge in the CFR. Simple linear
275 regression revealed no significant changes in the Σ21 PFAS concentration over time from 2018
276 to 2022 in either the CFR or LW alligators (LW F: 1.301, DF: 72, p-value 0.258; CFR F: 0.7962,
277 DF: 96, p-value 0.374). Only one year of FL data was available so no time trends could be
278 evaluated. Importantly, the 26 other PFAS identified through our NTA workflow (13 through library
279 screening and 12 through discovery) were not quantified, and the Σ21 PFAS concentrations
280 reported here are therefore an underestimate of the total PFAS burden in these animals.
281 Preliminary relative abundance data for the non-quantified PFAS are provided in the
282 **Supplemental Results** (**Figures S21-S37**) and **Table S5**. future work will evaluate semi-
283 quantitative methods for assessing spatial and temporal trends for the novel PFAS.
284

285 # DISCUSSION

286 Alligators live exclusively in aquatic environments and have no access to bottled water or
287 farm-raised fish, a worst-case scenario for exposure to toxic chemicals in water. PFAS have a
288 wide range of chemical properties and are known to partition differently between water and tissue;
289 therefore, our objective was to see if we could detect PFAS in alligator plasma which previously
290 had not been detected in water. Alligator plasma is a valuable biological matrix to examine for
291 PFAS that are bioaccumulating and therefore critical to prioritize. In this study, screening against
292 a library of CCS and RT values for over 100 PFAS allowed us to detect 34 PFAS, 21 of which
293 were quantified. Further, we adapted an NTA workflow previously used on water from this region,

294 which uniquely allowed us to discover 2 novel PFAS (the H-substituted polyfluorinated triether
295 acids) and detect 3 additional PFAS (PFHxESA, PFHpESA, and co-br-PFUdA) that could not
296 have been detected without NTA. We detected several other PFAS which have not previously
297 been reported in biota from this region, including 3 PFAS for which standards are not available
298 (U-PFOS, PFUdS, and PFPeDA), previously unreported isomers of Nafion Byproduct 2, and 1
299 fluorinated insecticide (fipronil sulfone). Detection of these compounds should be highly
300 concerning to PFAS researchers and residents of this region because they reveal ongoing
301 exposure and uptake of dozens of compounds with unknown toxicity, in addition to very high
302 plasma concentrations of the 21 quantified PFAS. There is an urgent need to monitor the novel
303 and known PFAS compounds we report here so we can better understand human and wildlife
304 exposure and increase our understanding of how dangerous these chemicals might be. Also of
305 note is the legality of alligator hunting in North Carolina; not only do these animals serve as useful
306 sentinels of human exposure to PFAS, they also are a source of dietary exposure for humans
307 who eat them.

308 This study illustrates a major advantage of IMS in PFAS applications, which is that all
309 detected features have a CCS value in addition to *m/z* and RT. This lends additional confidence
310 to molecular identifications when diagnostic evidence like homologous series or manufacturing
311 knowledge is available.[3, 17, 22] Several limitations are also noted for this study such as low detection
312 limits for several classes of PFAS, such as PFECAs. A lack of internal fragmentation for some of
313 the candidate structures also limited our ability to fully identify several of the flagged molecules.
314 Optimization of the fragmentation method could be beneficial if full structural elucidation of the
315 tentative candidates is required, though optimization for one class is likely to sacrifice sensitivity
316 for other PFAS classes. Additionally, NTA requires feature prioritization that may inherently
317 exclude certain molecules. Data analysis is highly resource intensive and time consuming, and it
318 is not always possible to re-analyze sample extracts once a feature of interest is discovered.
319 These challenges underscore the need for higher throughput workflows to more rapidly pull

features of interest out of complex data sets, a key area of improvement needed for improved NTA.[23] These challenges also illustrate the benefits of IMS CCS values, which are independent of feature concentration and highly useful in molecular identifications when used in combination with additional evidence.[3, 16, 17, 24]

Another major advantage of NTA data collection is the potential for retrospective data mining when new analytical techniques and software are developed.[23] We obtained data files that were collected by Donelson *et al.* from aquatic passive samplers deployed in the CFR and screened for the novel compounds detected in alligators. Notably, every novel PFAS reported here in alligators was detected retroactively in the water data. Work is ongoing to evaluate spatial and temporal trends for these novel PFAS in water. Similarly, our NTA data can be reassessed in the future as NTA tools improve. Other researchers might identify additional PFAS in our data set, which is publicly available on the MassIVE website. We urge regulators and researchers to begin monitoring the PFAS we report here in studies of food, water, wildlife, and people so we can begin to understand where these compounds come from, whether they are dangerous, and what to do about them.

MATERIALS AND METHODS

Study Area and Sample Collection

All animal procedures were performed with approval by the North Carolina State University Institutional Animal Care and Use Committee (protocol #18-085-O). Alligators were sampled using active capture methods described previously[25] and animals were released at the site of capture. Sampling locations included several sites within the lower Cape Fear River basin in North Carolina, one nearby reference site, Lake Waccamaw, in the Lumber River basin, and one site in Florida (St. John's River) (**Figure 1, Table S1**). Sampling occurred between July 24, 2018 and August 17, 2022.

345 Blood samples were collected from each animal immediately following capture as
346 described previously.[25] Plasma was collected in lithium heparin coated plasma tubes (Vacutainer,
347 BD, Franklin Lakes, NJ) ,stored on ice until centrifugation (1800x g for 10 min at 4C), and then
348 stored in polypropylene microcentrifuge tubes at -80C until analysis . Serum was used in the
349 original alligator study from 2018-2019; however, plasma was used in this study due to its easier
350 handling and higher yield volumes in the field compared to serum. Preliminary proof of concept
351 work (not published) comparing alligator serum and plasma indicated no significant differences in
352 PFAS detection between the two matrices.

353 ***Standards and Reagents***

354 A heavy-labeled PFAS standard mix (MPFAC-HIF-ES, Wellington Laboratories, Guelph,
355 Canada) was diluted 1:1 in methanol for use as internal standards. The Standard Reference
356 Material 1957 (SRM1957, human serum) was obtained from NIST (Gaithersburg, MD) and used
357 for quality control. Calibration curves were prepared in charcoal-stripped fetal bovine serum (part
358 #) using seven concentrations of native PFAS standards (PFAC-MXH and HFPO-DA, Wellington
359 Laboratories) ranging from 0.128 to 4,000 ng/ml. All solvents (acetonitrile, water, and methanol)
360 were Optima LC-MS grade from Fisher Scientific.

361 ***Plasma Sample Preparation***

362 Alligator plasma samples were blocked and blinded into four batches for extraction. A 50
363 µl aliquot of each sample was transferred into a polypropylene microcentrifuge tube, along with
364 three field blanks and three QCs (NIST SRM 1957 human serum) per extraction batch. Full
365 extraction method steps are detailed in the SI. In brief, internal standards (5 µl) were spiked into
366 each aliquot, proteins were precipitated using cold acetonitrile (300 µl), and a portion (200 µl) of
367 the acetonitrile layer was evaporated to dryness. Samples were reconstituted in 40:60
368 methanol:water buffered with 3 mM ammonium acetate (100 µl) and transferred to polypropylene
369 LC vial inserts. Pooled alligator plasma samples and matrix-matched calibration curve samples,

370 along with three method blanks and three QC samples, were prepared following the same
371 procedure in a separate batch.

372 ***LC-IMS-CID-HRMS Data Collection***

373 All samples were first analyzed on the LC-IMS-CID-HRMS platform in MS1-only mode (no
374 collision energy).[17] The pooled samples and several representative individual alligator samples
375 were reinjected using the size-dependent AIF method described previously by Donelson *et al*.[3]
376 Analytical methods are summarized briefly here and full details are provided in the **Supplemental**
377 **Methods**. A C18 column was used (Agilent Zorbax Eclipse Plus, 2.1 x 50 mm, 1.8 µm) with a 5
378 mm guard column inline. The LC method was a 0.4 mL/min gradient with water as mobile phase
379 A and 95% methanol and 5% water as mobile phase B, both buffered with 5 mM ammonium
380 acetate, and increased from 10 to 100% B over 16.5 minutes. The ion source was an Agilent
381 JetStream ESI operated in negative ion mode. The IMS drift tube used a constant voltage drop
382 and a 60 ms maximum drift time, and 4-bit multiplexing was used for the MS1-only injections. For
383 the size-dependent AIF injections, a drift time-based collision energy ramp from 2 to 60 V was
384 applied to alternating frames so that the larger molecules with higher drift times received a higher
385 voltage.[3] The QTOF was operated in *m/z* 50-1700 mode and a mass calibration was performed
386 every other day. MS1-only data files were demultiplexed in PNNL Preprocessor (v. 4.0)[26] with a
387 signal intensity threshold of 20 counts and 100% pulse coverage. To convert measured drift times
388 into CCS values, single-field CCS calibration was applied to all data files in Agilent IM-MS Browser
389 (v. 10.0) using the Agilent ESI tune mix.

390 ***Target Screening and Absolute Quantification***

391 Peak picking was performed in Skyline-daily (v. 24.1.1)[18, 27] using a mass resolving power
392 of 20,000 and a mobility resolving power of 50. All MS1-only files were screened using a transition
393 list from the Baker lab LC-IMS-MS PFAS library v1, which contains retention times and CCS
394 values for 168 PFAS standards collected under identical LC, IMS, and HRMS conditions.[28]

395 Surrogate standards were assigned based on structural similarity and nearest retention time
396 (**Table S4**). Peak areas were exported to Excel for further analysis. Detected PFAS were
397 assigned confidence levels based on a scale adapted from existing guidance incorporating PFAS
398 attributes and IMS-specific criteria.[19, 29-31] In brief, this includes Level 1 (confirmed by reference
399 standard analyzed under identical conditions), 2 (one probable structure supported by all available
400 evidence), 3 (one or more tentative candidate structures), 4 (unequivocal molecular formula), and
401 5 (feature of interest). Calibration curves for 26 PFAS were generated in Skyline using an
402 acceptable accuracy of 70-130%. Method reporting limit (MRLs) were defined as the average plus
403 3 standard deviations of the abundance in the blanks. Limits of quantitation (LOQs) were defined
404 as the lowest point on each calibration curve that was both above the MRL and had an acceptable
405 accuracy (**Table S6**).

406 ***PFAS Discovery and Non-Targeted Analysis***

407 Data files were mined for additional PFAS after completing initial target screening and
408 absolute quantification. Agilent Mass Profiler v10.0.2.202 was used to generate a feature list and
409 Fluoromatch-IM v1.2 was used to score potential PFAS features using homologous series and
410 mass defect flagging; relevant settings are provided in the **Supplemental Methods** (**Tables SM5-**
411 **SM6**). An in-house Python-based software was used to reduce data by eliminating spurious
412 detections from the feature list and to prioritize potential PFAS features which were then further
413 examined in Agilent IM-MS Browser v10.0. Identified features were added to the Skyline transition
414 list and then the target screening workflow described above was followed.

415 ***Statistical Analysis***

416 Statistical analyses were performed in GraphPad Prism (v10.3.1) and Python (3.12).
417 Correlation analysis (nonparametric Spearman correlation, two-tailed p-value, 95% confidence
418 interval) and multiple linear regression was performed to evaluate the relationship between Σ21
419 PFAS concentrations, sampling year, site, sex, body mass index (BMI), and life stage (adult or

juvenile). Significant relationships were found between Σ21 PFAS and site ($p < 0.0001$), life stage ($p = 0.0024$), and BMI ($p = 0.028$), A significant interaction was also found between life stage and BMI ($p < 0.0001$). We elected to group adults and juveniles together despite the known interaction to ensure we had an $n \geq 3$ for each site and year. A larger sample size of both age groups would be beneficial for future work; however, we note that catching even one alligator is quite challenging and sometimes you have to make the best of what you get.

ASSOCIATED CONTENT

Supplemental Document (PDF), including supplemental results and figures S1-S37, supplemental methods and tables SM1-SM6, and additional data in supplemental tables S1-S8.

Skyline files with all LC-IMS-MS data are available at https://panoramaweb.org/2025-alligator-PFAS.url

Raw data files are available at ftp://massive.ucsd.edu/v09/MSV000097330/

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AUTHOR CONTRIBUTIONS

Conceptualization: AKB, SMB, ESB

Methodology: AKB

Software: AKB, GPK

Investigation: AKB, KDR

Resources: MPG, FR, SMB, ESB

Visualization: AKB, GPK

Supervision: ESB

Writing—original draft: AKB

Writing—review & editing: AKB, GPK, KDR, SMB, ESB

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